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Cancer-associated fibroblast (CAF)-derived IL32 promotes breast cancer cell invasion and metastasis via integrin β3–p38 MAPK signalling

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ABSTRACT

Metastasis is the leading cause of breast cancer–related deaths. Cancer-associated fibroblasts (CAFs), the predominant stromal cell type in the breast tumour microenvironment, may contribute to cancer progression through interaction with tumour cells. Nonetheless, little is known about the details of the underlying mechanism. Here we found that interaction of interleukin 32 (IL32) with integrin β3 (encoded by *ITGB3;* a member of the integrin family) mediating the cross-talk between CAFs and breast cancer cells plays a crucial role in CAF-induced breast tumour invasiveness. IL32, an 'RGD' motif–containing cytokine, was found to be abundantly expressed in CAFs. Integrin β3 turned out to be up-regulated in breast cancer cells during epithelial–mesenchymal transition (EMT). CAF-derived IL32 specifically bound to integrin β3 through the RGD motif, thus activating intracellular downstream p38 MAPK signalling in breast cancer cells. This signalling increased the expression of EMT markers (fibronectin, N-cadherin, and vimentin) and promoted tumour cell invasion. Counteracting IL32 activity, a knockdown of IL32 or integrin β3 led to specific inactivation of p38 MAPK signalling in tumour cells. Blockage of the p38 MAPK pathway also diminished IL32-induced expression of EMT markers and breast cancer cell invasion and metastasis. Thus, our data indicate that CAF-secreted IL32 promotes breast cancer cell invasion and metastasis via integrin β3–p38 MAPK signalling.

1. Introduction

It has been well established that a tumour microenvironment is an active participant in the process of cancer progression [1–3]. Activated stromal cells [usually named as cancer-associated fibroblasts (CAFs) or myofibroblasts] are the major components in the tumour microenvironment [4,5]. Compared with normal fibroblasts (NFs), CAFs overexpress some biomarker proteins, such as α-smooth muscle actin (α-SMA), fibroblast activation protein (FAP), platelet-derived growth factor receptor α or β (PDGFR-α/β), or S100 calcium-binding protein A4 (S100A4), depend on the tumour type [6]. Generally, the cross-talk between CAFs and tumour cells contributes to tumour progression mainly via a release of various secretory proteins (e.g. TGF-β, IGF, and IL6), direct interactions with tumour cells, immune-response regulation, and extracellular matrix (ECM) remodelling [7].

Integrins belong to the transmembrane protein family and act as cell surface receptors that mediate cell–cell and cell–ECM adhesion [8]. Integrins are bidirectional signalling receptors [9] and can be activated by proteins binding to their extracellular domain (called outside-in signalling) or to their intracellular domain (named inside-out signalling); the signals are transmitted through tilting or pistoning movements of the transmembrane domain [10,11]. Integrin β3 in particular is a member of the RGD motif receptor family and plays a crucial pro-metastasis role in lung cancer, breast cancer, colorectal cancer, bladder cancer, melanoma, and glioblastoma [12–17]. Integrin β3 promotes cancer metastasis in part by phosphorylating a series of downstream proteins including Src, FAK, and Syk; this action enhances cell migration and invasion [18,19]. Some microRNAs (e.g. miR-let-7c and miR-30a-5p) can suppress metastasis by down-regulating integrin β3 [14,20]. In addition, a number of studies have revealed that actual participation of integrin β3 in metastasis depends on a series of downstream signalling activation events, affecting for example PI3-K–AKT, NF-κB, MAPK, and STAT1 pathways [19,21,22]. Nevertheless, all the aforementioned cascades belong to 'inside-out' transduction.

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Whether a component of a tumour microenvironment, especially CAFs, can facilitate breast cancer invasion and metastasis through integrin β3 in an 'outside-in' manner remains unclear.

Interleukin 32 (IL32), also known as NK4, contains an RGD cell attachment sequence and is a member of the cytokine family that can induce pro-inflammatory cytokines such as TNF-α, IL8, and MIP2 [23]. Expression of IL32 has been found in a variety of tissues, including lungs, the liver, colon, and pancreas [24–27]. In contrast to other inflammatory factors that are expressed predominantly in immune cells, IL32 is expressed in epithelial cells and fibroblasts [28,29]. Although some research has revealed participation of IL32 in immune diseases (e.g. rheumatoid arthritis), infectious diseases (e.g. hepatitis B), inflammatory reactions, and angiogenesis [30–33], whether IL32 is involved in biological behaviours of a tumour, particularly the aggressiveness mediated by the tumour microenvironment, is largely unknown.

