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ORIGINAL ARTICLE

Fibulin-3 is a novel TGF-β pathway inhibitor in the breast cancer microenvironment

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Transforming growth factor-β (TGF-β) is an important regulator of breast cancer progression. However, how the breast cancer microenvironment regulates TGF-β signaling during breast cancer progression remains largely unknown. Here, we identified fibulin-3 as a secreted protein in the breast cancer microenvironment, which efficiently inhibits TGF-β signaling in both breast cancer cells and endothelial cells. Mechanistically, fibulin-3 interacts with the type I TGF-β receptor (TβRI) to block TGF-β induced complex formation of TβRI with the type II TGF-β receptor (TβRII) and subsequent downstream TGF-β signaling. Fibulin-3 expression decreases during breast cancer progression, with low fibulin-3 levels correlating with a poorer prognosis. Functionally, high fibulin-3 levels inhibited TGF-β-induced epithelial–mesenchymal transition (EMT), migration, invasion and endothelial permeability, while loss of fibulin-3 expression/function promoted these TGF-β-mediated effects. Further, restoring fibulin-3 expression in breast cancer cells inhibited TGF-β signaling, breast cancer cell EMT, invasion and metastasis *in vivo*. These studies provide a novel mechanism for how TGF-β signaling is regulated by the tumor microenvironment, and provide insight into targeting the TGF-β signaling pathway in human breast cancer patients.

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INTRODUCTION

Breast cancer is the most common cancer among American women, with 12% of American women developing invasive breast cancer during their lifetime. Worldwide, breast cancer accounts for 22.9% of invasive cancers in women, with 425 000 women dying of breast cancer each year, underscoring the need to define mechanisms of breast cancer initiation and progression.

Cytokines and growth factors, including the transforming growth factor-β (TGF-β) superfamily, have important roles in breast cancer initiation and progression.[1–4] TGF-β is a prototypic family member of 33 secreted structurally related cytokines, including TGF-β, activin, nodal and bone morphogenetic proteins (BMPs). TGF-β signaling has dual roles during breast cancer progression.[5–7] In normal breast tissue and during the early stages of breast cancer initiation, the TGF-β signaling pathway functions as a tumor suppressor via suppressing proliferation, while promoting differentiation and apoptosis. However, in the later stages of breast cancer progression, the TGF-β signaling pathway acts as a tumor promoter, driving cell migration, invasion and metastasis, at least in part, via inducing epithelial–mesenchymal transition (EMT). The dual roles of the TGF-β signaling pathway during breast cancer progression suggest tight control and regulation of this pathway during breast cancer progression.

The canonical TGF-β superfamily signaling pathway is triggered when TGF-β superfamily ligands bind to cell surface receptors, including co-receptors, type II and type I receptors. Upon ligand binding, these receptors form complexes, which facilitate the transphosphorylation and activation of TβRI by TβRII; the TβRI then phosphorylates receptor-regulated Smads (R-Smads), which bind the co-Smad, Smad4, and accumulate in the nucleus where they act in concert with co-activators and co-repressors to regulate target gene expression.[8–14] Although mechanisms by which TGF-β signaling is regulated by the cellular machinery are widely and extensively studied,[5,6,15–18] how the breast cancer microenvironment, which has important roles in mediating breast cancer progression,[19–21] regulates TGF-β signaling during breast cancer progression remains largely unknown.

The breast and breast cancer microenvironment consists of extracellular matrix, stromal cells (for example, endothelial cells, fibroblasts, myofibroblasts and leukocytes), and cytokines, chemokine and proteins secreted by breast epithelial, breast cancer or stromal cells.[22] Both cell–cell and cell–microenvironment interactions modify the proliferation, survival, polarity, differentiation and invasive capacity of mammary epithelial cells. Most of these effects are mediated by these secreted proteins, including TGF-β superfamily cytokines.[23–25] In addition to cytokines, secreted proteins[21,26,27] and exosomes[28–30] can cooperate or regulate these cytokines to control breast cancer progression. Indeed, we have previously established that decreases in shed soluble type III TGF-β receptor (sTβRIII) increase TGF-β signaling in the tumor microenvironment to generate an immunotolerant tumor microenvironment in breast cancer,[26] and promote breast cancer metastasis.[27] In addition, the secreted protein Coco was recently demonstrated to inhibit BMP signaling to reactivate dormant breast cancer lung micrometastasis.[31] However, how secreted proteins in the breast cancer microenvironment regulate breast cancer progression remains poorly understood.

The fibulins are a family of secreted proteins that associate with the extracellular matrix scaffold, forming anchoring structures that

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can regulate cell proliferation and migration.[32–34] Seven fibulin family members have been identified, with family members defined by the presence of two structural modules, a tandem repeat of epidermal growth factor-like modules and a unique C-terminal fibulin-type module. The FBLN-3 gene (also called EFEMP1) encodes fibulin-3, and is a relatively recent addition to the family. Fibulin-3 has been reported to be downregulated in several solid tumors,[35] including breast cancer, via aberrant promoter methylation,[36–38] with loss of fibulin-3 expression associated with tumor progression, metastasis and a poor patient prognosis. Although fibulin-3 has been reported to regulate some signaling pathways including the MAPK,[39] Akt[39,40] Notch[41] and Wnt[42] signaling pathways, the role of fibulin-3 in the TGF-β signaling pathway has not been investigated. Here, we utilize a proteomic approach to investigate the role of secreted proteins in regulating TGF-β signaling in the breast cancer tumor microenvironment.

RESULTS

Conditioned media from breast cancer cells inhibits TGF-β signaling

To investigate whether there were components in the breast cancer microenvironment that regulate TGF-β signaling, we examined the effects of conditioned media from the estrogen receptor-negative human breast cancer cell line, MDA-MD-231 cells, on TGF-β and BMP2 signaling. Pretreating MDA-MB-231 cells with conditioned media dramatically inhibited TGF-β induced Smad2 phosphorylation and AKT phosphorylation (Figure 1a), while only slightly inhibiting BMP-2 induced Smad1/5/8 phosphorylation (Figure 1b). As stromal cells, including endothelial and immune cells, interact and regulate breast cancer cells, we next asked whether the breast cancer conditioned media also regulates TGF-β signaling in endothelial cells. Pretreating human microvascular endothelial cells (HMEC-1) with MDA-MB-231 conditioned media inhibited TGF-β induced Smad1/5/8 phosphorylation, increased basal Smad2 phosphorylation, inhibited TGF-β induced Smad2 phosphorylation (Figure 1c) and TGF-β induced transcription of its downstream genes, including Smad7 and plasminogen activator inhibitor-1 (PAI-1) (Figure 1d). However, MDA-MB-231 conditioned media had no effect on BMP-9 signaling in endothelial cells (Figures 1c and d). In addition, the conditioned media from the estrogen receptor-positive human breast cancer line, MCF-7, also inhibited TGF-β induced Smad1/5/8 phosphorylation in a dose-dependent manner in HMEC-1 cells (Supplementary Figure 1A). However, only high doses of conditioned media increased basal Smad2 phosphorylation, while

