TGFβ1 regulates HGF-induced cell migration and hepatocyte growth factor receptor MET expression via C-ets-1 and miR-128-3p in basal-like breast cancer

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Breast cancer is the most common cancer in women worldwide. The tumor microenvironment contributes to tumor progression by inducing cell dissemination from the primary tumor and metastasis. TGFβ signaling is involved in breast cancer progression and is specifically elevated during metastatic transformation in aggressive breast cancer. In this study, we performed genomewide correlation analysis of TGFBR2 expression in a panel of 51 breast cancer cell lines and identified that MET is coregulated with TGFBR2. This correlation was confirmed at the protein level in breast cancer cell lines and human tumor tissues. Flow cytometric analysis of luminal and basal-like breast cancer cell lines and examination of 801 tumor specimens from a prospective cohort of breast cancer patients using reverse phase protein arrays revealed that expression of TGFBR2 and MET is increased in basal-like breast cancer cell lines, as well as in triple-negative breast cancer tumor tissues, compared to other subtypes. Using real-time cell analysis technology, we demonstrated that TGFβ1 triggered hepatocyte growth factor (HGF)-induced and MET-dependent migration in vitro. Bioinformatic analysis predicted that TGFβ1 induces expression of C-ets-1 as a candidate transcription factor regulating MET expression. Indeed, TGFβ1-induced expression of ETS1 and breast cancer cell migration was blocked by knockdown of ETS1. Further, we identified that MET is a direct target of miR-128-3p and that this miRNA is negatively regulated by TGFβ1. Overexpression of miR-128-3p reduced MET expression and abrogated HGF-induced cell migration of invasive breast cancer cells. In conclusion, we have identified that TGFβ1 regulates HGF-induced and MET-mediated cell migration, through positive regulation of C-ets-1 and negative regulation of miR-128-3p expression in basal-like breast cancer cell lines and in triple-negative breast cancer tissue.

Abbreviations
CAF, cancer-associated fibroblast; ER, estrogen receptor; HGF, hepatocyte growth factor; RPPA, reverse phase protein array; TGFBR2, TGFβ receptor type-2; TGFβ1, transforming growth factor beta 1; TNBC, triple-negative breast cancer.
1. Introduction

Breast cancer is the most common cancer in women worldwide with over 1.5 million new breast cancer cases diagnosed every year, which makes up to 30% of all cancers (Siegel et al., 2017). It is a heterogeneous disease, and the appearance of drug resistance and formation of metastasis are leading causes of mortality (Di Cosimo and Baselga, 2010; Weigelt et al., 2005). The tumor microenvironment contributes to tumor progression by inducing cell dissemination from the primary tumor and metastasis. Various cytokines and growth factors are secreted by cells of the tumor microenvironment and are involved in processes that potently promote tumor growth as well as metastasis formation (Breunig et al., 2014; Korkaya et al., 2011).

Transforming growth factor beta 1 (TGFβ1) is one major player in this process and has been described as a double edged sword in breast cancer (Bierie and Moses, 2006). While TGFβ1 inhibits cell growth at an early stage of carcinogenesis, it supports metastasis formation in late-stage cancer (Imamura et al., 2012). We have previously shown that TGFβ signaling and TGFβ receptor type-2 (TGFBR2) seem to have a tumor promoting role in human estrogen receptor (ER)-negative breast cancer (Keklikoglou et al., 2012). In addition, clinical studies in ER-negative breast tumors indicated a correlation of elevated TGFBR2 levels with shorter overall breast cancer patient survival (Buck et al., 2004). However, the molecular mechanisms of TGFβ signaling and elevated TGFBR2 expression are likely diverse and have not been fully elucidated.

Transcriptional and post-transcriptional regulations of gene and protein expression are well-described mechanisms of metastasis regulation. Transcriptional regulation involves transcription factors, which are key regulators for gene expression. Several transcription factors are deregulated in human carcinomas and vastly contribute to tumor progression (Lee and Young, 2013; Willis et al., 2015). Due to this deregulation, transcription factors such as ER alpha (ERα) in breast cancer represent attractive targets for cancer therapy (Darnell, 2002). miRNAs regulate signaling pathways at the post-transcriptional level and are frequently deregulated in breast cancer. It has been shown that aberrantly expressed miRNAs promote cancer development, metastasis formation, and potently induce drug resistance in both tumor cells and cells of the tumor microenvironment (Bott et al., 2017; Breunig et al., 2017; Dvinge et al., 2013; Tang et al., 2012; Zhu et al., 2011). In a previous study, we have identified a tumor-suppressive function of the miR-520/miR373 family by targeting TGFBR2 in breast cancer cells (Keklikoglou et al., 2012).

The hepatocyte growth factor (HGF) is another major player promoting tumor growth as well as metastasis formation. HGF is secreted by stromal cells such as cancer-associated fibroblast (CAF) in the tumor microenvironment (Casbas-Hernandez et al., 2013). Upon binding of HGF to its receptor MET, it induces the activation of downstream FAK, MAPK, and PI3K/Akt signaling (Birchmeier et al., 2003) and thereby leads to the regulation of a wide range of cellular processes such as metastasis formation in breast cancer cells (Ho-Yen et al., 2015). MET expression is a prognostic factor in breast carcinoma, and high levels of MET have been shown to correlate with poor survival of patients with breast cancer (Zhao et al., 2017). Expression of MET is elevated in basal-like tumors and inflammatory breast carcinoma (Garcia et al., 2007; Ponzo and Park, 2010); however, it is still not fully understood how MET is regulated in breast cancer cells.

In this study, we performed a genomewide correlation analysis of TGFBR2 in a panel of 51 breast cancer cell lines (Riaz et al., 2013) to identify genes which are coregulated with TGFBR2 and to test their impact on cell migration. MET was one of the top positively correlated genes with TGFBR2 in these breast cancer cell lines. Clinical significance of our in vitro findings was validated by analyzing 801 breast cancer tissue samples of a multicenter prospective study (NCT01592825). There, the same correlation was observed also at the protein level. TGFBR2 and MET were both significantly stronger expressed in triple-negative breast tumors (TNBC) than in luminal-like specimen. We identified and characterized the transcription factor C-ets-1 and miR-128-3p as regulators of MET expression that are both driven by the TGFβ signaling pathway in vitro. Inhibition of C-ets-1 and overexpression of miR-128-3p significantly repressed MET expression and thereby inhibited the HGF/MET signaling pathway as well as migration toward HGF.

