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ANATOMICAL PATHOLOGY

Fibrillin-1, a novel TGF-beta-induced factor, is preferentially expressed in metaplastic carcinoma with spindle sarcomatous metaplasia

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Summary

TGF-β induces epithelial–mesenchymal transition (EMT), which is involved in tumour progression. This study aims to identify and characterise novel factors potentially related to TGF-β-mediated tumour aggression in breast cancer. We treated the human mammary epithelial cell line MCF10A with TGF-β and observed TGF-β-dependent upregulation of *FBN1*, involving demethylation of CpG sites, in MCF10A cells undergoing EMT. The biological importance of fibrillin-1, encoded by *FBN1*, was evaluated through immunohistochemistry on 225 breast cancer specimens of various subtypes. Fibrillin-1 expression was observed only in metaplastic carcinoma of the breast (MCB) (51.7%), and the expression was observed in spindle sarcomatous metaplasia (SSM), but not in other metaplasia, including matrix-producing, pleomorphic, and squamous metaplasia, and carcinomatous components of both MCB and non-MCB. Fibrillin-1 expression was also restricted to the SSM of non-mammary carcinosarcomas of various organs. Overall, fibrillin-1 expression was enriched in MCB and non-mammary carcinosarcoma with SSM (93.7% and 93.3%, respectively), but not in MCBs and non-mammary carcinosarcoma without SSM. *FBN1* knockdown in MDA-MB-231 cells with high *FBN1* expression did not compromise migration, invasion, and tumourigenesis, and did not alter the expression of other EMT-related markers. In conclusion, fibrillin-1 is a novel TGF-β-induced marker. Fibrillin-1 expression in SSM, but not in other metaplasia and carcinomatous components, in both MCBs and non-mammary carcinosarcomas, together with the inability of *FBN1*-knockdown to compromise migration and invasion, indicates that fibrillin-1 is a marker induced solely in spindle metaplasia during EMT and does not induce EMT nor lead to tumour aggressiveness.

Key words: TGF-β; fibrillin-1; epithelial-mesenchymal transition; metaplastic carcinoma; breast.

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INTRODUCTION

Breast cancer is the most prevalent cancer in women and is a major cause of mortality, with metastasis causing the majority of deaths. Because initial migration or invasion is a prerequisite for cancer progression and eventually for metastasis, further characterisation of molecular mechanisms regulating breast cancer invasion is required to improve breast cancer recovery.[1] Epithelial–mesenchymal transition (EMT) is the process of disaggregation of structured polarised epithelial units into single motile fibroblastoid cells to enable cell movement and morphogenesis.[2] EMT is characterised by protein loss associated with a polarised epithelial phenotype and protein synthesis associated with the mesenchymal and migratory morphology of transitioning cells.[3] EMT-like processes have been proposed to occur during tumour progression in carcinomas, particularly at the invasion and intravasation stages, during which tumour cells disassemble and migrate to sites distant from the primary tumours.[3,4]

EMT has been extensively studied in embryogenesis and cancer progression.[5] Several transcriptional factors, including Snail, Slug, ZEB1, ZEB2, and TWIST, and certain signalling pathways, such as TGF-β, EGF, Notch, and Wnt/beta-catenin, are known EMT inducers.[5–7] TGF-β is a multifunctional protein that suppresses tumours during early tumour development and promotes tumour growth during later stages.[8] Despite extensive knowledge of TGF-β biology in cancer development, several factors that mediate the cancer-promoting role of TGF-β continue to be identified.[9] For example, paired-related homeobox 1 and 2 have been reported to be induced by TGF-β and to increase cancer invasiveness through EMT.[10,11] These findings are consistent with the complex roles of TGF-β involving multiple aspects relating to cancer progression, and suggest the presence of additional uncharacterised factors associated with TGF-β-related cancer biology. This study aims to identify and characterise additional factors potentially related to TGF-β-mediated tumour aggression of breast cells to gain further insight into the complex role of TGF-β in cancer.

MATERIALS AND METHODS

This study was approved by the Research Ethics Committee of National Taiwan University Hospital.

Cell culture

MCF10A human mammary epithelial cells were cultured in DMEM/F12 medium (Life Technologies, USA) as previously described.[11] Breast cancer cell lines MDA-MB-231, T47D, and MCF-7 were grown in DMEM; MDA-

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MB-468 cells were cultured in Leibovitz's L-15 medium; and SK-BR3 cells were cultured in McCoy's 5A medium. For EMT induction, MCF10A cells were treated with recombinant human TGF-β1 (R&D Systems, USA) at a concentration of 5 ng/mL for 5 days before analysis. For TGF-β receptor blocking, the cells were preincubated with TGF-β R inhibitor SB431542 (10 μM, Sigma Aldrich, USA) for 1 h before TGF-β stimulation.

Microarray analysis

Total RNA for microarray analysis was prepared as previously described.[11] The microarray experiment and data analysis were done by Welgene Biotech (Taiwan) using the Agilent Oligo Chip (Agilent SurePrint G3 Human V2 GE 8×60K Microarray; Agilent Technologies, USA). Microarrays were scanned by laser scanner and the microarray signal intensities were measured to identify gene expression differences and ratios of gene expression.

RNA isolation and qRT-PCR

Total RNA was reverse transcribed into cDNA using standard protocols.[11] Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed with the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, USA) using the SYBR Green method. The quantitative values were calculated based on the threshold cycle number (Ct) and the fold-change in expression was calculated using the delta-delta Ct method. Target gene measurements in all samples were normalised to the internal control gene, GAPDH. The primers used in this study are listed in Supplementary Table 1 (Appendix A).

Bisulfite DNA sequencing analysis

The DNA methylation patterns in the CpG islands of *FBN1* gene were determined by bisulfite DNA sequencing analysis. Briefly, DNA was extracted with a QIAamp Mini kit (Qiagen GmbH, Germany). Bisulfite modification of DNA was performed with a CpGenome DNA modification kit (Chemicon, USA). The primer used is described in Supplementary Table 1 (Appendix A).