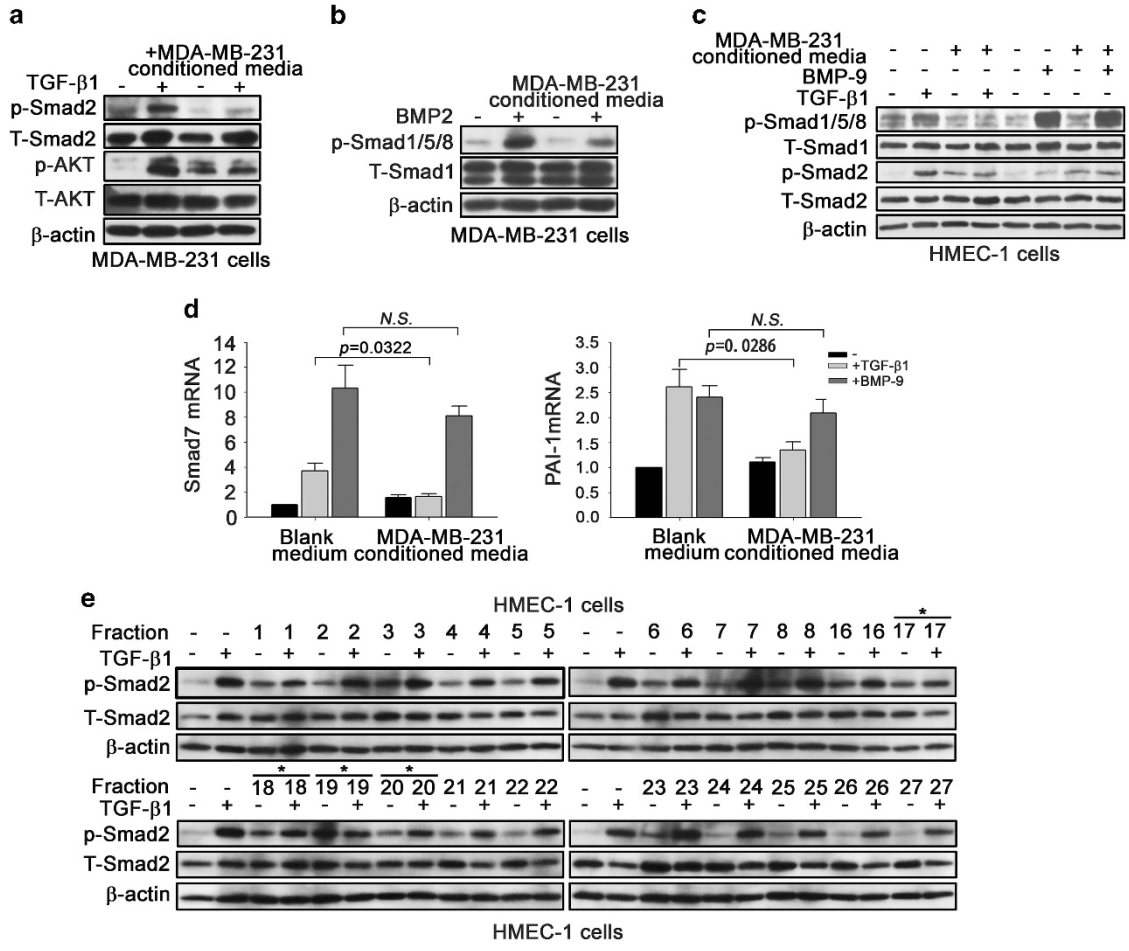


Figure 1. Conditioned media from breast cancer cells inhibits TGF-β signaling. (**a**, **b**) MDA-MB-231 cells were pretreated with conditioned media from MDA-MB-231 cells for 6 h, and then treated with 50 pM TGF-β1 (**a**) or 10 nM BMP2 (**b**) for 30 min, and the cell lysates analyzed with the indicated antibodies. (**c**) HMEC-1 cells were pretreated with the conditioned media from MDA-MB-231 cells for 6 h, and then treated with 50 pM TGF-β1 or 2 ng/ ml BMP9 for 30 min, and the cell lysates analyzed with the indicated antibodies. (**d**) HMEC-1 cells were cultured in blank medium or in conditioned media from MDA-MB-231 cells for 3 h, treated with 50 pM TGF-β1 or 2 ng/ml BMP9 for 4 h, Smad7 and PAI-1 mRNA were measured using qRT-PCR. Quantitated data from three experiments ± s.e.m. were indicated. (**e**) HMEC-1 cells were pretreated with the indicated individual FPLC fractions for 6 h, and then treated with 50 pM TGF-β1 for 30 min, and the cell lysates analyzed with the indicated antibodies. Results are representative of three independent experiments.

inhibiting TGF-β induced Smad2 phosphorylation in HMEC-1 cells (Supplementary Figure 1A), and having no effect on BMP-9 signaling (Supplementary Figure 1B). These results suggest that there are active components in the conditioned media from breast cancer cells that inhibit TGF-β signaling, without effecting BMP-9 signaling.

As the overall effect of conditioned media from breast cancer cells integrates the effects of secreted TGF-β ligands, TGF-β activators and repressors, to identify the active components responsible for inhibiting TGF-β signaling, the conditioned media from MDA-MB-231 cells was fractionated using FPLC gel filtration (Supplementary Figure 1C) and the fractions screened for inhibitory activity. The eluted fractions were combined into groups based on their elution peaks (Supplementary Figure 1C) and used to pretreat HMEC-1 cells. Compared with the other combined fractions, fractions 17–26 inhibited TGF-β induced Smad1/5/8 and Smad2 phosphorylation (Supplementary Figure 1D). Further, individual fractions 17, 18, 19 and 20 inhibited TGF-β induced Smad2 phosphorylation (Figure 1e), suggesting these fractions contained the active components responsible for mediating inhibition of TGF-β signaling. The proteins in fractions, and their adjacent fractions, were resolved by SDS-PAGE and detected by silver nitrate staining (Supplementary Figure 1E). The two bands that were enriched in fractions 17–20 relative to adjacent fractions were cut out and subjected to mass spectrometry analysis, resulting in the identification of 15 candidate proteins (Supplementary Table 1).

Fibulin-3 is the active component that inhibits TGF-β signaling

Three of the identified candidates proteins were secreted proteins (Supplementary Table 1), including epidermal growth factor-containing fibulin-like extracellular matrix protein 1 (fibulin-3, FBLN3), serum albumin and transforming growth factor-beta-induced protein ig-h3 (βIGH3). As exogenous serum albumin did not regulate TGF-β induced Smad2 phosphorylation in MDA-MB-231 cells (Supplementary Figure 2A), and decreasing βIGH3 levels in MDA-MB-231 conditioned media by either immunodepletion (Supplementary Figure 2B) or stable knockdown (Supplementary Figures 2C and D) further decreased TGF-β induced Smad1/2/5/8 phosphorylation in HMEC-1 cells (Supplementary Figures 2B and E), we turned our attention to fibulin-3, which has been demonstrated to regulate other signaling pathways.[39–41] As MDA-MB-231 cells express and secrete high levels of fibulin-3 (Figure 4h, Supplementary Figure 2H), we used shRNA to stably silence fibulin-3 expression and secretion (Supplementary Figures 2F–H) in MDA-MB-231 cells (shFBLN3-MDA-MB-231), with a non-targeted shRNA control (shNTC-MDA-MB-231). In the presence of blank medium, TGF-β induced Smad2 phosphorylation in both shNTC-MDA-MB-231 and shFBLN3-MDA-MB-231 cells (Figure 2a). However, consistent with our prior results, conditioned media from shNTC-MDA-MB-231 cells inhibited TGF-β induced Smad2 phosphorylation (Figure 2a, Supplementary Figure 2L) and TGF-β-induced transcription of its downstream genes, Smad7 (Figure 2b) and PAI-1 (Figure 2c), while conditioned media from shFBLN3-MDA-MD-231 cells failed to inhibit TGF-β induced Smad2 phosphorylation (Figure 2a) or TGF-β induced Smad7 (Figure 2b) or PAI-1 (Figure 2c) transcription. Similarly, conditioned media from shFBLN3-MDA-MD-231 cells did not inhibit TGF-β induced Smad1/5/8 and Smad2 phosphorylation in HMEC-1 cells (Figure 2d) or in colon fibroblast 18Co cells (Supplementary Figure 2I). Further, MDA-MB-231 conditioned media immunodepleted with two different anti-fibulin-3 antibodies did not inhibit TGF-β induced Smad1/2/5/8 phosphorylation (Figure 2e, Supplementary Figure 2J). In a reciprocal manner, the highly metastatic MDA-MB-231-4175 subline expressed and secreted low levels of fibulin-3 (Figure 4h, Supplementary Figure 2H), and as expected, its conditioned media did not inhibit TGF-β induced Smad phosphorylation or Smad7 and PAI-1 transcription in MDA-MA-MB-231-4175 (Figures 2f–h, Supplementary Figure 2L) or HMEC-1 cells (Figure 2i). However, the conditioned media from MDA-MB-231-4175 cells stably overexpressing fibulin-3 (Supplementary Figures 2H and K) inhibited TGF-β signaling (Figures 2f and i) and TGF-β induced genes transcription (Figures 2g and h). Moreover, exogenous fibulin-3 inhibited TGF-β induced Smad1/2/5/8 phosphorylation and Smad7 and PAI-1 transcription in HMEC-1 cells (Figure 2j) and in MDA-MB-231 cells (Figures 3a, 2k and l). In addition, pretreating MDA-MB-231 cells with recombinant fibulin-3 also inhibited TGF-β induced p38 phosphorylation (Supplementary Figure 2M). Consistent with the previous report,[39] fibulin-3 increased basal AKT and ERK1/2 phosphorylation, while inhibiting TGF-β induced AKT and ERK1/2 phosphorylation (Supplementary Figure 2M). As fibulin-3 inhibits lung cancer metastasis via suppressing the ERK-Wnt/β-catenin signaling axis,[42] we test whether fibulin-3 inhibits this pathway in breast cancer cells. Knockdown of fibulin-3 had no effect on β-catenin nuclear translocation (Supplementary Figure 2N) and ERK phosphorylation (Supplementary Figure 2O). Taken together, these data indicate that fibulin-3 is the active component in the breast cancer secretome that inhibits both canonical and non-canonical TGF-β signaling in breast cancer epithelial cells, endothelial cells and fibroblasts.

Fibulin-3 inhibits TGF-β signaling via interaction with TβRI to decrease TβRI/TβRII complex formation

Having defined fibulin-3 as an inhibitor of TGF-β signaling, we turned our attention to define the mechanism by which fibulin-3 inhibits TGF-β signaling. Fibulin-3-mediated inhibition of TGF-β signaling did not require protein synthesis or degradation, as pretreating cells with the protein synthesis inhibitor, cycloheximide, the proteasome inhibitor, MG132, or the lysosomal cysteine protease inhibitor, leupeptin, did not diminish the effect of conditioned media in inhibiting TGF-β signaling (Supplementary Figures 3A and B). We next assessed whether fibulin-3 inhibits TGF-β signaling by binding and sequestering TGF-β ligand. Compared with soluble TβRIII, which, as expected, efficiently bound TGF-β,[27] fibulin-3 did not directly bind TGF-β1 (Supplementary Figure 3C).