2. Materials and methods

2.1. Cell culture and cell lines

Cell lines MDA-MB-231 (HTB-26), MCF-7 (HTB-22), T47D (HTB-133), HCC1143 (CRL-2321), BT549 (HTB-122), UACC812 (CRL-1897), MDA-MB-468 (HTB-132), BT474 (HTB-20), HCC1954 (CRL-2339), SKBR-3 (HTB-30), HS578T (HTB-126), and MCF10A
(CRL-10317) were obtained from American Type Culture Collection (LGC Standards GmbH, Wesel, Germany). MDA-MB-231 cells were maintained in Leibovitz’s L-15 (Life Technologies, Carlsbad, CA, USA) medium [10% FBS, 1% L-glutamine, 1% nonessential amino acids (NEAA), 50 units·mL⁻¹ penicillin, 50 μg·mL⁻¹ streptomycin sulfate (all Life Technologies)]. MCF-7 cells were cultured in MEM (10% FBS, 1% NEAA, 0.01 mg·mL⁻¹ bovine insulin (Sigma-Aldrich, St. Louis, MO, USA), 50 units·mL⁻¹ penicillin, 50 μg·mL⁻¹ streptomycin sulfate). T47D cells were cultured in RPMI (Life Technologies) medium (10% FBS, 1% NEAA, 50 units·mL⁻¹ penicillin, 50 μg·mL⁻¹ streptomycin sulfate), and MDA-MB-468, BT549, UACC812, HCC1954, and HCC1143 cells were cultured in RPMI medium (10% FBS). HS5578T cells were cultured in DMEM (Life Technologies) (10% FBS). MCF10A cells were cultured in DMEM F12 medium (5% horse serum (Life Technologies) (10% FBS). MCF10A or HCC1143 cell lines were transfected with single or pooled siMET or siRNA control 24 h prior addition of TGFβ1.

2.3. Luciferase reporter assays
To validate direct targeting of miR-128-3p, pScheck2 vectors (Promega, Fitchburg, WI, USA), containing the respective 3’UTRs, were cotransfected with mimic miRNAs in MCF-7 cells. Forty-eight hours after transfection, Renilla and firefly luciferase activities were determined using a luminometer (Tecan, Männedorf, Switzerland). Mutations within the predicted target site of MET 3’UTRs were generated by site-directed mutagenesis using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions.

2.4. Antibodies, immunoblotting, and flow cytometry
For western blotting, cells were lysed in ice cold M-PER lysis buffer (Thermo-Fisher Scientific, Waltham, MA, USA) containing NaF (Th. Geyer), Na₃VO₄ (Sigma-Aldrich), and protease inhibitor Complete Mini and phosphatase inhibitor PhosSTOP (both Roche, Basel, Switzerland). Protein concentrations were determined by BCA Protein Assay Reagent Kit (Thermo-Fisher Scientific), and proteins were denatured with 4x Roti Load (Carl Roth, Karlsruhe, Germany) at 95 °C for 5 min. Depending on the size, proteins were separated by 10 and 15% SDS/PAGE, blotted onto a PVDF membrane Immobilon-FL (Merck Millipore, Darmstadt, Germany), and exposed to primary antibodies. The following antibodies were used: MET (clone L41G3; CST #3148), phospho-MET (clone D26; CST #3077), C-ets-1 (clone D808A; CST #14069), β-actin (polyclonal; Sigma-Aldrich #20-33), and β-actin (clone C4; MP Biochemicals #691001). Blots were probed with goat anti-rabbit or goat anti-mouse IgG (H+L) IRDye®-680- or IRDye®-780-conjugated secondary antibodies (Thermo-Fisher Scientific), and bands were visualized using an Odyssey scanner (LI-COR, Lincoln, NE, USA). Primary antibodies were used at a 1 : 1000 dilution and secondary antibodies at a 1 : 10 000 dilution.

For flow cytometry, cells were detached using Accutase (Life Technology), washed, and resuspended into

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FACS buffer (BD Biosciences). Cells were stained for MET and TGFBR2 expression using 5 μg·mL⁻¹ anti-MET (clone 95106; R&D systems #MAB3582) or anti-TGFBR2 primary antibodies (clone MM0056-4F14; Abcam #ab78419, Cambridge, UK). A mouse IgG isotype was used as a control (clone UPC-10; Sigma-Aldrich #M5409). Goat anti-mouse IgG/IgM antibody coated with FITC was used as a secondary antibody in a 1 : 100 dilution (polyclonal; BD Biosciences #555988). Specificity of MET and TGFBR2 primary antibodies was shown with knockdown experiments (Fig. S1). Cells were analyzed using FACSCalibur (BD Biosciences) and FLOWJO (Treestar, Ashland, OR, USA).

2.5. Patient samples

Tissue lysates from fresh-frozen breast cancer patient samples were derived from the multicenter prospective PiA study (NCT01592825) as previously described (Bernhardt et al., 2017). Breast cancer subtypes were defined to histopathological characteristics such as receptor status and grading according to the St. Gallen classification and by von Minckwitz and colleagues (Goldhirsch et al., 2013; von Minckwitz et al., 2012).

2.6. Ethics approval and consent to participate

Our study is in accordance with the Declaration of Helsinki. Institutional review board approval by the Ethics Committee of the Medical Faculty of the Martin-Luther-University Halle-Wittenberg was received, and informed consent was obtained from all patients.

2.7. Reverse phase protein array (RPPA)

The method used in this study has been described previously (Loebke et al., 2007). Briefly, RPPA was performed on 2 μg·mL⁻¹ of protein, lysed with tissue protein extraction reagent (50 mM Tris, pH 8.5, 138 mM NaCl, 2.7 mM KCl, 1% Triton X-100). Lysed protein was mixed with 4× SDS sample buffer (10% glycerol, 4% SDS, 10 mM DTT, 125 mM Tris/Cl, pH 6.8) and denaturated at 95 °C for 5 min. Protein samples were printed onto nitrocellulose-coated glass slides (Onyte Avid; Grace-Biolabs, Bend, OR, USA) with a Aushon 2470 contact spotter (Aushon BioSystems, Billerica, MA, USA) and stored at −20 °C until further use.

Slides were blocked with blocking buffer (Rockland Immunochemicals) in TBS (50%, v/v) containing 5 mM NaF and 1 mM Na3VO4 for 2 h at room temperature. Slides were incubated at 4 °C over night with primary antibodies diluted at 1 : 300 and subsequently washed four times for 5 min in 1× TBS with 0.1% Tween-20. The antibodies used were MET (CST 3148; Cell Signaling Technology, Danvers, MA, USA) and TGFBR2 (Santa Cruz Biotechnology, Heidelberg, Germany). Next, slides were incubated for 1 h with infrared-labeled secondary antibody, Alexa Fluor 680 F(ab’)2 fragments of goat anti-mouse IgG (Life Technologies) diluted at 1 : 8000 in 1× TBS with 0.1% Tween-20. Washing steps were performed as described above. All washing and incubation steps were carried out at room temperature with gentle shaking. Finally, slides were rinsed in water and air-dried at room temperature. Slides were scanned using the Odyssey Scanner (LI-COR, Biosciences) with 21 μm resolution. TIFF images of all slides were obtained at an excitation wavelength of 685 nm.