In this study, we demonstrate that CAFs (in contrast to NFs) can promote tumour invasion of integrin β3–positive breast cancer cells. The protein IL32 derived from CAFs binds to integrin β3 at the surface of breast cancer cells, thereby activating the downstream p38 MAPK pathway to enhance fibronectin, N-cadherin, and vimentin expression and strengthens breast cancer cell invasion and metastasis. Therefore, our data shed light on the mechanism underlying the cross-talk of stromal cells with tumour cells and offer a new strategy for the search for stromal therapeutic targets.

2. Materials and methods

2.1. Tissue samples and isolation of primary fibroblasts

Human breast tumour tissues and their corresponding normal breast tissues (at least 5 cm away from a tumour) were obtained from patients with breast tumours resected at the First Affiliated Hospital of Chongqing Medical University. All the patients who were involved in this study consented to participate in the study and to publication of its results, did not receive any radiotherapy or chemotherapy previously. The experiments were approved by the Ethics Committee of Chongqing Medical University.

Fibroblasts were isolated as previously described [34]. In brief, tumours and non-tumour tissues were minced and digested in type I collagenase (Sigma, USA), then cultured in DMEM with 10% of foetal bovine serum (FBS; GIBCO, USA) in a humidified atmosphere containing 5% of CO_2 at 37 °C until the fibroblasts got attached to the culture dish. The primary fibroblasts were used before passage 6.

2.2. Cell culture, plasmids, and reagents

Human mammary epithelial cell line MCF10A, breast cancer cells (including cell lines SKBr3, T47D, MDA-MB-453, MCF7, BT474, MDA-MB-468, BT549, and Hs578T), and HEK293T embryonic kidney cells were acquired from the American Type Culture Collection (ATCC). The cells were cultured according to standard protocols. Paired immortalised CAFs and NFs have been described elsewhere [35]. Cell lines MCF10A/Twist, MCF10A/Vector, BT549-shCtrl, and BT549-shTwist were established as described previously [34].

The human integrin β3-, integrin β4-, integrin β1-encoding vectors were obtained from Addgene (www.addgene.org) or Origene (www.origene.com.cn). For all the short hairpin RNA (shRNA) oligonucleotides, synthetic DNA inserts were cloned into the pGLVH1/GFP vector (GenePharma, China). The target sequences of integrin β3 shRNAs were shRNA-1: 5'-GAATTGTACCTATAAGAAT-3', and shRNA-2: 5'-GAAAAT CCGTTCTAAAGTA-3', the target sequences of IL-32 shRNA were 5′-CTCCTCTACTTGAAAAAGA-3′. The control shRNA, which did not match any known human cDNA, was 5′-TTCTCCGAACGTGTCACGT-3′. Human recombined IL32 (rIL32), TNF-α, MIP2, and IL8 were the products of R&D systems (Minnesota, USA); and mutant recombinant IL32 (RGD motif mutated to RGE) was purchased from Sangon (Shanghai, China). A p38 MAPK inhibitor, SB203580, was purchased from Selleck (Shanghai, China).

2.3. RNA preparation and quantitative reverse-transcription PCR (qRT-PCR)

Total-RNA samples were extracted using the TRIzol reagent (TAKARA, Japan) then subjected to reverse transcription with the PrimeScript RT Reagent Kit (TAKARA, Japan). qRT-PCR was performed by means of the SYBR Premix Ex Taq II kit (TAKARA, JPN). Standard curves were generated, and the relative amounts of target gene mRNA were normalised to β-actin. The utilised primer sequences are listed in Supplementary Table 1. All the experiments were performed at least three times.