Tumour samples, immunohistochemistry, and immunofluorescence staining

In total, 225 formalin-fixed, paraffin-embedded human breast samples, retrieved from the Department of Pathology, National Taiwan University Hospital, were used in this study. These included 144 invasive carcinomas of no special type (invasive ductal carcinoma, IDC), 28 invasive lobular carcinomas (23 classic types and five pleomorphic variants), 12 mucinous carcinomas, three invasive papillary carcinomas, six micropapillary carcinomas, three neuroendocrine carcinomas, and 29 metaplastic carcinomas of the breast (MCBs). The 144 cases of IDC were consecutively selected, and the carcinomas other than IDC were selected for contrasting purpose. The tumour samples were prepared as tissue microarrays (TMAs). For TMA construction, two to three representative areas were selected on haematoxylin and eosin stained sections in each case. Additional whole sections of tumour tissues were evaluated in most MCB samples due to histological diversity in this cancer subtype. The Scarff–Bloom–Richardson histological gradings of these 144 IDCs were 43.0% (62/144), 34.7% (50/144), and 22.2% (32/44) in grades 1, 2, and 3, respectively, and the biomarkers of these cases were 57.6% (83/144) ER positive, 51.3% (74/144) PR positive, 30.5% (44/144) HER2 positive, and 17.3% (25/144) triple-negative breast cancer. These 29 samples of MCB included 26 cases of biphasic type and three cases of pure epithelial type. Among the 26 biphasic MCBs, matrix metaplasia was observed in 11. The three pure epithelial MCBs were conventional high-grade ductal carcinomas with focal squamous metaplasia. An additional 21 specimens of non-mammary carcinosarcoma from various organs, including the uterus (n=6), oesophagus (n=4), ovary (n=2), oral cavity (n=2), lung (n=2), gall bladder (n=1), thyroid (n=1), pancreas (n=1), prostate (n=1), and salivary gland (n=1), were included in the additional immunohistochemical study. Sections of cancer tissues were stained through immunohistochemistry as previously described.[11] The slides were incubated with a rabbit polyclonal antibody against human fibrillin-1 (HPA021057, Sigma-Aldrich) and then incubated with polymer-HRP reagent (Dako Cytomation, Denmark). Unequivocal cytoplasmic staining in ≥10% of tumour cells was considered positive. All slides were reviewed by two pathologists (HCL and YHL). For immunofluorescence staining, slides were incubated with primary anti-fibrillin-1 antibody, washed, and incubated with Alexa Fluor 594 goat anti-rabbit IgG secondary antibody (Molecular Probes, USA), before counterstaining with 4′-6-diamidino-2-phenylindole (DAPI).

RNA interference and lentiviral infection

Lentivirus carrying *FBN1*-specific small hairpin RNA (shRNA; National RNAi Core Facility, Academia Sinica, Taiwan) were used to knockdown *FBN1*. The target sequence of shRNA is CCCAAGGGATTTATCTACAAA. To generate recombinant lentivirus, 293T cells were co-transfected with the packaging, enveloping, and shRNA expressing constructs (National RNAi Core Facility). The virus-containing supernatant was harvested and then used to infect cells.

Migration and invasion assays, and anchorage-independent growth assay

For migration assay, cells were grown to confluence in six-well culture plates. The cell layer was scraped with a sterile blue pipette tip and reincubated at 37°C. Migration from the edge of the injured monolayer was quantified by measuring the distance between the wound edges. For invasion assays, we used modified Boyden chambers with filter inserts coated with Matrigel in 24-well dishes. 1×10^4 cells were placed in the upper chamber and were fixed in 4% paraformaldehyde before being stained with DAPI and counted under a fluorescence microscope. For anchorage-independent growth, 5×10^3 cells were seeded into 0.3% soft agar and the resulting soft agar was layered onto 0.5% agar in six-well plates. The cells were grown at 37°C and the colonies were then stained with 0.05% *p*-iodonitrotetrazolium violet and counted by inverted microscopy.

Western blot

Proteins from cell lysates were resolved on 10% SDS-polyacrylamide gels and then electrotransferred onto polyvinylidene difluoride membranes (Millipore, UK). Immunoblotting was performed with the addition of antibodies to Akt, phospho-Akt (Ser473), phospho-ERK1/2 (Thr202/Tyr204), p38, phospho-p38 (Thr180/Tyr182), JNK, phospho-JNK (Thr183/Tyr185) (Cell Signaling Technology, USA) and ERK1/2 (GeneTex, USA). After incubation with a secondary antibody, the immunoreactive signals were detected using an enhanced chemiluminescence kit (T-Pro Biotechnology, Taiwan).

RESULTS

TGF-β-dependent *FBN1* upregulation in human mammary epithelial cell MCF10A

To identify the uncharacterised potential factors associated with TGF-β-mediated aggression, we treated human mammary epithelial MCF10A cells with TGF-β. As a well-known EMT inducer, TGF-β induction in MCF10A cells caused EMT-like morphological changes, evidenced by the reduced cohesiveness and increased spindle-like shape of the cells (Fig. 1A). The EMT-like morphological change was paralleled at the RNA levels by the downregulation of the epithelial marker *CDH1* and upregulation of known EMT markers, *CDH2*, *FN*, *VIM*, *SNAI1*, *SNAI2*, *TWIST1*, and *ZEB1*, as revealed through a microarray analysis (Fig. 1B). Some of the data from the same microarray analysis have been previously described.[11] Along with well-characterised EMT markers, *FBN1* was upregulated (Fig. 1B). *FBN1* gene encodes fibrillin-1, which is a large glycoprotein expressed in the extracellular matrix of tissues of mesenchymal origin.[12] Although fibrillin-1 has been reported to regulate TGF-β bioavailability and mutations in *FBN1* are responsible for Marfan syndrome pathogenesis,[13–15] the role of *FBN1* in tumourigenesis and its relationship with EMT remain elusive. Therefore, we focused on investigating the role of *FBN1*. Quantitative RT-PCR confirmed the TGF-β-induced time-dependent