Signal intensities of each individual spot were quantified using genePixPro 7.0 (Molecular Devices, LLC, San José, CA, USA). The R-package RPPA analyzer (version 1.4) was used for data preprocessing and quality control. Data were log2-transformed subsequently. For visualizing target expression levels in box plots, RPPA data were normalized by scaling into the range between 0 and 1.

2.8. RNA isolation and quantitative real-time PCR

Total RNA and miRNA were isolated from cells using miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. For mRNA, cDNA synthesis was carried out with the Revert Aid H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, USA). The quantitative RT-PCRs (qRT-PCRs) for target genes were performed using ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Waltham, MA, USA), using probes from the Universal Probe Library (Roche) (Table S2). The housekeeping genes ACTB and TFRC were used for normalization of mRNA analysis. For miRNAs, the TaqMan microRNA reverse transcription kit and TaqMan gene-specific microRNA assays (Applied Biosystems) were used. For the quantitative RT-PCRs (qRT-PCRs), RNU44 and RNU48 were used as housekeeping controls. Data were acquired using a HT-7900 TaqMan instrument (Applied Biosystems) and analyzed with the ΔΔCt algorithm (ddCt; Bioconductor).
Fig. 1. TGFBR2 is higher expressed in basal-like breast cancer cell lines and triple-negative breast cancer tissue. (A) Quantitative PCR for TGFBR2 mRNA expression in breast cancer cell lines using breast cancer cell line microarray database for TGFBR2 (Riaz et al., 2013) (luminal n = 18; luminal HER2 n = 9; basal-like n = 19; HER2 enriched n = 3; Student’s unpaired t-test). (B) Flow cytometry analysis for TGFBR2 of breast cancer cell lines of different subtypes and the fibrocystic cell line with the basal-like genotype MCF10A. (C) TGFBR2 protein expression in breast cancer patient subtypes luminal A-like (n = 510), luminal B-like (n = 104), luminal B-like HER2+ (n = 74), nonluminal HER2+ (n = 36), and triple-negative (n = 77) measured by RPPA. (D) TGFBR2 protein expression in ER-negative (ER neg.; n = 120) and ER-positive (ER pos.; n = 681) breast cancer specimens analyzed by RPPA. Data are expressed as mean for (A) and represented in a box and whiskers plot (in the style of Tukey) for (C) and (D). Key: *P < 0.05; **P < 0.01; ***P < 0.001.
2.9. RTCA migration assay

RTCA (real-time cell analyzer) cell migration assays were performed as previously described (Breunig et al., 2014). For RTCA migration experiments, transfections and/or treatment with inhibitors and TGFβ1 were performed as described above. Twenty-four hours prior experiment cells were starved with cell growth medium without FBS. Then, 100 000 cells were seeded in the upper chamber of the RTCA CIM-plate 16 (Roche) in starvation medium. The lower chamber was filled with starvation medium with or without 75 ng mL\(^{-1}\) HGF (R&D Systems). CIM-plate was inserted in the xCELLigence machine, and migration was measured every 15 min up to 30 h depending on the cell line.

2.10. Analysis of cell lines and breast cancer tissue and statistical analysis

A dataset containing mRNA expression of 51 breast cancer cell lines (Riaz et al., 2013) was used for mRNA expression analysis of TGFBR2 and MET. Cell lines were classified into luminal and basal according to their molecular characteristics (Neve et al., 2006). The microarray data were obtained from the Gene Expression Omnibus data repository (GEO: GSE41313). In addition, this dataset was used to calculate the Pearson product–moment correlation coefficient of all genes toward TGFBR2 and MET. An additional dataset containing mRNA expression data of 48 breast cancer cell lines (Kao et al., 2009) was used for validation. This microarray data were obtained from the Gene Expression Omnibus data repository (GEO: GSE15376). TGFBR2 and MET gene expression data from the NCI-60 panel, Sanger cell line panel as well as the TCGA datasets were obtained from the R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl). Two datasets which included mRNA and miRNA expression data for human primary breast tumors were obtained from the NCBI GEO database (GEO: GSE19783) and from the METABRIC dataset (EGAC0100000010) were used (Curtis et al., 2012). Potential transcription factor binding to the promoter of MET was analyzed using the Transcriptional Regulatory Element Database (http://rulai.cshl.edu/TRED). Correlations and statistical analyses were carried out with GRAPHPAD software (GraphPad software Inc., La Jolla, CA, USA) to generate Kaplan–Meier curves and boxplots. Unless otherwise stated, all P-values were calculated by means of a two-sided t-test where P-values < 0.05 were considered as significant.

3. Results

3.1. TGFBR2 is higher expressed in basal-like breast cancer and correlates with hepatocyte growth factor receptor expression

We recently described a tumor promoting role of TGFβ signaling and TGFBR2 in human ER-negative breast cancer (Keklikoglou et al., 2012). To further elaborate on the function of TGFBR2 within subtypes of human breast cancer, we initially analyzed a gene expression dataset from 51 breast cancer cell lines (Riaz et al., 2013). There, we identified TGFBR2 as being higher expressed in basal-like compared to luminal as well as higher in ER-negative compared to ER-positive breast cancer cell lines (Figs 1A and S2). To validate these findings, we measured surface expression of TGFBR2 at the protein level in several breast cancer cell lines confirming elevated expression in basal-like compared to luminal cell lines (Fig. 1B). Next, we measured TGFBR2 expression at the protein level in a set of 801 tissue samples of a prospective breast cancer cohort to investigate on TGFBR2 expression in different breast cancer subtypes in vivo. Here, we detected higher

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Fig. 2. MET correlates with TGFBR2 expression and is expressed at higher levels in basal-like breast cancer cell lines and triple-negative breast cancer tissue. (A, B) Correlation analysis of MET and TGFBR2 mRNA expression in (A) breast cancer cell lines with each data point representing a different breast cancer cell line (Pearson’s correlation, breast cancer cell line microarray database, n = 51) (Riaz et al., 2013); (B) breast cancer patients with each data point representing an individual sample (Pearson’s correlation (R); TCGA breast cancer dataset, n = 547). (C) Correlation of MET and TGFBR2 protein expression in breast cancer patients with each data point representing an individual sample (Pearson’s correlation (R); n = 801). Protein expression was measured with RPPA. (D) Quantitative PCR mRNA expression analysis in breast cancer cell lines using breast cancer cell line microarray database (Riaz et al., 2013). (E) Flow cytometry analysis of breast cancer cell lines of luminal and basal-like breast cancer subtypes and the fibrocystic cell line with the basal-like genotype MCF10A. (F) MET protein expression in breast cancer patient subtypes luminal A-like (n = 510), luminal B-like (n = 104), luminal B-like HER2+ (n = 74), nonluminal HER2+ (n = 36), and triple-negative (n = 77) measured by RPPA. (G) MET protein expression in ER-negative (ER neg.; n = 120) and ER-positive (ER pos.; n = 681) breast cancer specimens analyzed by RPPA. Data are expressed as mean for (D) and represented in a box and whiskers plot (in the style of Tukey) for (F) and (G). Key: *P < 0.05; **P < 0.01; ***P < 0.001.
TGFBR2 expression in triple-negative tumors compared to luminal-like tumors (Fig. 1C) as well as in ER-negative compared to ER-positive breast tumor tissue (Fig. 1D).