To investigate whether fibulin-3 inhibited TGF-β induced Smad2 phosphorylation upstream or downstream of TβRI, we investigated the effects of exogeneous fibulin-3 on ligand versus constitutively activated TβRI induced Smad2 phosphorylation. While exogeneous fibulin-3 inhibited TGF-β ligand induced Smad2 phosphorylation, it did not inhibit constitutively activated TβRI mediated phosphorylation of Smad2 (Figure 3a), suggesting that fibulin-3 inhibits TGF-β signaling upstream of TβRI activation. As fibulin-3 binds with receptors from other families, including the epidermal growth factor receptor,[39] we tested whether fibulin-3 could form complexes with TGF-β receptors, including TβRI and TβRII, to potentially inhibit TβRI activation. TβRI, and to a lesser extent for TβRII, co-immunoprecipitated with fibulin-3 in COS7 cells co-expressing fibulin-3 and TβRI or TβRII (Figure 3b). In a reciprocal manner, fibulin-3 also co-immunoprecipitated with TβRI but not with TβRII (Figure 3c). Further, purified recombinant fibulin-3 co-immunoprecipitated with TβRI but not with TβRII (Figure 3d). As fibulin-3 is a secreted protein, we expressed and purified cellular HA-TβRI and HA-TβRII from COS7 cells using anti-HA beads, and tested whether TβRI or TβRII formed a complex with fibulin-3 in the conditioned media. HA-TβRI, but not TβRII formed a higher molecular weight complex with fibulin-3 (Supplementary Figure 3D), suggesting that fibulin-3 may inhibit TGF-β signaling through interaction with TβRI.

We then investigated whether the interaction between fibulin-3 and TβRI blocked TGF-β induced TβRI/TβRII complex formation, which is an essential step for downstream TGF-β signaling.[11,43,44]

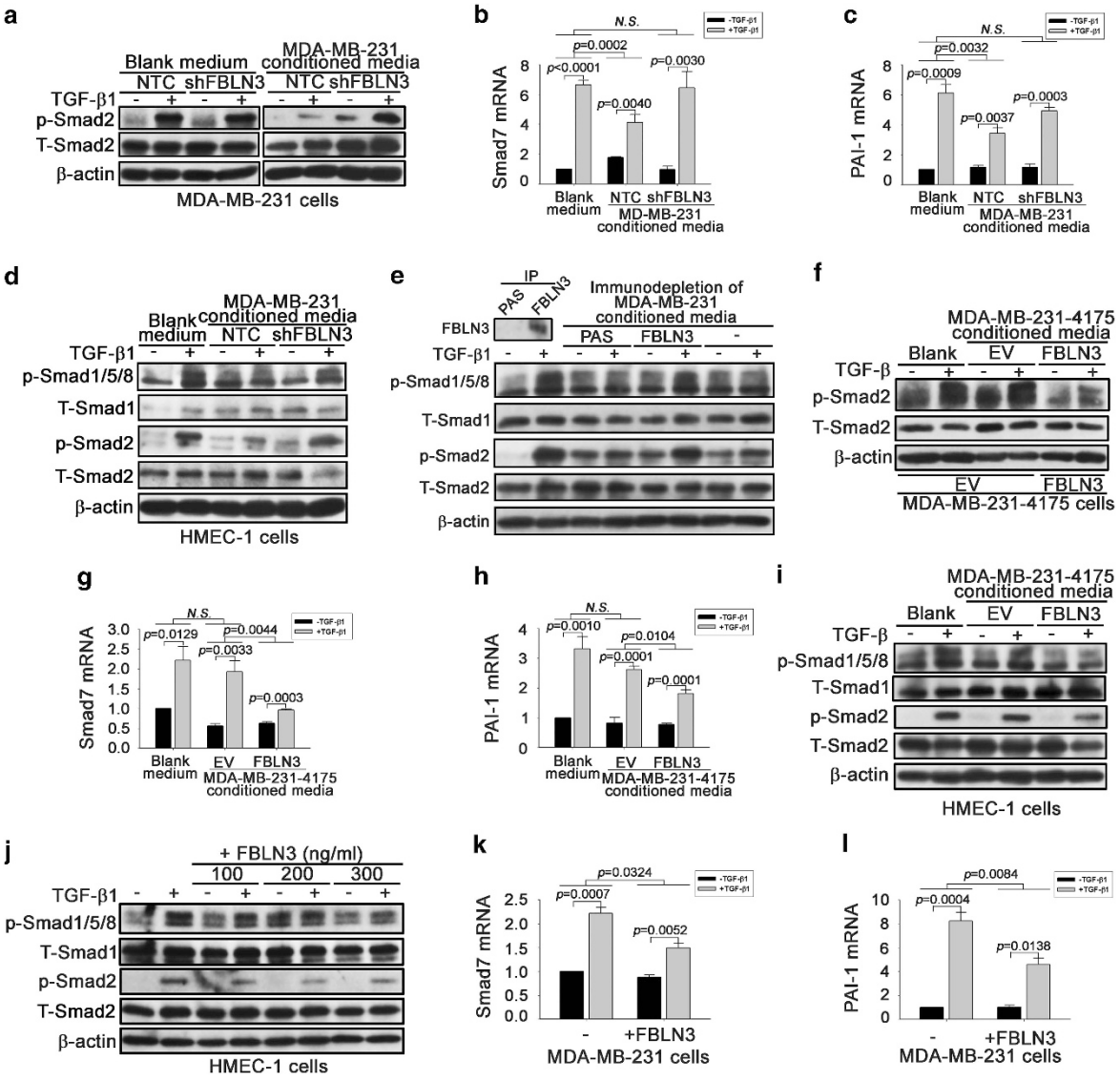


Figure 2. Fibulin-3 in conditioned media inhibits TGF-β signaling. (**a**) MDA-MB-231 cells were cultured in blank medium or in conditioned media from shNTC- or shFBLN3-MDA-MB-231 cells for 6 h, treated with 50 pM TGF-β1 for 30 min, and the cell lysates analyzed with the indicated antibodies. (**b**, **c**) MDA-MB-231 cells were cultured in blank medium or in conditioned media from shNTC- or shFBLN3-MDA-MB-231 cells for 3 h, treated with 20 pM TGF-β1 for 4 h, Smad7 (**b**) and PAI-1 (**c**) mRNA were measured using qRT-PCR. (**d**) HMEC-1 cells were cultured in blank medium or in conditioned media from shNTC- or shFBLN3-MDA-MB-231 cells for 6 h, and then treated with 50 pM TGF-β1 for 30 min, and the cell lysates analyzed with the indicated antibodies. (**e**) HMEC-1 cells were pretreated with the MDA-MB-231 conditioned media, which were immunodepleted with empty Protein A Sepharose (PAS) beads or fibulin-3 antibody (from Abcam) for 6 h, and then treated with 50 pM TGF-β1 for 30 min, and the cell lysates analyzed with the indicated antibodies. (**f**) MDA-MB-231-4175 cells or (**i**) HMEC-1 cells were pretreated with the conditioned media from empty vector or fibulin-3 overexpressing MDA-MB-231-4175 cells for 6 h, and then treated with 50 pM TGF-β1 for 30 min, and the cell lysates analyzed with the indicated antibodies. (**g**, **h**) MDA-MB-231-4175 cells were pretreated with the conditioned media from empty vector or fibulin-3 overexpressing MDA-MB-231-4175 cells for 3 h, and then treated with 20 pM TGF-β1 for 4 h, Smad7 (**g**) and PAI-1 (**h**) mRNA were measured using qRT-PCR. (**j**) HMEC-1 cells were pretreated with indicated doses of purified fibulin-3 for 6 h, and then treated with 50 pM TGF-β1 for 30 min, and the cell lysates analyzed with the indicated antibodies. Results are representative of three independent experiments. (**k**, **l**) MDA-MB-231 cells were pretreated with 200 ng/ml purified fibulin-3 for 3 h, and then treated with 20 pM TGF-β1 for 2 h, Smad7 (**k**) and PAI-1 (**l**) mRNA were measured using qRT-PCR.

While conditioned media from control MDA-MB-231 cells (Figure 3e) or recombinant fibulin-3 (Supplementary Figure 3E) inhibited TGF-β-induced TβRI/TβRII complex formation, conditioned media from shFBLN3-MDA-MB-231 cells failed to inhibit TGF-β induced TβRI/TβRII complex formation (Figure 3e). Finally, as the TGF-β signaling pathway is subject to multiple levels of regulation, we assessed the effects of TGF-β on fibulin-3 levels. TGF-β induced fibulin-3 expression in a time-dependent manner in both MDA-MB-231 cells and MDA-MB-231-4175 cells (Figure 3f). Collectively, these data indicate that fibulin-3 inhibits TGF-β signaling in a negative feedback loop through interaction with TβRI to inhibit TβRI/TβRII complex formation (Figure 8a).