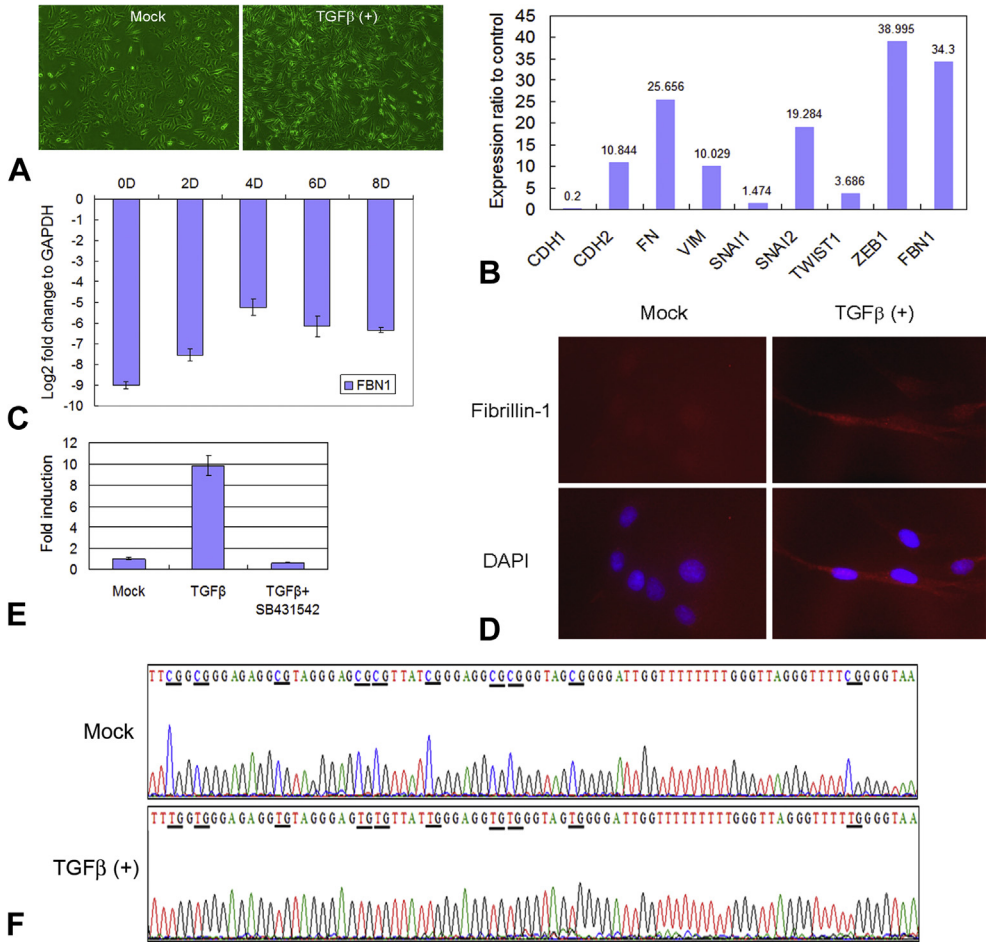

Fig. 1 TGF-β-dependent *FBN1* upregulation in human mammary epithelial cell MCF10A. (A) TGF-β induces EMT-like morphological changes in MCF10A cells. MCF10A cells treated with TGF-β (5 ng/mL) are more spindle shaped and less cohesive than epithelial-like cells in mock controls.[11] (B) EMT-like morphological change was accompanied by the downregulation of the epithelial marker *CDH1* and upregulation of EMT markers, *CDH2*, *FN*, *VIM*, *SNAI1*, *SNAI2*, *TWIST1*, and *ZEB1*, as evidenced by the microarray analysis on MCF10A cells treated with TGF-β (5 ng/mL) for 5 days, compared with mock control cells. (C) Treating MCF10A cells with TGF-β (5 ng/mL) time dependently upregulates *FBN1* mRNA levels, as demonstrated by qRT-PCR. (D) Immunofluorescence staining demonstrated fibrillin-1 staining in the spindle-shaped TGF-β-treated MCF10A cells, but not in the mock control cells. (E) Pretreatment with TGF-β R inhibitor SB431542 (10 μM) for 1 h before TGF-β stimulation significantly reduced *FBN1* upregulation. (F) Bisulfite treatment and sequencing showed a reversal of the hypermethylation of CpG sites in the *FBN1* gene promoter in TGF-β-treated MCF10A cells, compared with a mock control.

FBN1 upregulation (Fig. 1C) in MCF10A cells, and fibrillin-1 protein upregulation was demonstrated through immunofluorescence (Fig. 1D). Pretreatment with TGF-β receptor inhibitor SB431542 before TGF-β stimulation significantly reduced *FBN1* upregulation, indicating that the TGF-β-induced upregulation of *FBN1* was TGF-β receptor-dependent (Fig. 1E). Furthermore, bisulfite treatment and sequencing showed the reversal of CpG site hypermethylation in the *FBN1* gene promoter in TGF-β-treated MCF10A cells compared with control (Fig. 1F), suggesting that the TGF-β-induced upregulation of *FBN1* involves DNA demethylation of promoter. This result confirmed that *FBN1* upregulation was TGF-β-dependent.