We hypothesized that genes which are coexpressed with TGFBR2 could be involved in TGFβ-mediated progression of breast cancer. Therefore, a correlation analysis using gene expression data from a 51 cell line...
panel (Riaz et al., 2013) was performed to identify genes that are positively coexpressed with TGFBR2 in tumor cells. The hepatocyte growth factor receptor (MET) was among the top significant genes positively correlating with TGFBR2 expression (Fig. 2A and Table S3), which could be validated using an independent dataset of breast cancer cell lines (Fig. S3A) (Kao et al., 2009). Positive correlation of MET with TGFBR2 gene expression was also observed in breast cancer tissue using the breast cancer TCGA dataset (Fig. 2B) and, at the protein level, in 801 breast cancer tissue specimens (Fig. 2C). Besides breast cancer, a putative relationship between MET and TGFBR2 expression was observed also in cell lines from other tumor entities using the NCI-60 as well as the 789 cell line panels of the NCI and the Sanger Institute, respectively (Fig. S3B, C). These correlations could be validated by analyzing publicly available patient datasets. MET and TGFBR2 gene expressions were found to positively correlate in several other tumor entities, such as prostate adenocarcinoma, thymoma, glioblastoma, head and neck squamous cell carcinoma, testicular germ cell tumors, and esophageal carcinoma (Fig. S3D–I).

Next, we investigated whether the expression of MET is related to specific breast cancer subtypes and therefore checked its differential expression in cell lines of different breast cancer subtypes (Riaz et al., 2013). This analysis revealed that MET is higher expressed in basal-like compared to luminal as well as in ER-negative compared to ER-positive breast cancer cell lines (Figs 2D and S4A). To validate these findings, we analyzed surface expression of MET by flow cytometry. Luminal breast cancer cell lines MCF-7, T47D, and MDA-MB-453 as well as the luminal HER2+ breast cancer cell lines BT474 and SKBR-3 had very little surface expression of MET. In contrast, MET was expressed at substantial levels in basal-like breast cancer cell lines MDA-MB-468, BT549, HS578T, MDA-MB-231, and HCC1143, as well as the HER2-enriched cell line HCC1954 and in the immortalized breast epithelial cell line MCF10A (Figs 2E and S4B). Protein expression analysis revealed higher MET expression in triple-negative compared to luminal-like breast tumors in a set of 801 breast cancer tissue samples (Fig. 2F). As for the 51 breast cancer cell lines, MET was higher expressed in breast tumors lacking ER expression than in samples with ER expression (Fig. 2G).

3.2. TGFβ1 induces cell migration via upregulation of MET

We hypothesized that the observed correlation between TGFBR2 and MET is based on a common regulatory mechanism. To this end, we activated MET and TGFβ signaling pathways with HGF and TGFβ1, respectively, and tested their influence on TGFBR2 or MET expression in the model cell line MCF10A as well as in basal-like breast cancer cell lines HS578T and HCC1143. TGFβ1 significantly increased gene and surface protein expression of MET (Figs 3A, B and S5), while HGF did not affect TGFBR2 expression (Fig. S6). Next, we blocked TGFβ signaling with the highly selective TGFBR2 inhibitor ITD1 (Willems et al., 2012), which obstructed TGFβ1-induced MET expression (Fig. 3B) and proved a regulation of MET by the TGFβ signaling pathway.

Besides TGFβ, MET signaling importantly contributes to cell migration and metastasis formation (Birchmeier et al., 2003; Ho-Yen et al., 2015). Thus, we hypothesized that HGF could also influence cell migration in vitro. Indeed, we could show that HGF induces cell migration (Fig. 3C) and that this phenotype could be blocked by knockdown of MET using single as well as pooled anti-MET siRNAs (Figs 3D and S7A, B).

Next, we hypothesized that the strong upregulation of MET by TGFβ would also impact on HGF-mediated migration in vitro. To this end, we treated MCF10A, HS578T, and HCC1143 cells with TGFβ1 and could show stronger migration capabilities toward HGF (Figs 3E and S7C), which could be downregulated by the addition of the TGFBR2 inhibitor ITD1 (Figs 3F and S7D).

In conclusion, these results demonstrate that TGFβ1 upregulates MET and thereby leads to increases in migration in an HGF-dependent manner.

3.3. MET expression is regulated via C-ets-1

Next, we wanted to identify the mechanism of TGFβ1-induced expression of MET. To this end, we bioinformatically searched for putative transcription factor binding sites in the MET promoter and combined this with an analysis of genes that are coexpressed with either TGFBR2 or MET in breast cancer cell lines. Several transcription factors were found to have predicted binding sites in the MET promoter (Table S4). C-ets-1 was the top candidate as this transcription factor has previously been described to synergize with SMAD3 (Linde mann et al., 2001) and its expression also correlated the most with expression of TGFBR2 as well as MET in breast cancer cell lines (Fig. 4A, B, Tables S3 and S5). This strong correlation was validated in an independent dataset of breast cancer cell lines (Fig. S8A, B) (Kao et al., 2009). Correlation of ETS1 with MET and TGFBR2 was observed as well in breast cancer tissue using the TCGA breast cancer dataset (Fig. 4C, D).
**Fig. 3.** TGFβ1 elevates expression of MET and induces cell migration in MCF10A cell line. (A) Gene expression analysis using quantitative PCR in MCF10A cells, which were treated with 10 ng·mL⁻¹ TGFβ1 or untreated (medium) (n = 3; Student’s unpaired t-test). (B) Flow cytometry analysis of MCF10A cells after TGFβ1 treatment in combination with or without 2.5 μM TGFBR2 inhibitor ITD1 (n = 3). (C) Migration analysis of MCF10A cells toward HGF using the RTCA. (n = 4). (D) Migration analysis of MCF10A cells toward HGF using the RTCA. MCF10A cells were transfected with a pool of anti-MET siRNAs or siRNA control prior to the migration assay. Cells were seeded in starvation medium, and HGF was used as chemoattractant (n = 3). (E) MCF10A cells were treated with or without TGFβ1 for 72 h prior to the migration assay. Cells were seeded in starvation medium, and HGF was used as chemoattractant (n = 4). (F) MCF10A cells were treated with TGFβ1 in combination with or without ITD1 for 72 h prior to the migration assay. Cells were seeded in starvation medium, and HGF was used as chemoattractant (n = 3). Data are expressed as mean ± SD. The significance was determined using Student’s unpaired t-test. Key: *P < 0.05; ***P < 0.001.
We thus hypothesized that C-ets-1 is regulated by TGFβ1. To test this, we stimulated MCF10A, HS578T, and HCC1143 cells with TGFβ1 and then measured the expression levels of ETS1. We observed increases in ETS1 expression (Figs 4E and S9A), supporting a potential role of C-ets-1 as regulator of TGFβ1-induced MET expression. To ascertain this regulation of C-ets-1 by TGFβ1, we knocked down C-ets-1 leading to decreased basal MET expression at the RNA level in MCF10A, HS578T, and HCC1143 cells (Figs 4F and S9B–D) and resulted in a reduction of HGF-induced MET phosphorylation (Fig. S9E). C-ets-1 knockdown also decreased TGFβ1-induced MET expression (Figs 4F and S9F). These results suggest that C-ets-1 is involved in HGF-mediated cell migration. Accordingly, we knocked down C-ets-1 in MCF10A as well as HCC1143 cells and then analyzed HGF-dependent cell migration. As expected, knock down of C-ets-1 completely blocked or reduced cell migration toward HGF in MCF10A and HCC1143 cell, respectively (Figs 4G and S9G). These results show that TGFβ1 regulates MET expression at least in part via upregulation of C-ets-1 and defines both molecules as critical factors for HGF-induced cell migration.