Fibulin-3 expression is decreased during breast cancer progression

Having identified fibulin-3 as a novel and key suppressor of TGF-β signaling in the breast cancer microenvironment, we next investigated whether fibulin-3 expression changes during breast

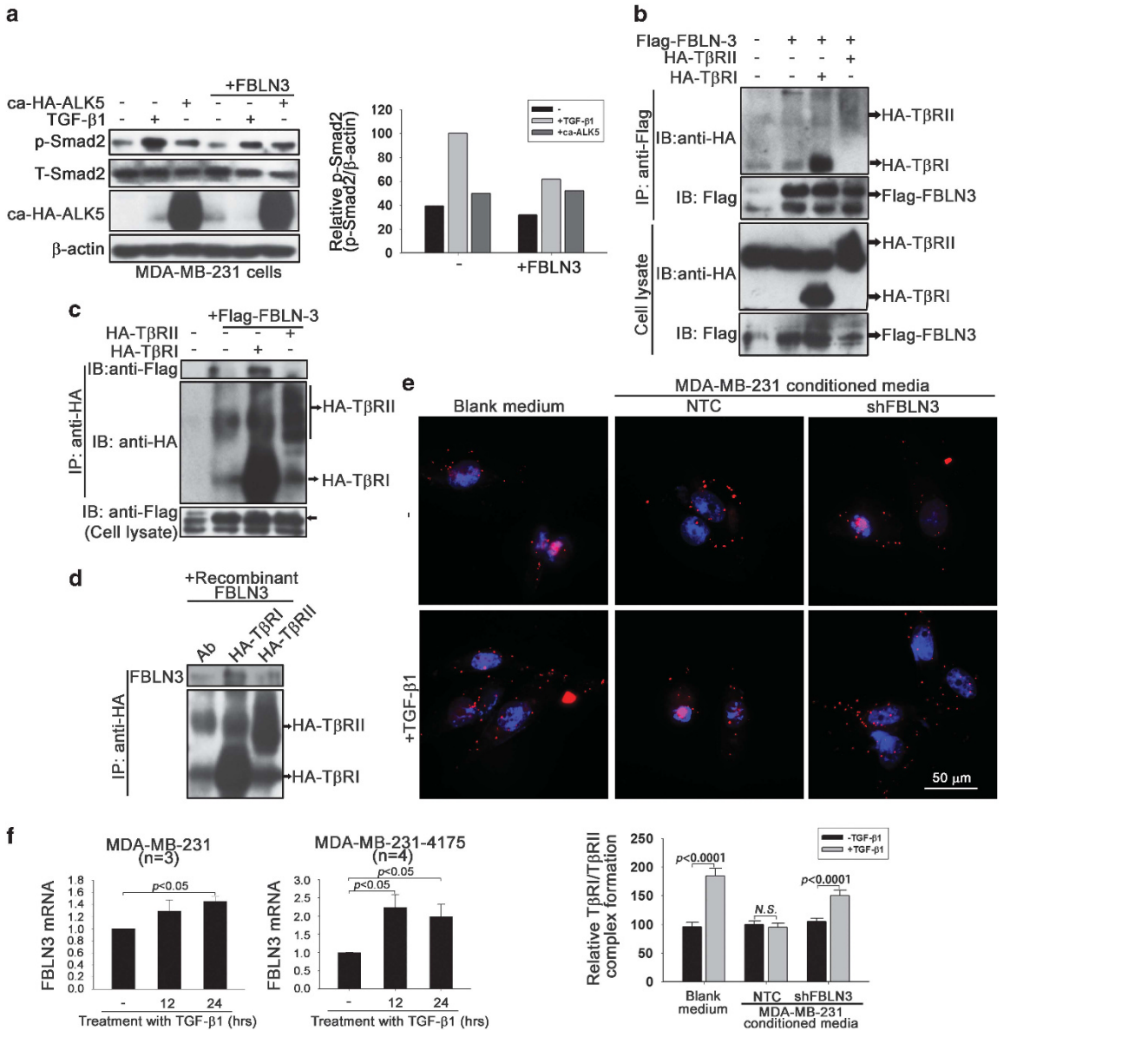


Figure 3. Fibulin-3 inhibits TGF-β signaling through interaction with TβRI. (**a**) MDA-MB-231 cells were either infected with ca-ALK5 adenovirus for 24 h, serum starved and treated with 200 ng/ml exogeneous fibulin-3 for 6 h, or serum starved, pretreated with 200 ng/ml exogeneous fibulin-3 for 6 h, then treated with 50 pM TGF-β1 for 30 min, and the cell lysates analyzed with the indicated antibodies. (**b**, **c**) Anti-Flag (**b**) or anti-HA (**c**) immunoprecipitate (IP) was prepared from COS7 cells expressing Flag-fibulin-3 and HA-TβRI or HA-TβRII. Flag-fibulin-3, HA-TβRI and HA-TβRII were detected in IP and total cell lysates by western blot analysis. (**d**) HA-TβRI or HA-TβRII was immunoprecipitated in HA-TβRI or HA-TβRII overexpressing COS7 cells with anti-HA crosslinked beads, washed, incubate with 100 ng recombinant fibulin-3. Fibulin-3, HA-TβRI or HA-TβRII was detected by western blot analysis. (**e**) shNTC- or shFBLN3-MDA-MB-231 cells were pretreated with their conditioned media for 6 h respectively, and treated with 50 pM TGF-β1 for 10 min. Interaction between TβRI and TβRII was assessed by Duolink assay. Nuclei were stained using DAPI. Quantitated data were from three experiments ± s.e.m. (**f**) MDA-MB-231 cells (left panel) or MDA-MB-231-4175 cells (right panel) were treated with 50 pM TGF-β1 for 12 h or 24 h. mRNA was extracted and converted to cDNA, and quantified using qRT-PCR. Quantitated data were from three or four experiments ± s.e.m.

cancer progression. In a meta-analysis of 32 publically available data sets on Oncomine, fibulin-3 expression was decreased in breast cancer compared with normal breast (Figure 4a). Further, while normal mammary epithelial tissue expressed high levels of fibulin-3, human breast cancer orthotopic xenograft expressed very low levels of fibulin-3 (Figure 4b). In addition, compared with normal breast, fibulin-3 levels were decreased in basal, Her2, luminal A and luminal B subtypes of breast cancers (Supplementary Figure 4A), suggesting that decreased fibulin-3 expression is general feature of breast cancer progression. Moreover, relative to normal breast, fibulin-3 expression decreased beginning in stage I breast cancer, with more pronounced decreases in stage IV breast cancer (Figure 4c), and relative to pre-invasive ductal carcinoma *in situ*, fibulin-3 expression decreased in both invasive ductal carcinoma and invasive lobular carcinoma (Figure 4d). Further, low expression level of fibulin-3 correlated with worse survival in breast cancer patients (http://kmplot.com/analysis/) (Figure 4e). These data support an important role for fibulin-3 in breast cancer progression. Consistent with this hypothesis, in the MCF10A breast cancer progression series,[45] with MCF10A cells being non-tumorigenic, MCF10A-T1k cells forming hyperplastic lesions, MCF10A-CA1h