Fibrillin-1 expression was enriched in metaplastic breast carcinoma with SSM

To investigate the biological role of fibrillin-1 in human breast cancer, we evaluated fibrillin-1 expression in 225 primary breast cancer specimens of different histological subtypes, which are summarised in Table 1. Consistent with its function as a structural glycoprotein in the extracellular matrix, fibrillin-1 was widely expressed in the stroma of non-tumoural breast tissue and in the stroma surrounding tumour cells. Invasive carcinomas, including various subtypes, were consistently devoid of fibrillin-1 staining with the exception of metaplastic carcinomas (Fig. 2). In contrast to non-metaplastic carcinomas, cytoplasmic fibrillin-1 staining was observed in 57.7% (15/26) of biphasic MCBs, but in none of the three squamous MCBs of pure epithelial type. Notably, among all the 26 biphasic MCBs evaluated, fibrillin-1 staining was exclusively observed in the spindle sarcomatous metaplastic tumour component, but not in other metaplastic tumour components, including matrix-producing, pleomorphic, and squamous metaplastic tumour components (Fig. 3). Consistent with the lack of fibrillin-1 staining in non-metaplastic carcinoma, fibrillin-1 was not observed in the conventional ductal carcinomatous components in all biphasic MCBs. In total, fibrillin-1 expression was identified in 51.7% (15/29) of all MCBs and was enriched in 93.8% (15/16) of MCBs with SSM, but was not observed in MCBs without SSM (0/10) and monophasic squamous MCB (0/3).

Table 1 Fibrillin-1 expression in 225 primary human breast carcinoma samples

	Fibrillin-1 expression		
	Positive	Negative	Total
	n (%)	*n* (%)	*n* (%)
Invasive ductal carcinoma	0 (0)	144 (100)	144 (100)
Invasive lobular carcinoma	0 (0)	28 (100)	28 (100)
Mucinous carcinoma	0 (0)	12 (100)	12 (100)
Invasive papillary carcinoma	0 (0)	3 (100)	3 (100)
Micropapillary carcinoma	0 (0)	6 (100)	6 (100)
Neuroendocrine carcinoma	0 (0)	3 (100)	3 (100)
Metaplastic carcinoma	15 (51.7)	14 (48.3)	29 (100)
Biphasic MCB with SSM	15 (93.8)	1 (6.2)	16 (100)
Biphasic MCB without SSM	0 (0)	10 (100)	10 (100)
Monophasic squamous MCB	0 (0)	3 (100)	3 (100)

MCB, metaplastic carcinoma of the breast; SSM, spindle sarcomatous metaplasia.

Fibrillin-1 expression was enriched in non-mammary carcinosarcoma with SSM

To investigate whether the restriction of fibrillin-1 expression in the spindle sarcomatous metaplastic tumour component of metaplastic carcinoma is organ specific, we further evaluated fibrillin-1 staining in 21 non-mammary carcinosarcomas from various organs, which exhibited biphasic carcinomatous and sarcomatous features similar to MCBs. Similarly, the enrichment of fibrillin-1 staining was observed in all but one non-mammary carcinosarcoma with SSM (14/15), but not in

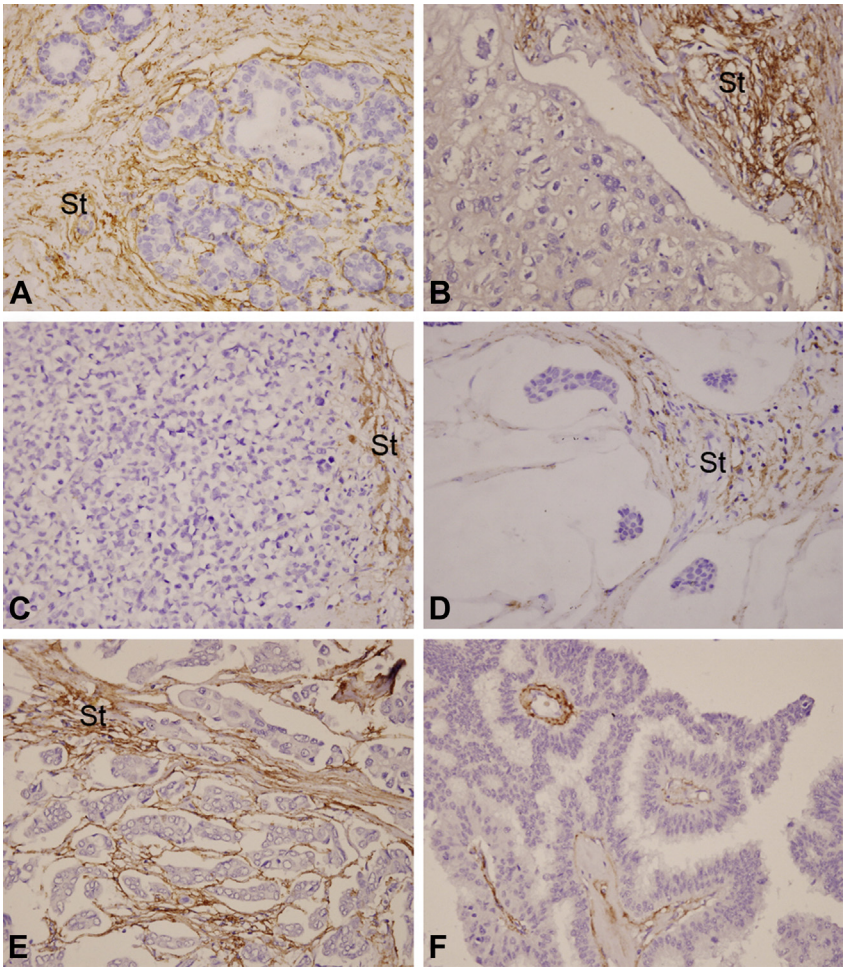

Fig. 2 Immunohistochemical staining for fibrillin-1 in normal breast tissue and various subtypes of non-metaplastic invasive breast carcinoma. (A) In normal breast tissue, fibrillin-1 staining was observed in the nonepithelial stromal connective tissue, but not in ductal and lobular epithelial cells in the ductal lobular unit. Fibrillin-1 was consistently negative in all non-metaplastic carcinomas. One representative sample each of invasive carcinoma of no special type (B), invasive lobular carcinoma (C), mucinous carcinoma (D), micropapillary carcinoma (E), and invasive papillary carcinoma (F) are shown. Note that staining in the stromal connective tissue (St) served as an internal positive control.