3.4. MiR-128-3p negatively correlates with MET expression in breast cancer and inhibits HGF-induced cell migration by directly targeting MET

We next wanted to know whether also miRNAs would potentially contribute to coordinated MET regulation via TGFβ1 to better reflect the complexity of MET expression regulation.
transcriptional and post-transcriptional regulation. To this end, we first screened the TargetScan database for miRNAs that could regulate MET expression. miR-128-3p was among the top predicted candidates, and its expression was found to negatively correlate with MET in a cell line panel (Fig. 5A). The same was observed upon analysis of the METABRIC breast tumor tissue dataset (Fig. 5B), which also showed lower expression of miR-128-3p in more aggressive ER-negative and basal-like breast cancer compared to ER-positive and luminal A and B breast cancer (Fig. S10A, B).

We thus hypothesized that miR-128-3p directly targets MET, as there is one highly conserved and predicted binding site for miR-128-3p in the 3'UTR of the MET mRNA (Fig. 6A). The 3'UTR of MET was cloned into a luciferase reporter system, and luciferase-based reporter assays were performed with wild-type or mutated binding sites for miR-128-3p. Luciferase activity of the MET wild-type 3'UTR construct was significantly decreased when miR-128-3p was cotransfected. This effect could be rescued by cotransfection of the plasmid containing the mutated 3'UTR, which proved direct targeting of miR-128-3p at the predicted binding site within the MET 3'UTR (Fig. 6B). It has been reported that TGFβ1 downregulates miR-128-3p expression in breast cancer cell lines (Qian et al., 2012), which could be confirmed in our model system MCF10A (Fig. S11A). We thus hypothesized that TGFβ1-mediated downregulation of miR-128-3p would increase MET expression. To this end, we tested whether antagonirs against miR-128-3p (Fig. S11B) could mimic the effect of TGFβ1 on increasing MET expression. Indeed, inhibition of miR-128-3p significantly induced MET expression in MCF10A, HS578T, and HCC1143 cells (Figs 6C and S11C). Based on these results, we hypothesized that miR-128-3p inhibition could also modulate HGF-induced cell migration in MCF10A cells. Therefore, the cells were transfected with miR-128-3p antagonirs and HGF-induced migration was measured 48 h later. miR-128-3p antagonirs increased migration of MCF10A cells toward HGF and confirmed the activity of miR-128-3p in this process (Fig. 6D). Next, we tested the impact of miR-128-3p on MET expression in MET-positive breast cancer cell lines. As expected, surface protein levels of MET were reduced in MCF10A as well as in all tested basal-like breast cancer cell lines HS578T, MDA-MB-468, MDA-MB-231, BT549, and HCC1143 upon overexpression of miR-128-3p (Fig. 6E). Ectopic overexpression of miR-128-3p using respective mimics (Fig. S11D) strongly reduced HGF-mediated migration in MCF10A as well as in HS578T cell lines (Fig. 6F).

Altogether, these data demonstrate that TGFβ1 modulates the expression levels of C-ets-1 and miR-128-3p, thereby triggering promigratory stimuli from the tumor microenvironment in breast epithelial and cancer cells.

4. Discussion

Breast cancer is a highly heterogeneous disease and the clinical outcome strongly correlates with the respective tumor subtype (Jemal et al., 2011). The high mortality rate in patients diagnosed with specific subtypes of breast cancer derives from the limited therapeutic success and a rapid onset of metastasis. Tumor cells and cells of the tumor microenvironment secrete diverse growth factors and cytokines and establish a specific milieu fostering tumor growth, tumor cell migration, and angiogenesis (Quail and Joyce, 2013). In this context, TGFβ1 and MET form key players and act on both cancer cells and the tumor stroma. In breast cancer, TGFβ1 is upregulated compared to its adjacent nonmalignant tissue and it impacts on various cells of the tumor microenvironment. In the tumor stroma, TGFβ1 gets secreted by tumor-associated macrophages and CAFs (Kojima et al., 2010; Yu et al., 2014). It has been shown that TGFβ1 directly acts on the basal-like breast tumor cell line MDA-MB-231 and that this drives metastatic processes of breast cancer cells by its binding to TGFBR2 (Willis et al., 2015). In line with these findings, we demonstrate that TGFBR2 is expressed at higher levels in more aggressive basal-like breast cancer cell lines as well as in triple-negative breast cancer tissue than in luminal breast cancer cell
lines and luminal-like tumor tissue. This subtype-specific expression of TGFBR2 might explain why TGFBR2 expression correlates with reduced overall survival of patients with ER-negative but not with ER-positive breast cancer (Buck et al., 2004). Nevertheless, the exact mechanisms of TGFβ1/TGFBR2-driven breast cancer progression and metastasis have not been fully elucidated until now.
Here, we highlight the role of TGFβ1 as inducer of tumor cell migration by increasing HGF/MET signaling in vitro. We could establish a positive correlation of TGFBR2 and MET protein expression in breast cancer by analyzing 801 tumor tissues samples. In line with our findings for TGFBR2, we observed higher MET expression in nonluminal compared to luminal breast cancer cell lines as well as nonluminal-like compared to luminal-like breast tumors. These findings are supported by Xu et al. (2012) who identified elevated MET signaling and caveolin accumulation in human basal-like breast tumors. In addition, high MET expression has been shown to be associated with poor patient survival in breast cancer (Ponzo et al., 2009; Raghav et al., 2012). MET signaling initiates tumorigenesis in early progenitor cells and induces an invasive growth phenotype, which can be explained by its capacity to increase tumor cell motility, invasion, and resistance toward apoptosis induction (Comoglio and Trusolino, 2002; Gravel et al., 2009). In renal epithelial cells, Sp1 and Smad are involved in TGFβ1-induced MET expression (Zhang et al., 2005). However, little is known about how MET expression is regulated downstream of TGFβ signaling in breast cancer.