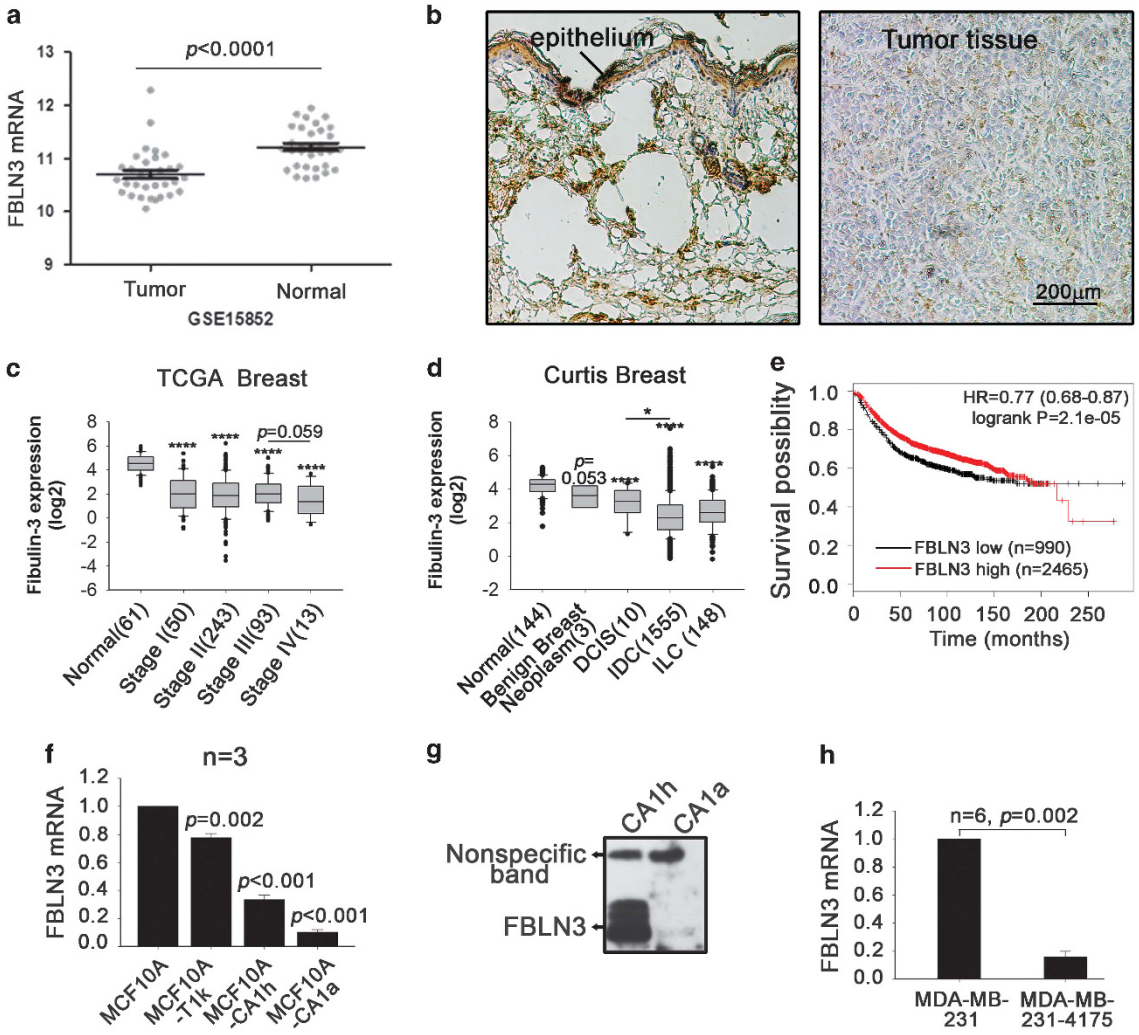


Figure 4. Fibulin-3 expression is decreased during breast cancer progression. (**a**, **c** and **d**) Analysis of publicly available microarray expression data for fibulin-3 mRNA expression in normal breast and breast cancer (**a**), in different types of breast cancer (**c**), and by breast cancer stage (**d**). (**b**) Representative sections of murine mammary epithelium and MDA-MB-231-4175 orthotopic tumor xenograft immunostained for fibulin-3. (**e**) Kaplan–Meier plot of survival based on low (below mean) or high (above mean) fibulin-3 expression. (**f**) Quantitative PCR analysis of fibulin-3 mRNA expression level in MCF10A breast cancer progression series. Results from three independent experiments were averaged and normalized relative to expression in MCF10A cells. (**g**) Conditioned media from MCF10A-CA1h and MCF10A-CA1a cells analyzed for fibulin-3 expression. (**h**) Quantitative PCR analysis of fibulin-3 mRNA expression level in MDA-MB-231 and MDA-MB-231-4175. Results from six independent experiments were averaged and normalized relative to expression in MDA-MB-231 cells.

cells forming low-grade, well-differentiated carcinomas and MCF10A-CA1a cells forming poorly differentiated, metastatic carcinomas, fibulin-3 mRNA levels progressively decreased with cancer progression (Figure 4f). In addition, compared with the non-metastatic cell line, MCF10A-CA1h, fibulin-3 protein levels in the conditioned media were dramatically decreased in metastatic MCF10A-CA1a cells (Figure 4g). Consistent with a prior report,[46] fibulin-3 mRNA levels were also decreased in the metastatic MDA-MB-231-4175 subline compared with the poorly-metastatic parental MDA-MB-231 cells (Figure 4h). In addition, in the isogenic murine mammary carcinoma progression series, composed of four murine mammary carcinoma cell lines (67NR, 168FARN, 4TO7 and 4T1), each with progressive metastatic potential but all derived from same parental breast tumor,[47] fibulin-3 mRNA levels were decreased in metastatic 4T1 cells relative to the other non-metastatic cells (Supplementary Figure 4B). Thus, in all series, fibulin-3 expression was lost as cells acquired metastatic capability.

As the fibulin-3 promoter has CpG islands (Supplementary Figure 4C) and has been reported to be regulated by promoter methylation,[36–38] we investigated the potential for epigenetic regulation in our model systems. Treating 4T1 cells (Supplementary Figure 4D) or MDA-MB-231-4175 cells (Supplementary Figure 4E) with the promoter methylation inhibitor, 5-azacytidine, increased fibulin-3mRNA levels in both cell lines. These data suggest that fibulin-3 expression is decreased via either direct or indirect epigenetic silencing during breast cancer progression.

Decreased fibulin-3 promotes TGF-β induced EMT, migration and invasion *in vitro*

As fibulin-3 inhibits TGF-β signaling in breast cancer cells and fibulin-3 expression decreases during breast cancer progression, especially in invasive breast cancer and at the later stages of breast cancer progression, we next investigated the effects of fibulin-3 on TGF-β-induced migration and invasion. While conditioned media from poorly-metastatic parental MDA-MB-231 cells with high fibulin-3 expression[46] (Figure 4h, Supplementary Figure 2H) inhibited TGF-β induced migration (Figure 5a, Supplementary Figure. 5A) and invasion (Figure 5b), conditioned media from shFBLN3-MDA-MB-231 failed to inhibit TGF-β's effects

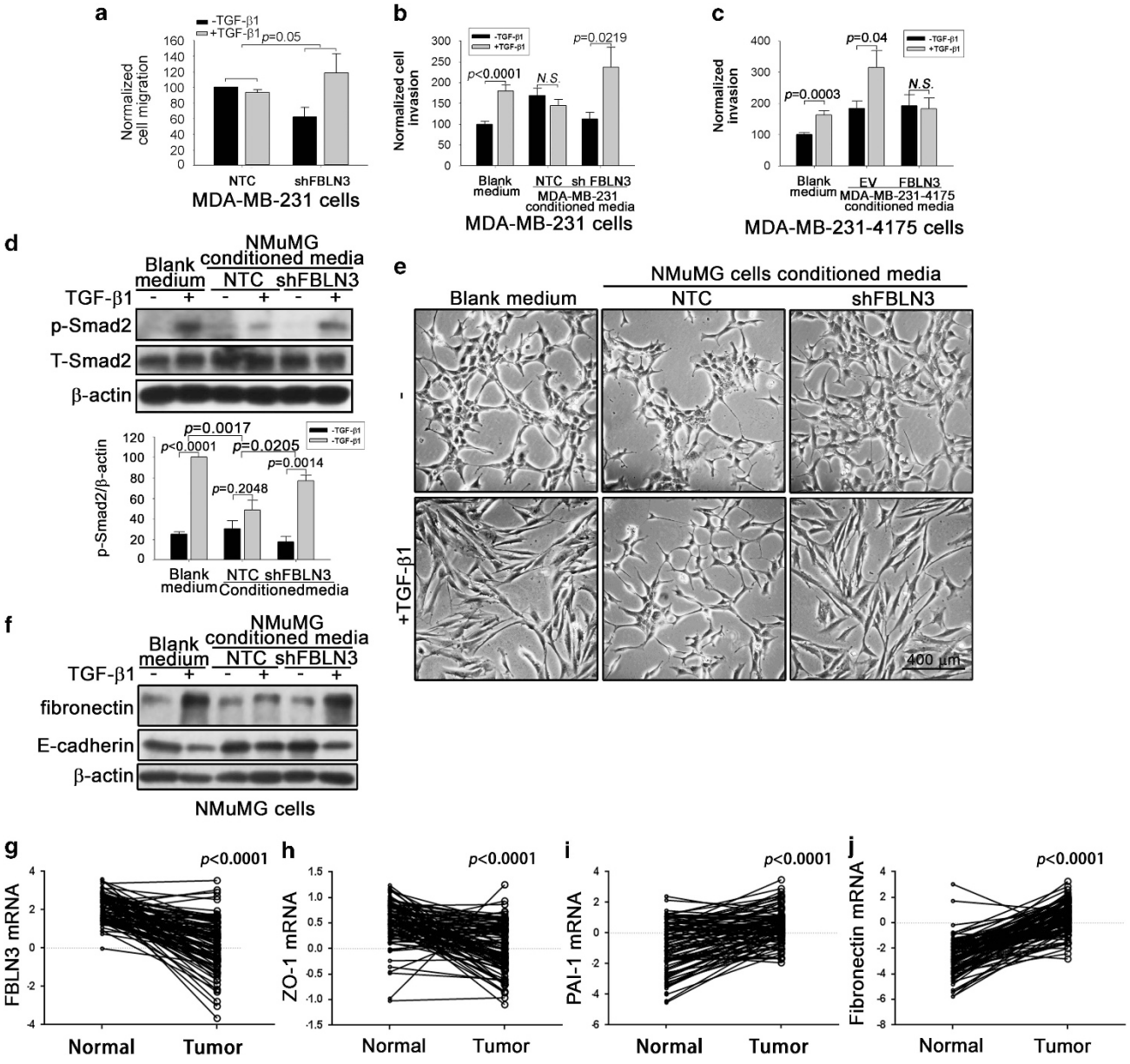


Figure 5. Fibulin-3 inhibits TGF-β induced EMT, migration and invasion. (**a**, **b**) MDA-MB-231 cells incubated with blank medium or with conditioned media from shNTC- and shFBLN3-MDA-MB-231 cells, were plated on non-coated (**a**) or Matrigel-coated (**b**) transwells, treated with or without 50 pM TGF-β1 and assessed for migration/invasion after 12 h. Quantitated data were from three experiments ± s.e.m. (**c**) MDA-MB-231-4175 cells were incubated with blank medium or with conditioned media from empty vector or fibulin-3 stable overexpressing MDA-MB-231-4175 cells, plated on Matrigel-coated transwells, treated with or without 50 pM TF-β1 and assessed for migration/invasion after 12 h. Quantitated data were from three experiments ± s.e.m. (**d**) NMuMG cells were cultured in blank medium or in conditioned media from shNTC- or shFBLN3-NMuMG cells for 6 h, treated with 50 pM TGF-β for 30 min, and the cell lysates analyzed with the indicated antibodies. Quantitated data below were from three experiments ± s.e.m. (**e**, **f**) NMuMG cells were cultured in regular medium or conditioned media from empty vector or fibulin-3 stable overexpressing NMUMG cells, treated with or without 50 pM TGF-β for 24 h, the cell morphology assessed by phase contrast microscopy (**e**), and the cell lysates analyzed with the indicated antibodies (**f**). Fibulin-3 (**g**), ZO-1 (**h**), PAI-1 (**i**) and fibronectin (**j**) mRNA expression in 94 normal-tumor pairs from the TCGA data set.