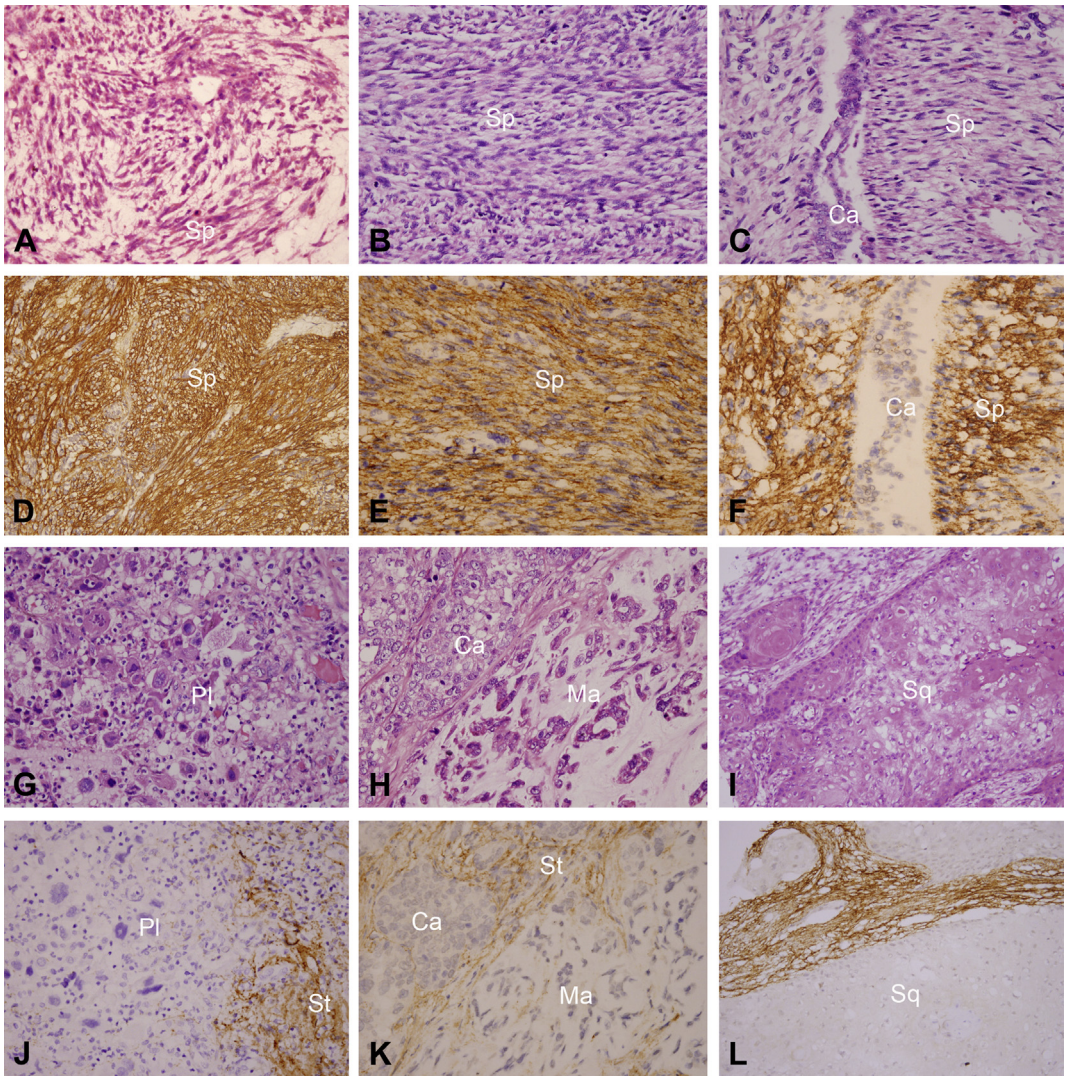

Fig. 3 Immunohistochemical staining for fibrillin-1 in metaplastic breast carcinomas. (A), (B), (C), (G), (H), and (I) are haematoxylin and eosin stained. (D), (E), (F), (J), (K), and (L) are corresponding immunohistochemical stainings of (A), (B), (C), (G), (H), and (I). Intense fibrillin-1 staining was observed in a representative sample of biphasic MCB with solely spindle sarcomatous metaplasia (A,D). Fibrillin-1 staining was observed only in the spindle sarcomatous metaplastic component (Sp) (B,E), but not in the conventional ductal carcinomatous (Ca) (C,F) and the pleomorphic sarcomatous metaplastic (Pl) (G,J) components present in a representative sample of biphasic MCB with multiple metaplastic components. A representative sample of biphasic MCB with solely matrix-producing metaplasia showed a lack of fibrillin-1 staining both in the carcinomatous (Ca) and matrix-producing metaplastic sarcomatous (Ma) components (H,K). Note that intense fibrillin-1 staining in the peritumoral stromal tissue (St) served as a positive control. The squamous metaplastic carcinomatous component (Sq) in a representative sample of squamous MCB of the pure epithelial type stained negatively for fibrillin-1 (I,L).

other metaplastic tumour components, including matrix-producing and pleomorphic carcinomas, and not in all conventional carcinomatous components within carcinosarcomas (Table 2 and Fig. 4). These findings indicate that the restriction of fibrillin-1 in SSM is not tissue-specific.