2.4. Immunohistochemistry (IHC) and immunofluorescent staining (IF)

Tumourous and normal tissues were fixed with 10% formalin. Paraffin-embedded specimens were sectioned at 4 μm thickness. The immunohistochemical protocols were described previously [35]. Tissue sections were incubated with a rabbit polyclonal antibody against integrin β3 (1:200; Abcam, UK). The normal rabbit IgG served as a negative control to eliminate non-specific staining. Integrin β3-positive cells were counted in at least 20 fields of view, each section at 200 × magnification. Image-Pro plus 6.0 software (Media Cybernetics, USA) was employed to quantify the IHC staining. Mean optical density (MOD: IOD/area) was used to evaluate integrin β3 expression levels. p-p38 (1:100; Bioworld, USA), Ki67 (1:200; Abcam, UK) were conducted in the same manner.

For IF staining, cells were grown on pre-prepared coverslips in a 24-well plate and were co-cultured with CM from CAFs for 3 h at 37 °C, then fixed with 4% paraformaldehyde. The subsequent staining was described in detail as done previously [35]. The specific antibodies against IL32 (Rockland, USA) and integrin β3 (Santa Cruz, USA) were employed in the IF staining. The normal rabbit or mouse IgG served as a negative control. A fluorescein isothyonate-labelled goat anti-rabbit and Cy3-labelled goat anti-mouse IgG antibodies served as a secondary antibody (Sigma, USA).

2.5. Western blotting

Western blotting analysis was performed as described previously [36]. Briefly, total cell proteins were extracted in RIPA lysis buffer (Beyotime, China), quantified with BCA protein assay kit (Beyotime, China), resolved in a 6–12% SDS-PAGE gel, and then were incubated with appropriate primary antibodies. β-Actin (ZSGBBIO, China) was used as a loading control. The specific primary antibodies employed in this study were as follows: anti–integrin β3 (Abcam, UK), anti-IL32 (Rockland, USA), anti-p38, anti-AKT, anti-STAT1, and the corresponding phosphorylated proteins (Bioworld, USA), anti-fibronectin (Bioworld, USA), anti–N-cadherin (Bioworld, USA), and anti-vimentin (Bioworld, USA). Horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG antibody (ZSGBBIO, China) served as a secondary antibody, and the protein expression levels were visualised by the enhanced chemiluminescence system (Bio-Rad, Hercules, EDA USA). Images were captured using Scion image software.

2.6. Western blotting of immunoprecipitates (IP-WB)

IP-western blotting was performed with the lysates from HEK293T and BT549 cells. The cells were incubated with rIL32 (20 ng/ml) or CM derived from CAFs for 3 h. The extracts were cleared by centrifugation and then pre-cleared by gentle rocking at 4 °C with washed protein G-Dynabeads (Abcam, UK). The pre-cleared extracts subjected to immunoprecipitation with 2 μg of an anti-IL32 or anti–integrin β3

antibody and 20 μl Protein G Dynabeads overnight. Equivalent amounts of immunoglobulin G (IgG) served as the control. The Protein G Dynabead–IgG conjugates were washed five times with lysis buffer and boiled in SDS sample buffer, and the released proteins were resolved by SDS-PAGE. The immunoprecipitates were analysed by western blotting with a primary antibody against IL32 or integrin β3.

2.7. Enzyme-linked immunosorbent assay (ELISA)

IL-32 protein levels in CAFs and NFs supernatants were measured by ELISA (R&D, USA). The absorbance (450 nm) of each sample was detected on a standard automatic microplate reader (BioTek, USA).

2.8. A transwell assay

CAFs or NFs at approximately 80% confluence were washed with PBS, and then cultured in a fresh serum-free DMEM medium for 48 h. The conditioned medium (CM) was collected and filtered for further analysis. For neutralisation experiments, a neutralizing antibody against human IL32 (Rockland, USA, 500 ng/ml) was pre-incubated at 37 °C with the supernatant for 1 h before invasion assays.

For the Transwell assay, breast cancer cells (2×10^4) were re-suspended in 200 μl of the CM and were seeded into the upper well of 8-μm-pore Boyden chambers (Millipore, GER) coated with Matrigel (Corning BioCoat, USA, 1:7.5), and 500 μl medium with 10% FBS was added into the lower well. After incubation at 37 °C and 5% CO_2 for 12 h, the cells that adhered to the upper surface of the filter were removed with a cotton applicator. Stained with 0.5% crystal violet, the cells that invaded to the opposite side of the filter were counted under the microscope (Nikon, Japan). The data represent at least three experiments conducted in triplicate (mean ± standard error).