(Figures 5a and b). Although TGF-β was not able to induce migration in MDA-MB-231-4175 cells (Supplementary Figure 5B), TGF-β induced their invasion (Figure 5c), and conditioned media from MDA-MB-231-4175 cells with low fibulin-3 expression[46] (Figure 4h; Supplementary Figure 2H) was not able to inhibit TGF-β induced invasion (Figure 5c). However, the conditioned media from MDA-MB-231-4175 stably overexpressing fibulin-3 inhibited TGF-β's effects (Figure 5c), supporting that fibulin-3 mediated inhibition of TGF-β signaling is sufficient to block TGF-β functions during breast cancer progression. In addition, consistent with fibulin-3 having no effect on the ERK-Wnt signaling axis in breast cancer cells (Supplementary Figures 2N and O), silencing fibulin-3 expression had no significant effects on MDA-MB-231 cell migration in the presence or absence of the Wnt pathway inhibitor, IWP-2 (Supplementary Figure 5C).

As EMT is important for breast cancer metastasis and TGF-β is a strong EMT inducer, we next investigated whether decreased fibulin-3 promotes EMT by enhancing TGF-β signaling in normal mouse mammary epithelial cells (NMuMG), which have been widely used as an EMT model. TGF-β induced Smad2

phosphorylation (Figure 5d) and EMT in blank medium as detected by changes in cell morphology (Figure 5e) and EMT marker expression, including upregulation of fibronectin and downregulation of E-cadherin (Figure 5f). While conditioned media from NMuMG cells decreased TGF-β induced Smad2 phosphorylation (Figure 5d) and EMT (Figures 5e and f), the conditioned media from shFBLN3-NMUMG cells was not able to inhibit TGF-β's effects (Figures 5d–f). Consistent with an effect on EMT, fibulin-3 (Figure 5g) and ZO-1 (Figure 5h) mRNA levels decreased, with their expression positively correlating (TCGA data base: *Q*-value = 2.6e-11; METABRIC database: *Q*-value = 2.5e-02), while PAI-1, a TGF-β downstream gene (Figure 5i), and fibronectin (Figure 5j) mRNA levels increased in breast tumor tissue compared with their paired normal breast tissue in 92 normal-tumor pairs from the TCGA data set. Collectively, these data indicate that decreased fibulin-3 expression during breast cancer progression may promote TGF-β induced breast cancer cell EMT, migration and invasion.

Decreased fibulin-3 enhances TGF-β's effects on endothelial sprouting and transendothelial cell migration

As fibulin-3 inhibits TGF-β signaling in endothelial cells, we investigated fibulin-3's roles in endothelial biology. While conditioned media from metastatic MDA-MB-231-4175 cells[46] with very low fibulin-3 expression (Figure 4h, Supplementary Figure 2H) did not inhibit TGF-β-mediated decreases in sprouting as detected by spheroid-based sprout assay, the conditioned media from MDA-MB-231-4175 stably overexpressing fibulin-3 inhibited TGF-β-mediated decreases in sprouting (Figures 6a and b). In addition, while TGF-β did not decrease sprouting in the conditioned media from poorly-metastatic parental MDA-MB-231 cells[46] with high fibulin-3 expression (Figure 4h, Supplementary Figure 2H), TGF-β decreased sprouting in the presence of conditioned media from shFBLN3-MDA-MB-231 cells (Figure 6c).

As breast cancer cell intravasation into or extravasation out of blood vessels are pivotal steps for cancer metastasis, and both processes require transendothelial cell migration, we studied the effect of fibulin-3 on transendothelial cell migration. While TGF-β increased transendothelial cell migration in blank medium and in the conditioned media from metastatic MDA-MB-231-4175 cells (Figures 6d and e) with very low fibulin-3 expression[46] (Figure 4h, Supplementary Figure 2H), conditioned media from MDA-MB-231-4175 cells stably expressing fibulin-3 (Figures 6d and e) or from poorly-metastatic parental MDA-MB-231 cells (Figure 6f) with high fibulin-3 expression[46] (Figure 4h, Supplementary Figure 2H) inhibited TGF-β-increased transendothelial cell migration (Figures 6d–f), while conditioned media from MDA-MB-231-shFBLN3 cells restored TGF-β-increased transendothelial cell migration (Figure 6f). These data indicate that decreased fibulin-3 promotes TGF-β regulation of endothelial sprouting and transendothelial cell migration to facilitate breast cancer metastasis.

Overexpression of fibulin-3 inhibits breast cancer metastasis *in vivo*

To investigate whether the ability of fibulin-3 to inhibit TGF-β signaling in both breast cancer and endothelial cell function *in vitro* had effects on breast cancer progression *in vivo*, we injected MDA-MB-231-4175 stably expressing fibulin-3 into the inguinal mammary fat pads of nude mice. While there were no effects on primary tumor growth (Figure 7a), fibulin-3 expression inhibited and delayed breast cancer metastasis (Figures 7b and c). Further, fibulin-3 expression also inhibited micrometastasis to the liver as detecting by staining with anti-human vimentin (Figure 2d). Expression of fibulin-3 also appeared to decrease cancer cell invasiveness *in vivo*, decreasing the extent to which MDA-MB-231-4175 cancer cells penetrated through smooth muscle layers (black arrows, Figure 7e). In addition, while the control MDA-MB-231-4175 cells displayed an elongated, mesenchymal phenotype, fibulin-3 expression promoted a more cobblestone epithelial morphology (Figures 7f and g), and increased pan-cytokeratin and decreased fibronectin expression relative to control MDA-MB-231-4175 cells (Figure 7g). Finally, fibulin-3 expressing MDA-MB-231-4175 tumors had reduced nuclear p-Smad2 staining relative to control MDA-MB-231-4175 tumors (Figure 7h). Taken together, these data indicate that expression of fibulin-3 decreased TGF-β/Smad2 signaling and inhibited breast cancer EMT and metastasis *in vivo*.

DISCUSSION

Here, we used an unbiased approach to identify fibulin-3 as a novel and key regulator of the TGF-β signaling pathway in the breast cancer microenvironment (Figure 8a). Fibulin-3 expression decreases during breast cancer progression, with decreased fibulin-3 promoting TGF-β signaling and in turn promoting EMT, migration, invasion and transendothelial cell migration of breast cancer cells (Figure 8b). Mechanistically, fibulin-3 inhibits the TGF-β pathway through interaction with TβRI and inhibition of TGF-β induced TβRI/TβRII complex formation (Figure 8a).

The TGF-β pathway is tightly regulated to have dual tumor suppressor and tumor promoter roles during breast cancer progression.[5–7] Although mechanisms regulating the TGF-β pathway within the cancer cell have been extensively studied, mechanisms by which the tumor microenvironment might regulate TGF-β signaling are less defined. Importantly, secreted proteins have the potential to coordinately regulate TGF-β signaling in the multiple different cell types represented in the tumor microenvironment, including cancer cells, endothelial cells, fibroblasts and immune cells. Here, we identify fibulin-3 as one of those secreted proteins, which can inhibit TGF-β signaling in cancer cells, endothelial cells and fibroblast cells. Whether fibulin-3 also inhibits TGF-β signaling in other components of the tumor microenvironment, including immune system cells, to mediate its effects on inhibiting cancer progression remains to be explored. In addition, while the current studies focused on breast cancer, fibulin-3 expression is also decreased in other cancers,[36,48,49] suggesting that loss of fibulin-3 expression may be a common mechanism by which TGF-β signaling is increased to promote cancer progression.

In the context of breast cancer progression, the source, timing and mechanisms for loss of fibulin-3 expression are critical and of potential translational relevance. Fibulin-3 is ubiquitously expressed and secreted from all types of cells, including epithelial cells, endothelial cells and fibroblast cells. However, in the context of the breast cancer microenvironment, as breast cancer cells constitute the majority of cells, breast cancer cells are likely the major source of fibulin-3. Here, we demonstrate that fibulin-3 expression decreases during breast cancer progression, particularly from breast cancer cells and in human breast cancer specimens, with this loss occurring primarily as the breast cancer cell acquires invasive capabilities (Figure 4). In this context, high levels of fibulin-3 expressed and secreted by normal breast epithelial and pre-invasive ductal carcinoma *in situ* cells may function to inhibit the effects of TGF-β on the stroma in the early tumor microenvironment, with losses in expression during cancer progression promoting TGF-β signaling at the same time as the cancer cells become unresponsive via mutation or loss of receptor or Smad expression, increasing the tumor promoting effects of TGF-β signaling. This scenario is consistent with the role of enhanced TGF-β signaling in promoting tumor metastasis in the late stages of breast cancer. We provide preliminary evidence for epigenetic regulation mediating loss of fibulin-3, as has been reported in other tumor types.[36,37,48,49] These results raise the exciting possibility that DNA methyltransferase inhibitors in clinical use could increase fibulin-3 levels and provide therapeutic benefit in this manner.