FBN1 knockdown did not compromise migration, invasion, and tumourigenesis

Because *FBN1* was identified along with other well-known EMT markers (Fig. 1B) in MCF10A cells treated with TGF-β, we hypothesised that *FBN1* could be correlated with EMT features, such as increased migration and invasiveness. To characterise the functional role of *FBN1* in breast cancer, we first examined *de novo FBN1* expression in a panel of human breast cancer cell lines encompassing major molecular subtypes, including MDA-MB-231, MDA-MB-468, SK-BR-3, MCF-7, and T47-D. As shown in Fig. 5A, all the tested breast cancer cell lines, except for MDA-MB-231, expressed low levels of *FBN1*. Because MDA-MB-231 is highly invasive, we then stably knocked down *FBN1* in MDA-MB-231 cells using lentivirus carrying *FBN1* shRNA to examine the impact of *FBN1* on tumour migration and invasiveness. To minimise the effect of clone heterogeneity, we used pooled transductants in the following assays. The knockdown efficiency of *FBN1* was measured using qRT-PCR (Fig. 5B). In contrast to our hypothesis, we found no significant difference in migration and invasiveness, as measured by a wound healing assay and Boyden chamber assay with Matrigel coating, between *FBN1*-knockdown MDA-MB-231 cells (shFBN1) and the luciferase knockdown control (shLuc) (Fig. 5C,D). Similarly, the knockdown of *FBN1* did not compromise tumourigenesis, as measured by soft agar colony-forming assay (Fig. 5E). Consistently, we observed no significant difference in the phosphorylated

Table 2 Fibrillin-1 expression in 21 non-mammary carcinosarcoma samples

	Fibrillin-1 expression		
	Positive	Negative	Total
	n (%)	*n* (%)	*n* (%)
NMC with SSM	14 (93.3)	1 (6.7)	15 (100)
NMC without SSM	0 (0)	6 (100)	6 (100)
Total	14 (66.7)	7 (33.3)	21 (100)

NMC, non-mammary carcinosarcoma; SSM, spindle sarcomatous metaplasia.

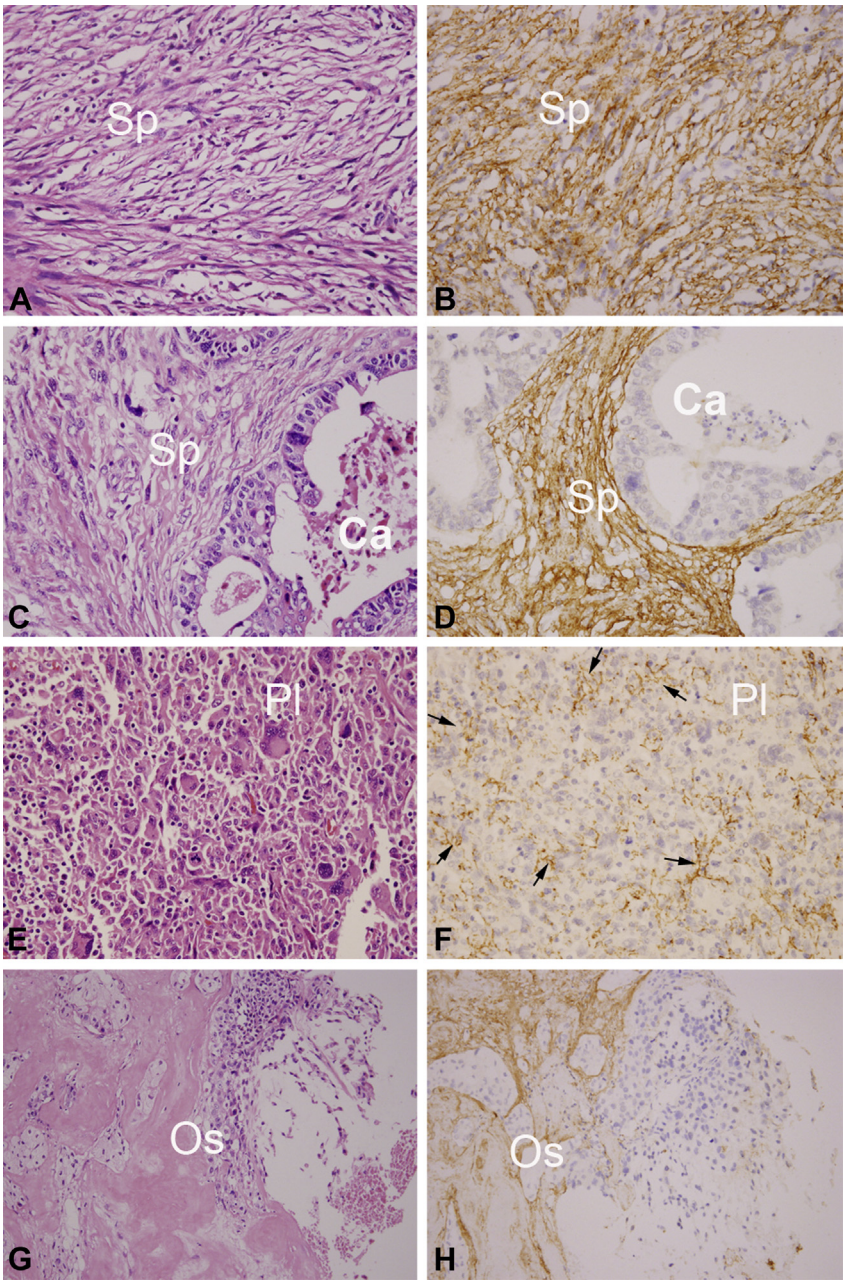

Fig. 4 Immunohistochemical staining for fibrillin-1 in non-mammary carcinosarcomas. (A), (C), (E), and (G) are haematoxylin and eosin stained. (B), (D), (F), and (H) are corresponding immunohistochemical stainings of (A), (C), (E), and (G). Intense fibrillin-1 staining was observed in the spindle sarcomatous component (Sp) of two representative samples of non-mammary carcinosarcoma with SSM (A,B and C,D). The carcinomatous component (Ca) had a complete lack of staining (D). In contrast to the spindle sarcomatous metaplastic component, fibrillin-1 staining was not detected in the pleomorphic sarcomatous component (Pl) of a representative sample of non-mammary carcinosarcoma with solely pleomorphic sarcomatous metaplasia (E,F) or in the sarcomatous component with osteoid matrix (Os) production in a representative sample of matrix-producing non-mammary carcinosarcoma (G,H). Note that intense fibrillin-1 staining observed in the peritumoural stromal tissue (arrow) and in the osteoid matrix served as internal positive controls.