2.9. An integrin-binding assay

These assays were performed as described previously [37]. Briefly, 96-well microtiter plates were coated with 1 μg/ml recombinant αvβ3 (R&D, USA) or α5β1 (R&D, USA). The plates were incubated with different concentrations (0–160 ng/ml) of recombinant IL32 (R&D, USA) diluted in PBS. Bound proteins were quantified by ELISA using an HRP-conjugated anti-IL32 (R&D, USA) antibody at 450 nm. The data represent the means of triplicate experiments. Saturation binding assays were conducted to calculate apparent dissociation constants as previously described [38].

2.10. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] MTT assay

Cell growth was assessed by the MTT assay as previously described [35]. Briefly, 3×10^3 cells were seeded in 96-well plates containing 200 μl of complete medium. The cells were treated with recombinant IL32 (20 ng/ml) or vehicle (PBS) after attachment. Cell culture for the specified time, MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h. After DMSO (ThermoFisher, USA) was added into each well, the absorbance was recorded on a microplate reader (BioTek, USA) at 490 nm.

2.11. Tumour xenografts

Animal experiments were approved by the animal care ethics committees at Chongqing Medical University. Breast cancer BT549 cells (10^6) were mixed or not mixed with an equal number of NFs or CAFs in 200 μl PBS: Matrigel at a 1:1 ratio and were subcutaneously injected into 4-week-old female nude mice. Tumour growth was evaluated by monitoring tumour volume (V = length × width^2 × 0.5) every 4 days. For the mice injected with BT549 cells alone, when the xenografts were palpable (~3 mm in diameter), intra-tumour injection of vehicle or rIL32 at 0.1 μg/kg was performed twice weekly for 5 consecutive weeks. The animals were euthanised on the 45th day after introduction of xenografts, and the tumours and mouse lungs were harvested for further research. Cryosections (4 μm) of the tissues were subjected to H &E staining for histological assessment.

2.12. Statistical analysis

This analysis was performed in SPSS standard version 19.0 software. The data are presented as mean ± SD from at least three independent experiments. Student's *t*-test was performed for single comparisons between two groups, and ANOVA followed by the Student–Newman–Keuls multiple-comparison test was conducted for a comparison between multiple groups. A P value < 0.05 in all cases was assumed to indicate statistical significance.

3. Results

3.1. Integrins are dysregulated during the epithelial–mesenchymal transition (EMT) of mammary cells

Our previous study and other reports have revealed that EMT is critical for tumour cell invasion and metastasis [39–41]. Using a cDNA array and proteomic analyses, we identified a series of dysregulated genes of the integrin family in mammary cells during EMT (Fig. 1A). Some of these integrins (ITGB1, ITGB3, ITGB4, ITGB5, ITGAV, ITGA5, ITGA3, ITGA9, and ITGA11), which were simultaneously identified by the cDNA array analysis and proteomics, were next validated by qRT-PCR. Growing evidence suggests that Twist is an inducer of EMT [40,42], and ITGB1, ITGB3, and ITGB4 were proved here to be significantly up-regulated during EMT in mammary cells (Fig. 1B).

In line with this finding, the integrin family [integrin β1 (encoded by *ITGB1*), integrin β3 (encoded by *ITGB3*), and integrin β4 (encoded by *ITGB4*)] mRNA expression levels were found to be significantly elevated in breast cancer cells overexpressing Twist (e.g. BT549 and Hs578T cells) as compared with those with low expression of Twist (e.g. MCF10A and MCF7 cells; Fig. 1C). Furthermore, the loss of Twist apparently down-regulated ITGB1, ITGB3, and ITGB4 in BT549 cells (Fig. 1D).

It has been reported that breast CAFs, not NFs, can promote breast cancer cell migration and invasion [43,44]. To test whether CAFs can promote mammary cell invasion by means of the integrin family, MCF10A cells were transfected with ITGB1, ITGB3, or ITGB4 and co-cultured with CAFs or NFs. The potentials for cell invasion were tested. Although ITGB1, ITGB3, and ITGB4 increased the invasive capacity of MCF10A (Fig S1A), only ITGB3 strengthened the cell invasion of MCF10A cells during co-culture with CAFs (Fig. 1E). Similarly, a loss of Twist in BT549 cells attenuated the invasion ability (Fig S1B), but silencing of Twist in BT549 cells dramatically reduced their invasive capacity in co-culture with CAFs (Fig. 1F).