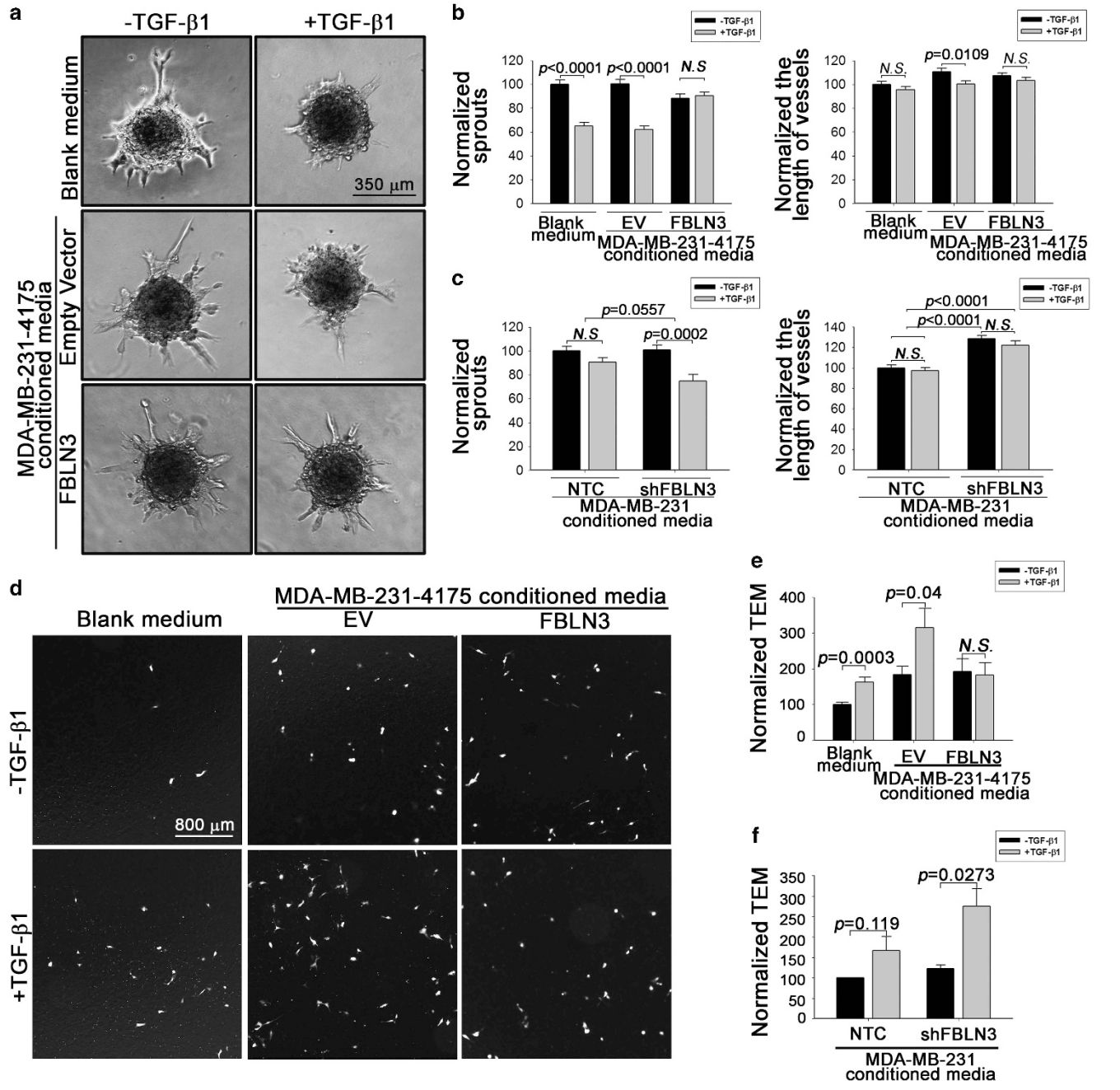
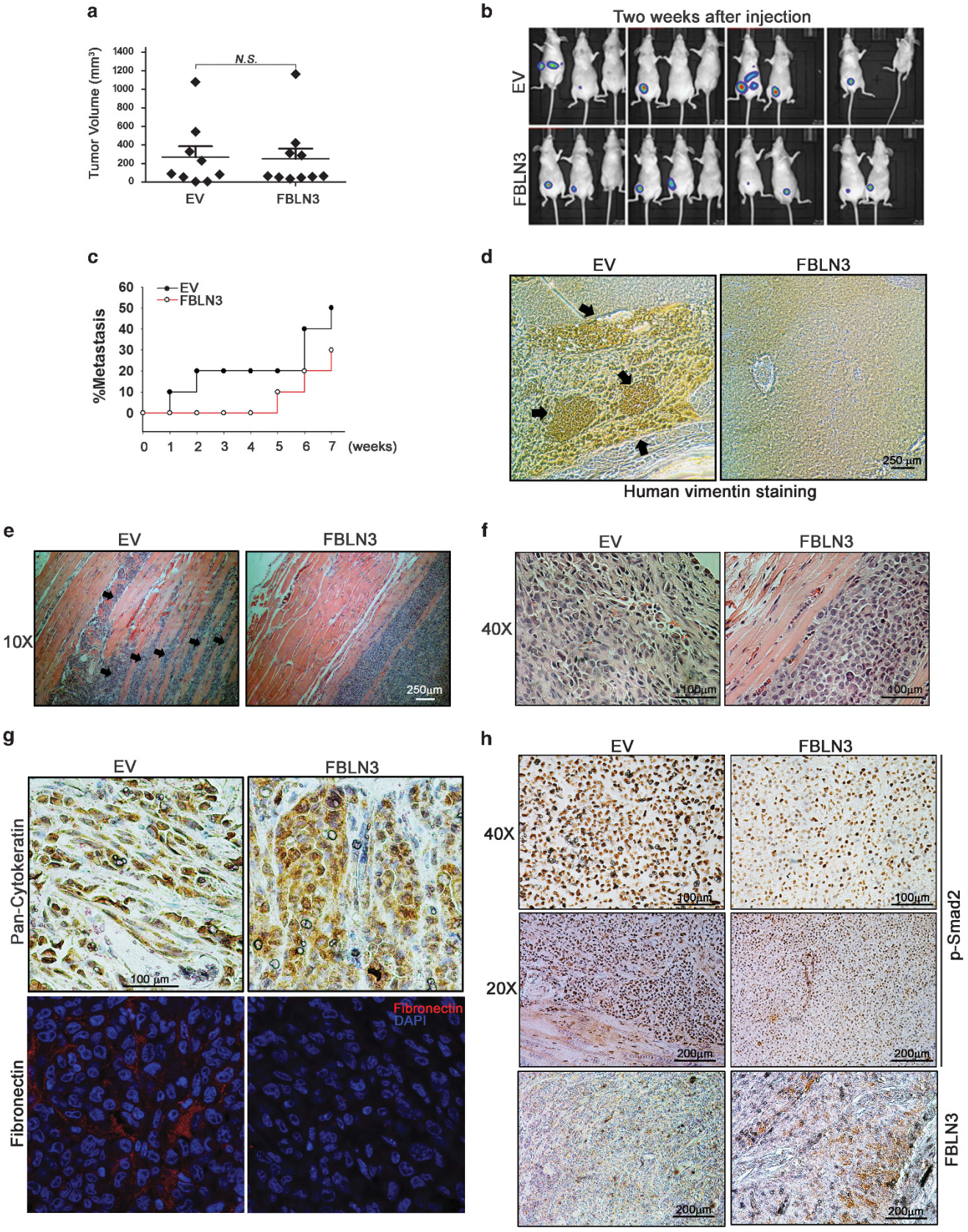


Figure 6. Loss of fibulin-3 enhances TGF-β induced transendothelial cell migration. (**a**–**c**) HMEC-1 cells were cultured in hanging drops to make spheroids. Endothelial spheroids were seeded on Matrigel with conditioned media from empty vector or fibulin-3 stably overexpressing MDA-MB-231-4175 cells (**a**, **b**), or from shNTC-or shFBLN3-MDA-MB-231 cells (**c**), treated without or with 50 pM TGF-β1, and cultured overnight. The number of sprouts was counted and the length of every vessels was measured using Image J. Quantitated data were from three experiments ± s.e.m.. (**d**–**f**) A confluent endothelial monolayer growing in transwells was cultured in conditioned media from empty vector or fibulin-3 stably overexpressing MDA-MB-231-4175 cells (**d**, **e**), or from shNTC- and shFBLN3-MDA-MB-231 cells (**f**), treated without or with 50 pM TGF-β1 for 12 h. GFP labelled breast cancer cells were then plated on the top of transwell and allowed to migrate for 24 h, with the number of cells undergoing transendothelial cell migration (TEM) quantitated. Quantitated data were from three experiments ± s.e.m.

Our studies with conditioned media from breast cancer cells demonstrated a predominant effect on inhibiting TGF-β signaling, with more subtle effects on BMP-2 signaling (Figure 1b), and no effects on BMP-9 signaling (Figure 1c, Supplementary Figure 1B). However, in many cases we also observed an effect of conditioned media on increasing basal p-Smad2 levels (Figures 1c and e; Supplementary Figure 1A). As the conditioned media contains secreted TGF-β superfamily ligands, shed TGF-β superfamily receptors and other TGF-β superfamily activators and repressors, the observed effects of conditioned media may be due to the integrated effects of these components, in particular, TGF-β superfamily ligands that can stimulate Smad2 phosphorylation (that is, activin and BMP). In addition to fibulin-3, we also identified another TGF-β regulator in the breast cancer conditioned media, βIGH3. Although its effects on TGF-β signaling were subtle, knockdown of βIGH3 suppressed TGF-β signaling, suggesting that it may function to promote TGF-β signaling. How the effects of the complex cancer microenvironment secretome



are integrated to regulate cancer progression is currently being explored.