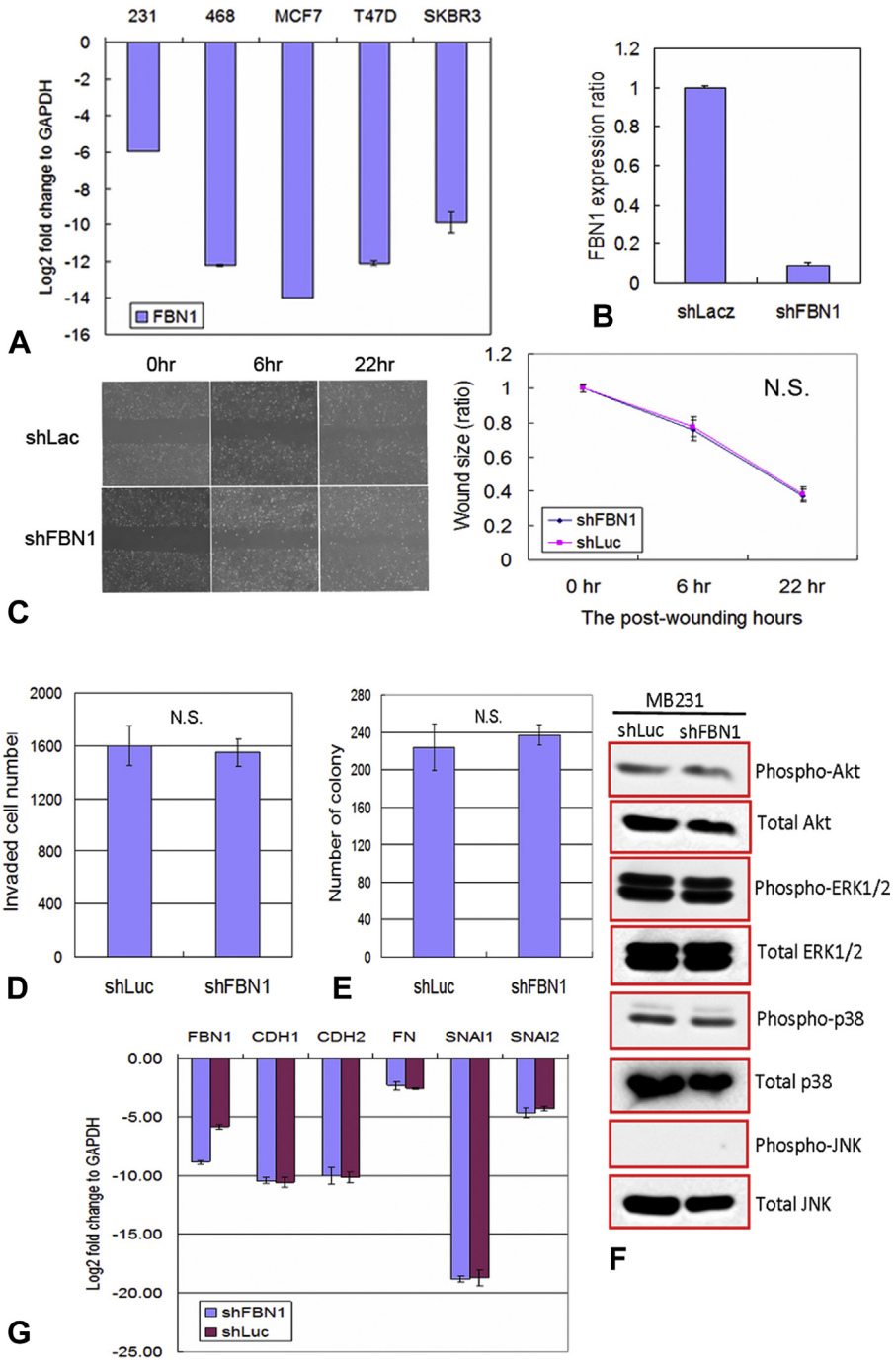

Fig. 5 *FBN1* knockdown does not compromise migration, invasion, and tumourigenesis and does not alter the expression of other EMT-associated markers. (A) qRT-PCR showed the highest expression of *FBN1* in MDA-MB 231 cells, compared with other breast cancer cell lines. (B) qRT-PCR confirmed the knockdown of *FBN1* mRNA levels in MDA-MB 231 cells infected with lentivirus carrying shRNA for *FBN1* (shFBN1) compared with the luciferase knockdown control (shLuc). *FBN1* knockdown did not significantly compromise the capability to migrate into the wounded area in the wound healing assay (C) and the invasiveness through Matrigel in the Boyden chamber assay (D) of shFBN1 cells, compared with shLuc cells. A colony-forming assay did not show a significant difference in the number of colonies between shFBN1 cells and shLuc cells (Student's *t* test) (E). *FBN1* knockdown did not alter the phosphorylated protein levels of Akt, ERK1/2, p38, and JNK (F). qRT-PCR showed no significant differences in the RNA levels of epithelial marker *CDH1* and mesenchymal markers *CDH2* and *FN*, and EMT regulators *SNAI1* and *SNAI2* between shFBN1 and shLuc (G).

protein levels of some kinases reported to be involved in cancer progression, including Akt, ERK1/2, p38, and JNK,[16] between shFBN1 and shLuc (Fig. 5F). These results indicate that *FBN1*, despite being a potential EMT marker, does not enhance mammary cell migration, invasion, and tumourigenesis.

FBN1 knockdown did not alter the expression of other EMT-associated markers

Some critical EMT-initiating transcriptional factors, such as Snail and SIP1, induce EMT phenotypes through the downregulation of epithelial markers and upregulation of

mesenchymal markers. However, it has been reported that vimentin, a mesenchymal maker but not a transcriptional factor, induces EMT in human breast cells.[17] Therefore, we investigated whether the knockdown of the potential EMT marker *FBN1* would affect other well-defined EMT markers. As shown in Fig. 5G, the mRNA levels of these EMT markers, including *CDH1*, *CDH2*, *FN*, *SNAI1*, and *SNAI2*, showed no significant change between the shFBN1 and shLuc control. Thus, the EMT-associated marker fibrillin-1 does not alter the expression levels of other EMT markers.