Next, we investigated the expression levels of integrin β3 in normal breast tissue samples, ductal carcinoma in situ (DCIS), and in invasive and metastatic breast tumour tissues by qRT-PCR and IHC staining. We found that integrin β3 was undetectable or under-expressed in normal mammary tissues, but integrin β3 levels gradually increased during progression of tumour tissues from DCIS to metastasis (Fig. 1H and I). Collectively, these data indicated that the expression of integrin β3 is a pro-invasive or pro-metastatic factor toward breast cancer.

3.2. CAFs promote breast cancer cell invasion via integrin β3

To confirm the correlation between integrin β3 expression and the invasive ability of breast cancer, normal mammary epithelial cells MCF10A, breast cancer cells with a low invasive potential (e.g. cell lines SKBr3, T47D, MDA-MB-453, BT474), and breast cancer cells with a high invasive potential (e.g. MDA-MB-468, BT549, and Hs578T) were

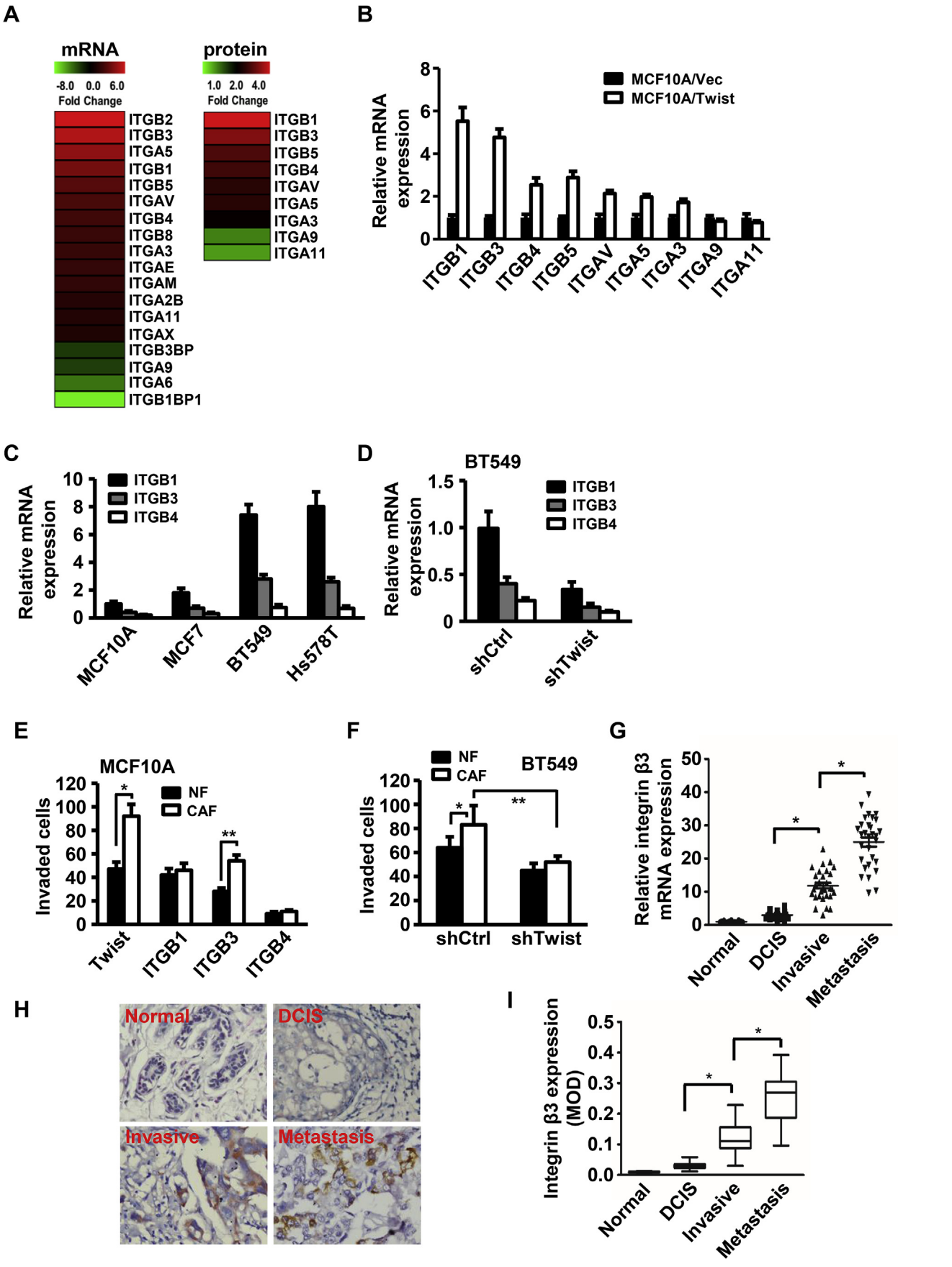

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Fig. 1. Integrins are dysregulated during EMT in mammary cells. (**A**) The expression levels of the integrin family in MCF10A/Twist and MCF10A control cells were identified by cDNA array and proteomics analyses. Data are presented as relative fold changes (> 1.2) of MCF10A/Twist vs MCF10A/Vector. (**B**) mRNA expression levels of integrins were determined by qRT-PCR analysis in