In summary, these studies provide a novel mechanism for how TGF-β signaling is regulated by the tumor microenvironment, and provide insight into targeting the TGF-β signaling pathway for the benefit of human cancer patients.

MATERIALS AND METHODS

Cell culture

MDA-MB-231 cells were from American Type Culture Collection (ATCC) and MDA-MB-231-4175 cells were acquired from Dr Joan Massage at Memorial Sloan Kettering Cancer Institute and grown in MEM medium, supplemented with 10% fetal bovine serum (FBS), 5 ml sodium pyruvate and 5 ml non-essential amino-acid. NMuMG cells were purchased from ATCC and grown in DMEM supplemented with 10% FBS and 10 μg/ml insulin. MCF-10A human breast cancer progression series of cell lines were acquired from Dr William P Schiemann at Case Western Reserve University and grown in DMEM/F12 medium, supplemented with 5% horse serum, 10 μg/ml insulin, 20 ng/ml epidermal growth factor, 100 ng/ml hydrocortisone,100 ng/ml cholera toxin and 100 U/ml penicillin and 100 mg/ml streptomycin. 4T1 mouse breast cancer series of cell lines were acquired from Dr William P Schiemann at Case Western Reserve University and grown in DMEM, supplemented with 10% FBS. HMEC-1s were acquired from Dr Edwin Edes at CDC and grown in MCDB-131 medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% FBS, 1 μg/ml hydrocortisone (Sigma, St Louis, MO, USA), 10 ng/ml epidermal growth factor (Sigma) and 2 mM L-glutamine (Invitrogen). COS7 cells were purchased from ATCC and were grown in DMEM, supplemented with 10% FBS. 18Co cells were from ATCC via Cell Culture Facility at Duke and grown in EMEM medium (Sigma), supplemented with 2 mM glutamine, 1% NEAA and 10% FBS. EFEMP1 (Human) Recombinant Protein was purchased from Abnova (Taipei, Taiwan, China).

Spheroid-based sprout assay

Endothelial spheroids were prepared as previously reported.[50] Briefly, 1×10^3 HMEC-1 cells were cultured in hanging drops of 25 μl medium containing 20% methocel and 80% culture medium, and allowed to aggregate as spheroids. After 24 h, the spheroids were collected using conditioned media from breast cancer cells and plated on 24-well plates coated with growth factor reduced Matrigel and treated as indicated. Sprouts were digitally imaged after the indicated times and the number and length of sprouts per spheroid quantitated.

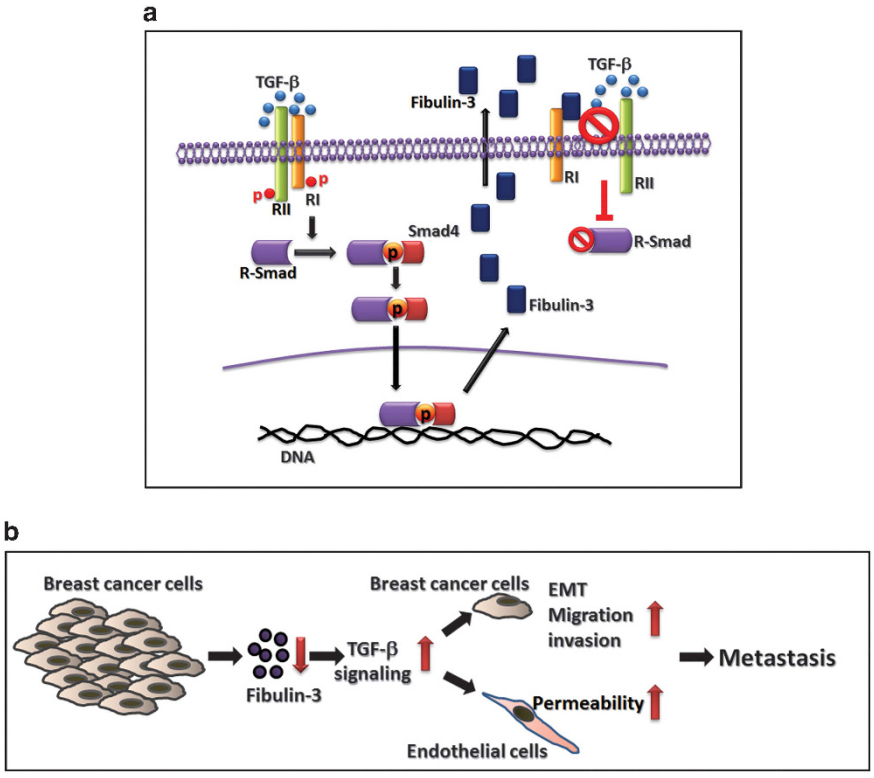


Figure 8. Fibulin-3-mediated regulation of TGF-β signaling and biology. (**a**) TGF-β induces fibulin-3 expression and secretion. Fibulin-3 inhibits TGF-β signaling by interaction with TβRI, blocking complex formation with TβRII and downstream signaling. (**b**) Fibulin-3 expression decreases during breast cancer progression, with decreased fibulin-3 promoting TGF-β signaling in breast cancer cells and endothelial cells, and in turn promoting EMT, migration, invasion in breast cancer cells and endothelial permeability, collectively promoting metastasis.

Figure 7. Fibulin-3 inhibits breast cancer EMT, invasiveness and metastasis *in vivo*. (**a**) MDA-MB-231-4175-vector (EV) or MDA-MB-231-4175-fibulin-3 cells (FBLN3) (50 000 cells/mouse) were implanted into the inguinal mammary fat pads of female nude mice. Primary tumor growth was recorded by measuring tumor size at 6 weeks after injection presented as mean ± s.e.m. (**a**). Metastasis was followed by bioluminescence imaging every 7 postoperative days (POD) (**b**, **c**). (**d**) Tissue sections of liver from mice implanted with MDA-MB-231-4175-vector (EV) or MDA-MB-231-4175-fibulin-3 cells (FBLN3) were immunostained with anti-human vimentin antibody. (**e**, **f**) Primary tumors from mice implanted with MDA-MB-231-4175-vector cells exhibiting local invasion (black arrows) of tumor cells into the adjacent smooth muscle layers (**e**) and mesenchymal morphology (**f**). (**g**, **h**) Tissue sections of primary tumors from mice implanted with MDA-MB-231-4175-vector (EV) or MDA-MB-231-4175-fibulin-3 cells (FBLN3) were immunostained for pan-cytokeratin (**g**, upper panel), fibronectin (**g**), bottom panel), p-Smad2 (**h**) and FBLN3 (**h**), respectively. Representative staining frequency and intensity is shown.

Transendothelial migration

1×10^5 HMEC-1 cells were cultured on top of 24-well membrane of transwells until they formed a monolayer, and treated with conditioned media from breast cancer cells with or without 50 pM TGF-β1 for 12 h. In all, 1×10^5 GFP-labelled breast cancer cells were plated with serum-free medium on the top of endothelial layers in a transwell. Media containing 0.5% bovine serum albumin was placed in the lower chamber as a chemoattractant. Twenty-four hours later, the cells on the upper surface of the filter were removed by gently scrubbing with a cotton swab. Transendothelial migration of breast cancer cells was then assessed by fluorescent microscopy.

Duolink assay

The Duolink assay (Olink Bioscience, Uppsala, Sweden) was performed according to the manufacturer's protocol. Briefly, MDA-MB-231 cells were pretreated with breast cancer conditioned media or 200 ng/ml recombinant fibulin-3, and treated with 50 pM TGF-β1 for 10 min. Cells were then washed with PBS (phosphate-buffered saline), fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X-100/PBS for 5 min and then blocked with 5% bovine serum albumin in PBS for 1 h. Slides were incubated with anti-TβRI, and anti-TβRII (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies for 1 h in room temperature and then incubated with PLA probe MINUS and PLUS mixture for 1h at 37 °C. After ligation for 30 min, and amplification for 100 min at 37 °C, the slides were labeled with DAPI, mounted with Prolong Anti-Fade (Sigma), digitally imaged and counted for number of the red dots per cells manually using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

In vivo tumorigenicity and metastasis

MDA-MB-231-4175-Luc cells stably expressing firefly luciferase gene were infected with either an empty vector (EV) as control or fibulin-3 overexpression pBABE retrovirus, and stably infected cells were selected using 1 μg/ml puromycin for 72 h. EV and fibulin-3 overexpressing cells were implanted (50 000 cells/mouse) into the right-side inguinal mammary fat pads of female BALB/c mice. Starting from day 7, tumor growth and tumor metastasis were recorded by bioluminescence.

Statistical analysis

Quantitative data are expressed as mean ± s.e.m. Statistical significance was determined by the two-tailed Student's *t*-test or one-way ANOVA, followed by the LSD-*t* test for multiple comparisons. A *P*-value of less than 0.05 was considered as statistically significant. To examine the statistical interaction between fibulin-3 expression and ligand treatment, two-way ANOVA was performed with specific interest in the interaction term.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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