DISCUSSION

TGF-β is a multifunctional protein that plays critical roles in a broad spectrum of developmental, physiological, and pathological processes.[9,18] In this study, we identified fibrillin-1 as a novel TGF-β-induced factor in human breast cells. Fibrillin-1, which is encoded by the *FBN1* gene in humans, is a large glycoprotein produced by fibroblasts, and is the main component of microfibril expressed in the extracellular matrix.[12,19] It contributes to the strength and elasticity of tissues and regulates TGF-β bioavailability.[13–15,20] Marfan syndrome is an autosomal dominant connective tissue disorder with multiple organ manifestations. The genetic cause of this syndrome is mutation in the *FBN1* gene, leading to impaired fibrillin-1 protein synthesis and subsequent upregulation of the TGF-β signalling pathway due to the lack of sequestration of TGF-β by fibrillin-1.[21–23] In this study, we demonstrated the induction of fibrillin-1 by TGF-β in human mammary epithelial cells. Previous findings on bioavailability regulation of TGF-β by fibrillin-1, and the lack of sequestration of TGF-β by fibrillin-1 leading to the upregulation of TGF-β signalling pathway in the pathological state of Marfan syndrome, together with fibrillin-1 induction by TGF-β as demonstrated in the present study, suggest a potential interaction between fibrillin-1 and TGF-β. It remains to be investigated whether the upregulation of the TGF-β signalling pathway induces fibrillin-1 expression in fibroblasts and epithelial cells and acts as a negative feedback loop of TGF-β signalling under certain physiological states.

The biological importance of fibrillin-1 in EMT was supported by its differential expression in up to 51.7% of the MCBs and its lack of expression in all other breast cancer subtypes. Consistently, the conventional ductal carcinomatous elements in all cases of metaplastic carcinoma completely lacked fibrillin-1 expression. Metaplastic carcinoma, classically characterised by the coexistence of carcinomatous and sarcomatous components, has been shown through transcriptional profiling and immunohistochemical studies to pathogenetically involve the EMT in which the sarcomatous component is converted from a carcinomatous component through an EMT-induced metaplastic process.[24,25] The differential expression of fibrillin-1 in over half of metaplastic carcinomas suggests an association between fibrillin-1 expression and the EMT.

Although fibrillin-1 was differentially expressed in the MCB subtype, notably, fibrillin-1 expression was solely observed in the spindle sarcomatous metaplastic tumour component, but not in other metaplastic tumour components, including matrix-producing, pleomorphic, and squamous metaplasia, resulting in an overall fibrillin-1 positivity in 93.7% of MCBs with SSM, compared with none in MCBs without SSM. Furthermore, the restriction of fibrillin-1 expression to SSM was observed in non-mammary carcinosarcomas, a biphasic carcinoma with a sarcomatous component converted from a carcinomatous component through an EMT-induced metaplastic process,[26] with fibrillin-1 expression in 93.3% of the non-mammary carcinosarcomas with SSM, but in none of the non-mammary carcinosarcomas without SSM. Although these metaplastic components are considered metaplastic elements of conventional carcinoma, the underlying pathogenesis and marker expressions in each metaplastic histological element remains elusive and may be distinctive. In fact, a recent study of genomic profiling of MCB has revealed genetic heterogeneity that correlates with metaplastic histological subtype.[27] Our findings of the enrichment of fibrillin-1 expression solely in spindle sarcomatous tumour elements suggests that fibrillin-1 expression may be pathogenetically associated with spindle metaplasia, but not other metaplasia, in metaplastic carcinoma across different organs. Such *in vivo* observations are consistent with the upregulation of fibrillin-1 expression in the spindle metaplastic cells in the present TGF-β-induced EMT model where only spindle metaplastic cells, but not other types of metaplastic cells, were observed. Whether upregulation of TGF-β signalling and resultant fibrillin-1 expression is pathogenetically associated spindle metaplasia in MCB remains to be investigated.

EMT-initiating transcriptional factors, such as Snail, Slug, ZEB-1, and TWIST, induce the EMT phenotype through the downregulation of epithelial markers (e.g., E-cadherin) and upregulation of mesenchymal markers (e.g., N-cadherin, fibronectin and vimentin).[17,28,29] Vimentin is an intermediate filament and is considered an EMT marker.[30] Although it is not a transcriptional factor, it induces changes in cell shape, motility, and adhesion during EMT.[31] However, in the present study, *FBN1* knockdown in *FBN1*-expressing MDA-MB-231 breast cancer cells did not compromise migration, invasion, and tumourigenesis and did not alter the expression levels of other EMT markers. This finding implies that fibrillin-1 may represent a marker induced during spindle metaplasia in the process of EMT and that it does not induce EMT features. Despite the presence of EMT markers in carcinomas being associated with poorer prognosis, notably, in this study, fibrillin-1 staining was consistently negative for carcinomatous components in both non-metaplastic and metaplastic breast cancer subtypes. Our finding is in contrast to the reports that have shown fibrillin-1 immunostaining in >80% of the tested samples of ovarian serous carcinoma, thyroid papillary carcinoma, and germ cell carcinoma.[32–34] Whether tissue-specific distribution or differences in antibodies used accounted for the discrepancy remains to be investigated.

In summary, fibrillin-1 is a novel TGF-β-induced marker. Fibrillin-1 expression in SSM but not in other metaplasia and carcinomatous components, in both MCBs and non-mammary carcinosarcomas, together with the inability of *FBN1* knockdown to compromise migration, invasion, and tumorigenesis, indicates that fibrillin-1 represents a marker induced solely in spindle metaplasia during EMT and does not induce EMT, nor does it lead to tumour aggressiveness.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pathol.2019.02.001.

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