MCF10A/Twist and MCF10A/vector cells. The data are presented as mean ± SD. (**C, D**) mRNA expression levels of ITGB1, ITGB3, and ITGB4 were assessed by qRT-PCR analysis of normal breast cells MCF10A and breast cancer cell lines (MCF7, BT549, and Hs578T) (C) and in BT549 cells transfected with shRNA against Twist (BT549/shTwist) or control shRNA (BT549/shCtrl) (D). The data represent mean ± SD. (**E, F**) A Transwell assay was carried out to test cell invasion abilities of MCF10A cells transfected with Twist, ITGB1, ITGB3, and ITGB4 (E); or BT549 cells transfected with shRNA against Twist (BT549/shTwist) or control shRNA (BT549/shCtrl) (F) in the presence of a culture supernatant derived from CAFs or NFs (*$P < 0.05$, **$P < 0.01$). (**G**) Integrin β3 mRNA levels were detected by qRT-PCR in normal breast tissues, ductal carcinoma in situ (DCIS), and samples of breast cancer tissues with invasive and metastatic lymph nodes. Data are shown as relative fold changes of each group compared with the normal group (n = 30 per group). (**H**) Representative images of IHC staining of integrin β3 in normal breast tissues, DCIS, and in breast cancer tissues with invasion and metastatic lymph nodes (n = 27 per group). Magnification: × 200. (**I**) Semi-quantitative analysis of integrin β3 expression presented in (H); the MOD represents the mean value of integrin β3 staining at different clinical stages (*$P < 0.05$).