We are the first to show that two different mechanisms of MET regulation are merged by TGFβ1 in aggressive breast cancer cell lines and tumor tissue. The first mechanism describes TGFβ1 as regulator of the transcription factor C-ets-1, which mediates elevated expression of MET. This finding is supported by Gambarotta et al. (1996) as well as by Kubic et al., (2015), who both described transcriptional regulation of MET by C-ets-1, however, not in a TGFβ1-dependent context. C-ets-1 overexpression has been shown to strongly promote malignant invasiveness of breast cancer cells, which is in line with our findings (Furlan et al., 2008). Further, it is prognostic marker for poor prognosis of human breast cancer (Span et al., 2002). The second mechanism of MET regulation by TGFβ1 is related to suppression of miR-128-3p which, in turn, directly targets MET, which is in line with Jiang and colleagues non-small cell lung cancer (Jiang et al., 2016), and thereby impairs HGF/MET-mediated cell migration. Migliore and colleagues support the idea of post-transcriptional regulation of MET by showing that also miR-34b, miR-34c, and miR-199a-5p negatively regulate MET expression and thereby inhibit invasive growth in different cancer cell lines (Migliore et al., 2008). In bovine skeletal muscle satellite cells, miR-128-3p directly target SP1 through this inhibiting cell proliferation (Dai et al., 2016). As SP1 is involved in TGFβ1-induced MET expression (Zhang et al., 2005) in renal epithelial cells, MET might be also regulated by miR-128-3p via SP1 in breast cancer. The regulatory mechanism of miR-128-3p seems to be relevant also in breast cancer patients as our analysis suggests that miR-128-3p is lower expressed in nonluminal breast cancer compared to luminal-like subtypes. Low-level expression of miR-128-3p also correlates with poor clinical outcome of patients with breast cancer (Qian et al., 2012; Zhu et al., 2011). Other studies have shown that miR-128-3p is also lower expressed in other tumor tissues and that its overexpression inhibits
tumor cell proliferation, angiogenesis, and invasion (Evangelisti et al., 2009; Hu et al., 2014; Palumbo et al., 2013). Hence, we define TGFβ1 as a regulator of MET expression in breast cancer by upregulating C-ets-1 and downregulating miR-128-3p expression, which results in cell migration, invasion, and metastasis in a HGF-dependent manner (Fig. 7). Regulation of MET expression by TGFβ1 seems to be tumor-type-specific as in glioblastoma, TGFβ suppresses HGF/MET pathway activity (Papa et al., 2017).

Recent data suggest that miR-128-3p is involved in a negative feedback loop of TGFβ signaling in non-small cell lung cancer (Cai et al., 2017). While we have shown that TGFβ1 results in downregulation of miR-128-3p, Cai and colleagues demonstrated that the negative regulators of TGFβ signaling, SMURF2 and PP1c, are regulated by miR-128-3p (Cai et al., 2017). However, aggressive breast cancer carries elevated TGFβ signaling activities, which indicates that this negative feedback loop is not effective in this cancer entity (Dunning et al., 2003). Further, expressions of SMURF2 as well as of PP1c neither correlated with miR-128-3p expression nor were these genes differentially expressed in the different breast cancer subtypes (data not shown). Hence, the regulation of events that are controlled by TGFβ signaling seems to be intricately controlled and this regulation appears to be context-dependent.

Currently, several TGFβ and MET pathway inhibitors are under development. A few have already been tested in clinical trials and show promising results in various cancer entities, including metastatic breast cancer (Ganapathy et al., 2010; Gherardi et al., 2012). By revealing TGFβ1 as a regulator of HGF/MET-induced cell migration, our findings suggest TGFβ1 with its downstream mediators C-ets-1 and miR-128-3p as potential targets for therapy of basal-like breast cancer.

5. Conclusions

In this study, we demonstrate that TGFβ signaling leads to increased MET signaling activity and that this results in HGF-dependent migration of breast epithelial and cancer cells. We could explain this migratory phenotype by the action of TGFβ1 leading to both upregulation of C-ets-1 and inhibition of miR-128-3p expression. Higher C-ets-1 and lower miR-128-3p expression levels intensify MET signaling and thereby suggest TGFβ1 as a regulator of HGF-mediated migration in MCF10A and breast cancer cells.

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Author contributions

CB designed the study. CB, NE, AB, JG, SB, CG, and ER performed experiments. AW, TB, and SB analyzed RPPA data of patient samples. AW, TB, and SB interpreted the results, and CB, AB, and SW wrote the manuscript. All authors have reviewed and approved the manuscript for submission.

References

Bernhardt S, Bayerlova M, Vetter M, Wachter A, Mitra D, Hanf V, Lantzsch T, Uleer C, Peschel S, John J et al. (2017) Proteomic profiling of breast cancer metabolism identifies SHMT2 and ASCT2 as prognostic factors. Breast Cancer Res 19, 112.

Bierie B and Moses HL (2006) Tumour microenvironment: TGFbeta: the molecular Jekyll and Hyde of cancer. Nat Rev Cancer 6, 506–520.
Birchmeier C, Birchmeier W, Gherardi E and Vande Woude GF (2003) Met, metastasis, motility and more. Nat Rev Mol Cell Biol 4, 915–925.

Bott A, Erdem N, Lerrer S, Hotz-Wagenblatt A, Breunig C, Abnaof K, Worner A, Wilhem H, Munstermann E, Ben-Baruch A et al. (2017) miRNA-1246 induces pro-inflammatory responses in mesenchymal stem/stromal cells by regulating PKA and PP2A. Oncotarget, 8, 43897–43914.

Breunig C, Mueller BJ, Umansky L, Wahl K, Hoffmann K, Lehner F, Manns MP, Bantel H and Falk CS (2014) BRaf and MEK inhibitors differentially regulate cell fate and microenvironment in human hepatocellular carcinoma. Clin Cancer Res 20, 2410–2423.

Breunig C, Pahl J, Kubbeck M, Miller M, Antonelli D, Erdem N, Wirth C, Will R, Bott A, Cai J, Fang L, Huang Y, Li R, Xu X, Hu Z, Zhang L, Casbas-Hernandez P, D'Arcy M, Roman-Perez E, Brauer Dai Y, Zhang WR, Wang YM, Liu XF, Li X, Ding XB and Guo H (2016) MicroRNA-128 regulates the recognition by natural killer cells. Cell Death Dis 8, e2973.

Buck MB, Fritz P, Dippon J, Zugmaier G and Knabbe C (2004) Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients. Clin Cancer Res 10, 491–498.

Cai J, Fang L, Huang Y, Li R, Xu X, Hu Z, Zhang L, Yang Y, Zhu X, Zhang H et al. (2017) Simultaneous overactivation of Wnt/beta-catennin and TGFbeta signalling by miR-128-3p confers chemoresistance-associated metastasis in NSCLC. Nat Commun 8, 15870.