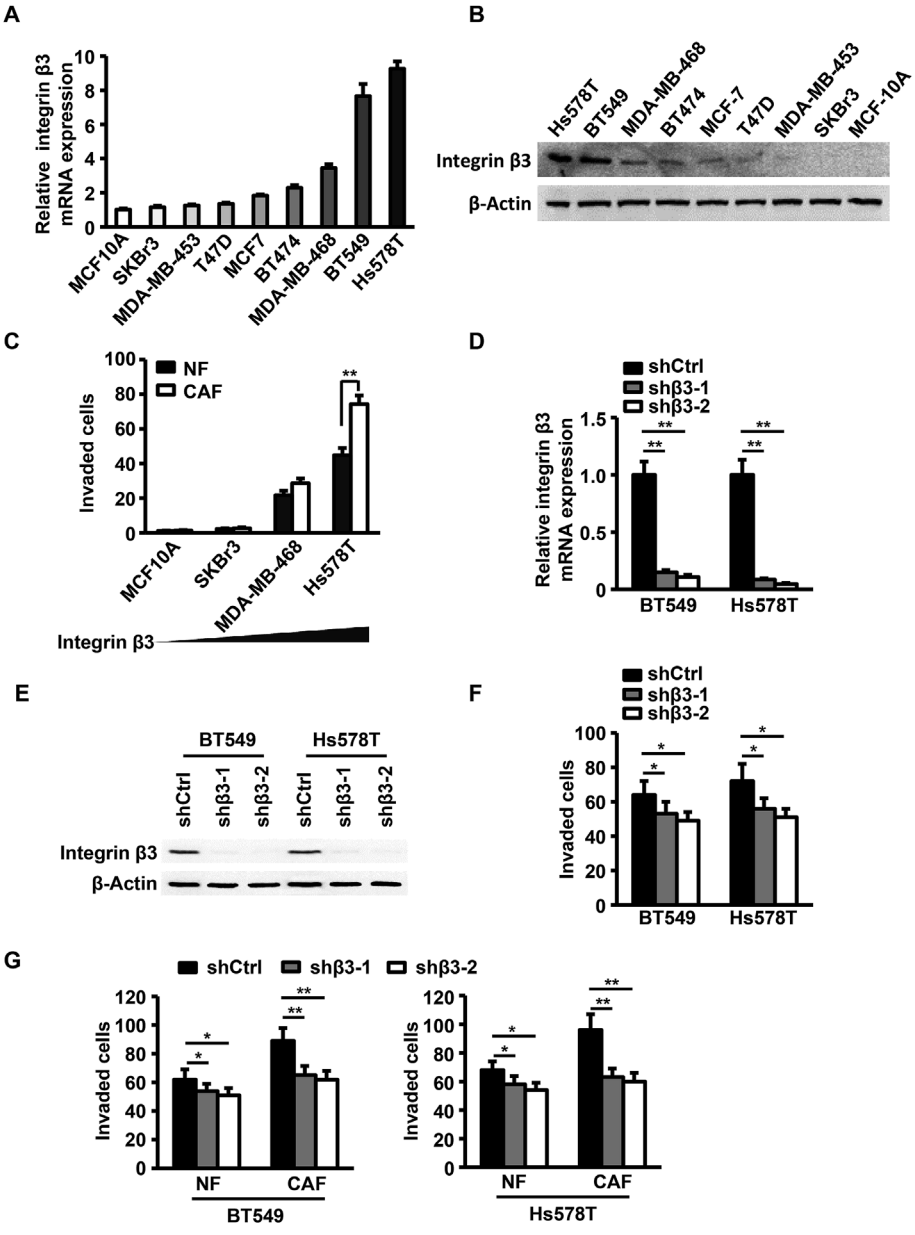

Fig. 2. CAFs promote breast cancer cell invasion via integrin β3 (**A, B**) Integrin β3 mRNA (A) and protein (B) levels in normal mammary epithelial cells and in different breast cancer cell lines. (**C**) The invasion potentials of normal mammary epithelium and of the indicated breast cancer cell lines in the presence of a culture supernatant (CM) derived from CAFs or NFs (**$P < 0.01$). (**D, E**) Integrin β3 mRNA (D) and protein expression (E) was detected in BT549 and Hs578T cells transfected with shCtrl, shβ3 #1, and shβ3 #2, respectively. Data are given as mean ± SD (**$P < 0.01$). (**F**) Cell invasion of integrin β3 knockdown BT549 and Hs578T cells (BT549-shβ3 and Hs578T-shβ3) (*$P < 0.05$). (**G**) Cell invasion abilities of integrin β3–deficient BT549 and Hs578T cells in the presence of a culture supernatant (CM) derived from CAFs or NFs (*$P < 0.05$; **$P < 0.01$). (Here, shβ3: shRNA against integrin β3.)

subjected to determination of integrin β3 levels. Consistent with the above findings, the mRNA and protein expression levels of integrin β3 turned out to significantly increase in the malignant breast cancer cells (Fig. 2A and B). Indeed, the invasive ability of breast cancer cells co-cultured with CAFs significantly increased (Fig. 2C), this phenomenon was closely associated with their integrin β3 levels (Fig. 2A).

To clarify whether CAFs can promote breast cancer cell invasion via integrin β3, the invasive potentials of integrin β3 knockdown BT549 and Hs578T cells were evaluated next. The knockdown of integrin β3 (Fig. 2D and E) reduced tumour cell invasion (Fig. 2F). Notably, during co-culture of integrin β3 knockdown tumour cells with CAFs or NFs, a significant attenuation of the invasive ability was detected only in tumour cells co-cultured with CAFs (not with NFs; Fig. 2G), suggesting that integrin β3 is essential for CAF-stimulated tumour cell invasion.