Casbas-Hernandez P, D'Arcy M, Roman-Perez E, Brauer HA, McNaughton K, Miller SM, Chhetri RK, Oldenburg AL, Fleming JM, Amos KD et al. (2013) Role of HGF in epithelial-stromal cell interactions during progression from benign breast disease to ductal carcinoma in situ. Breast Cancer Res 15, R82.

Comoglio PM and Trusolino L (2002) Invasive growth: from development to metastasis. J Clin Investig 109, 857–862.

Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, Speed D, Lynch AG, Samarajiva S, Yuan Y et al. (2012) The genomic and transcriptomic architecture of 2,000 breast tumours reveals novel subgroups. Nature 486, 346–352.

Dai Y, Zhang WR, Wang YM, Liu XF, Li X, Ding XB and Guo H (2016) MicroRNA-128 regulates the proliferation and differentiation of bovine skeletal muscle satellite cells by repressing Sp1. Mol Cell Biochem 414, 37–46.

Darnell JE Jr (2002) Transcription factors as targets for cancer therapy. Nat Rev Cancer 2, 740–749.

Di Cosimo S and Basela J (2010) Management of breast cancer with targeted agents: importance of heterogeneity. [corrected]. Nat Rev Clin Oncol 7, 139–147.

Dunning AM, Ellis PD, McBride S, Kirschenslohn HL, Healey CS, Kemp PR, Luben RN, Chang-Claude J, Mannermaa A, Kataja V et al. (2003) A transforming growth factor beta1 signal peptide variant increases secretion in vitro and is associated with increased incidence of invasive breast cancer. Cancer Res 63, 2610–2615.

Dvinge H, Git A, Graf S, Salmon-Divon M, Curtis C, Sottoriva A, Zhao Y, Hirst M, Armisen J, Miska EA et al. (2013) The shaping and functional consequences of the microRNA landscape in breast cancer. Nature 497, 378–382.

Evangelisti C, Florian MC, Massimi I, Dominici C, Giannini G, Galardi S, Bae MC, Massalini S, McDowell HP, Messi E et al. (2009) MiR-128 up-regulation inhibits Reelin and DCX expression and reduces neuroblastoma cell motility and invasiveness. FASEB J 23, 4276–4287.

Furlan A, Vercamer C, Desbiens X and Pourtier A (2008) Ets-1 triggers and orchestrates the malignant phenotype of mammary cancer cells within their matrix environment. J Cell Physiol 215, 782–793.

Gambarotta G, Boccaccio C, Giordano S, Ando M, Stella MC and Comoglio PM (1996) Ets up-regulates MET transcription. Oncogene 13, 1911–1917.

Ganapathy V, Ge R, Grazioli A, Xie W, Banach-Petrosky W, Kang Y, Lonning S, McPherson J, Yingling JM, Biswas S et al. (2010) Targeting the Transforming Growth Factor-beta pathway inhibits human basal-like breast cancer metastasis. Mol Cancer 9, 122.

Garcia S, Dales JP, Jacquemier J, Charafe-Jauffret E, Birnbaum D, Andrac-Meyer L, Lavaut MN, Allasia C, Carpenter-Meunier S, Bonnier P et al. (2007) c-Met overexpression in inflammatory breast carcinomas: automated quantification on tissue microarrays. Br J Cancer 96, 329–335.

Gherardi E, Birchmeier W, Birchmeier C and Vande Woude G (2012) Targeting MET in cancer: rationale and progress. Nat Rev Cancer 12, 89–103.

Goldhirsh A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thurlimann B, Senn HJ, Panel M. (2013) Personalizing the treatment of women with early breast cancer: highlights of the St Gallen International Expert Consensus on the Primary Therapy of Early Breast Cancer 2013. Ann Oncol 24, 2206–2223.

Graveel CR, DeGroot JD, Su Y, Koeman J, Dykema K, Leung S, Snider J, Davies SR, Swiatek PJ, Cottingham S et al. (2009) Met induces diverse mammary carcinomas in mice and is associated with human basal breast cancer. Proc Natl Acad Sci USA 106, 12909–12914.

Ho-Yen CM, Jones JL and Kermorgant S (2015) The clinical and functional significance of c-Met in breast cancer: a review. Breast Cancer Res 17, 52.

Hu J, Cheng Y, Li Y, Jin Z, Pan Y, Liu G, Fu S, Zhang Y, Feng K and Feng Y (2014) microRNA-128 plays a
critical role in human non-small cell lung cancer tumourigenesis, angiogenesis and lymphangiogenesis by directly targeting vascular endothelial growth factor-C. 

Imamura T, Hikita A and Inoue Y (2012) The roles of TGF-beta signaling in carcinogenesis and breast cancer metastasis. Breast Cancer 19, 118–124.

Jemal A, Bray F, Center MM, Ferlay J, Ward E and Forman D (2011) Global cancer statistics. CA Cancer J Clin 61, 69–90.

Jiang J, Feng X, Zhou W, Wu Y and Yang Y (2016) MiR-128 reverses the gefitinib resistance of the lung cancer stem cells by inhibiting the c-met/PI3K/AKT pathway. Oncotarget 7, 73188–73199.

Kao J, Salari K, Bocanegra M, Choi YL, Girard L, Gandhi J, Kwei KA, Hernandez-Boussard T, Wang P, Gasdar AF et al. (2009) Molecular profiling of breast cancer cell lines defines relevant tumor models and provides a resource for cancer gene discovery. PLoS One 4, e6146.

Keklikoglou I, Koerner C, Schmidt C, Zhang JD, Heckmann D, Shavinskaya A, Allgayer H, Guckel B, Fehm T, Schneeweiss A et al. (2012) MicroRNA-520/373 family functions as a tumor suppressor in estrogen receptor negative breast cancer by targeting NF-kappaB and TGF-beta signaling pathways. Oncogene 31, 4150–4160.

Kojima Y, Acar A, Eaton EN, Mellody KT, Scheel C, Ben-Porath I, Onder TT, Wang ZC, Richardson AL, Weinberg RA et al. (2010) Autocrine TGF-beta and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. Proc Natl Acad Sci USA 107, 20009–20014.

Korkaya H, Liu S and Wicha MS (2011) Breast cancer stem cells, cytokine networks, and the tumor microenvironment. J Clin Investig 121, 3804–3809.

Kubic JD, Little EC, Lui JW, Iizuka T and Lang D (2015) PAX3 and ETS1 synergistically activate MET regulation of tumor progression and metastasis. Proc Natl Acad Sci USA 106, 12903–12908.

Korkaya H, Liu S and Wicha MS (2011) Breast cancer stem cells, cytokine networks, and the tumor microenvironment. J Clin Investig 121, 3804–3809.

Lindemann RK, Ballischmietner P, Nordheim A and Dittmer J (2001) Transforming growth factor beta regulates parathyroid hormone-related protein expression in MDA-MB-231 breast cancer cells through a novel Smad/Ets synergism. J Biol Chem 276, 46661–46670.

Loebke C, Sueltmann H, Schmidt C, Henjes F, Wiemann S, Poustka A and Korf U (2007) Infrared-based protein detection arrays for quantitative proteomics. Proteomics 7, 558–564.

Migliore C, Petrelli A, Ghiso E, Corso S, Capparuccia L, Eramo A, Comoglio PM and Giordano S (2008) MicroRNAs impair MET-mediated invasive growth. Cancer Res 68, 10128–10136.

von Minckwitz G, Untich M, Blohmer JU, Costa SD, Eidmann H, Fasching PA, Gerber B, Eiermann W, Hilfrich J, Huober J et al. (2012) Definition and impact of pathologic complete response on prognosis after neoadjuvant chemotherapy in various intrinsic breast cancer subtypes. J Clin Oncol 30, 1796–1804.

Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Terri T, Clark L, Bayani N, Coppe JP, Tong F et al. (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell 10, 515–527.

Palumbo T, Faurez F, Azevedo M, Xekouki P, Iliopoulos D and Stratakis CA (2013) Functional screen analysis reveals miR-26b and miR-128 as central regulators of pituitary somatomammotrophic tumor growth through activation of the PTEN-AKT pathway. Oncogene 32, 1651–1659.

Papa E, Weller M, Weiss T, Ventura E, Burghardt I and Szabo E (2017) Negative control of the HGF/c-MET pathway by TGF-beta: a new look at the regulation of stemness in glioblastoma. Cell Death Dis 8, 3210.

Ponzo MG, Lesurf R, Petkiewicz S, O’Malley FP, Pinnaduwage D, Andrulis IL, Bull SB, Chughtai N, Zuo D, Souleimanova M et al. (2009) Met induces mammary tumors with diverse histologies and is associated with poor outcome and human basal breast cancer. Proc Natl Acad Sci USA 106, 12903–12908.

Ponzo MG and Park M (2010) The Met receptor tyrosine kinase and basal breast cancer. Cell Cycle 9, 1043–1050.

Qian P, Banerjee A, Wu ZS, Zhang X, Wang H, Pandey V, Zhang WJ, Lv XF, Tan S, Lobie PE et al. (2012) Loss of SNAIL regulated miR-128-2 on chromosome 3p22.3 targets multiple stem cell factors to promote transformation of mammary epithelial cells. Cancer Res 72, 6036–6050.

Quail DF and Joyce JA (2013) Microenvironmental regulation of tumor progression and metastasis. Nat Med 19, 1423–1437.

Raghav KP, Wang W, Liu S, Chavez-MacGregor M, Meng X, Hertobagyi GN, Mills GB, Meric-Bernstam F, Blumenschein GR Jr and Gonzalez-Angulo AM (2012) cMET and phospho-cMET protein levels in breast cancers and survival outcomes. Clin Cancer Res 18, 2269–2277.

Riaz M, van Jaarsveld MT, Hollestelle A, Prager-van der Smissen WJ, Heine AA, Boersma AW, Liu J, Helmi J, Ozturk B, Smid M et al. (2013) miRNA expression profiling of 51 human breast cancer cell lines reveals...
subtype and driver mutation-specific miRNAs. Breast Cancer Res 15, R33.

Siegel RL, Miller KD and Jemal A (2017) Cancer statistics, 2017. CA Cancer J Clin 67, 7–30.

Span PN, Manders P, Heuvel JJ, Thomas CM, Bosch RR, Beex LV and Sweep CG (2002) Expression of the transcription factor Ets-1 is an independent prognostic marker for relapse-free survival in breast cancer. Oncogene 21, 8506–8509.

Tang J, Ahmad A and Sarkar FH (2012) The role of microRNAs in breast cancer migration, invasion and metastasis. Int J Mol Sci 13, 13414–13437.

Weigelt B, Peterse JL and van ’t Veer LJ (2005) Breast cancer metastasis: markers and models. Nat Rev Cancer 5, 591–602.

Willis S, De P, Dey N, Long B, Young B, Sparano JA, Wang V, Davidson NE and Leyland-Jones BR (2015) Enriched transcription factor signatures in triple negative breast cancer indicates possible targeted therapies with existing drugs. Meta gene 4, 129–141.

Xu K, Usary J, Kousis PC, Prat A, Wang DY, Adams JR, Wang W, Loch AJ, Deng T, Zhao W et al. (2012) Lunatic fringe deficiency cooperates with the Met/ Caveolin gene amplicon to induce basal-like breast cancer. Cancer Cell 21, 626–641.

Yu Y, Xiao CH, Tan LD, Wang QS, Li XQ and Feng YM (2014) Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-beta signalling. Br J Cancer 110, 724–732.

Zhang X, Yang J, Li Y and Liu Y (2005) Both Sp1 and Smad participate in mediating TGF-beta1-induced HGF receptor expression in renal epithelial cells. Am J Physiol Renal Physiol 288, F16–F26.

Zhao X, Qu J, Hui Y, Zhang H, Sun Y, Liu X, Zhao X, Zhao Z, Yang Q, Wang F et al. (2017) Clinicopathological and prognostic significance of c-Met overexpression in breast cancer. Oncotarget 8, 56758–56767.

Zhu Y, Yu F, Jiao Y, Feng J, Tang W, Yao H, Gong C, Chen J, Su F, Zhang Y et al. (2011) Reduced miR-128 in breast tumor-initiating cells induces chemotherapeutic resistance via Bmi-1 and ABCC5. Clin Cancer Res 17, 7105–7115.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Specificity of MET and TGFBR2 antibodies.

Fig. S2. TGFBR2 is higher expressed in estrogen receptor negative breast cancer cell lines.

Fig. S3. MET correlates with TGFBR2 expression.

Fig. S4. MET is higher expressed in estrogen receptor negative breast cancer cell lines.

Fig. S5. TGFβ1 elevates expression of MET in HCC1143 and HS578T cell lines.

Fig. S6. HGF does not induce TGFBR2 expression.

Fig. S7. Cell migration is regulated by MET and TGFβ1.

Fig. S8. ETS-1 correlates with TGFBR2 and MET expression.

Fig. S9. Interference of MET by C-ets-1.

Fig. S10. miR-128-3p is lower expressed in basal-like breast cancer.

Fig. S11. Regulation of miR-128-3p and MET expression by TGFβ1 and miR-128-3p.

Table S1. Sequences of siRNAs targeting MET, TGFBR2, ETS1 as well as non-targeting siRNA control.

Table S2. Sequences and Universal Probe Library (UPL) probe numbers for genes quantified by TaqMan qRT-PCR.

Table S3. Correlation analysis of TGFBR2 in a data set (Riaz et al.) containing mRNA expression of 51 breast cancer cells. Shown are the top 33 correlated genes.

Table S4. Transcription factors correlated with (A) TGFBR2 and (B) MET having putative binding site in the MET promoter.

Table S5. Correlation analysis of MET in a data set (Riaz et al.) containing mRNA expression of 51 breast cancer cells. Shown are the top 33 correlated genes.