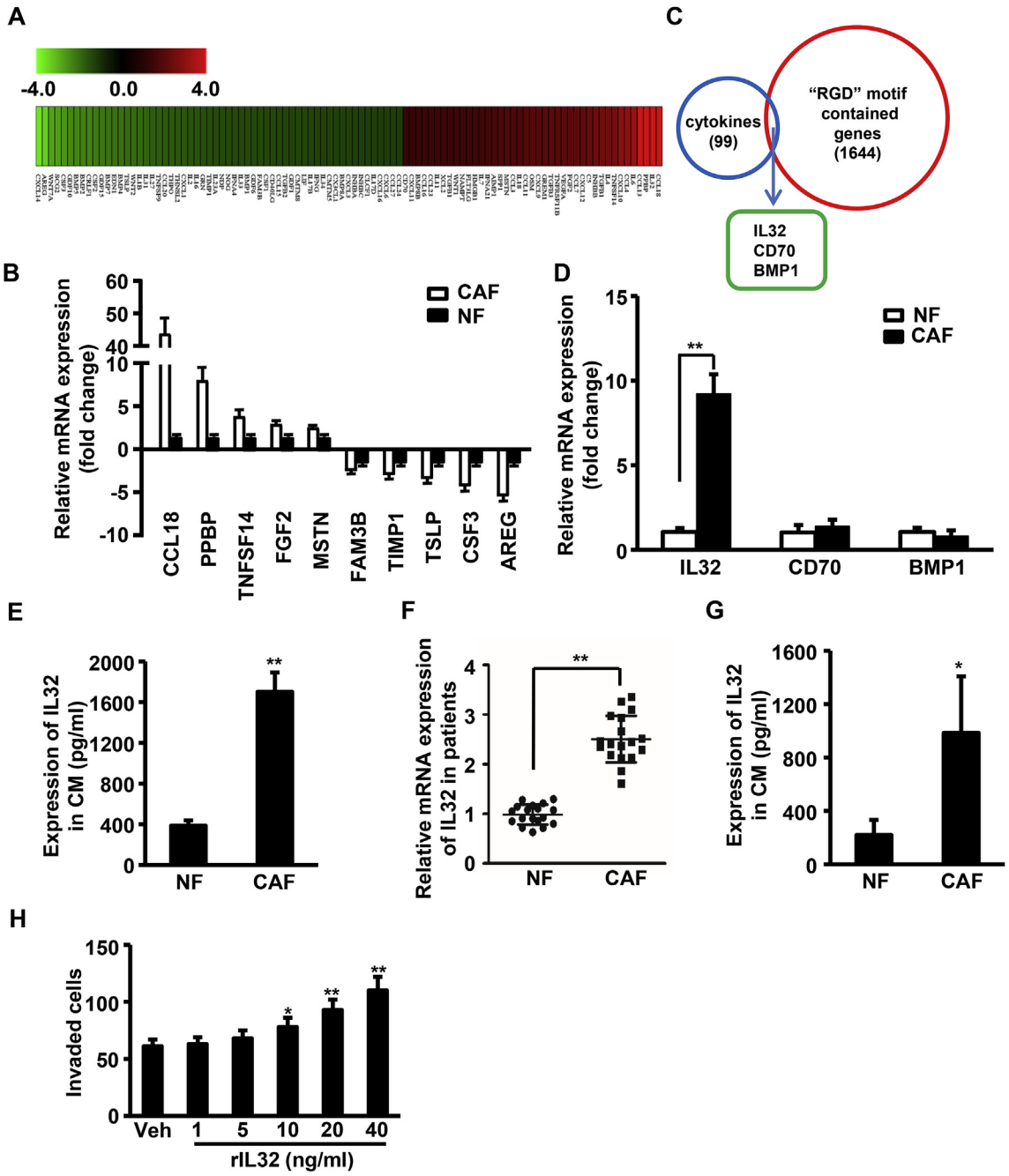

Fig. 3. Differential cytokine levels between CAFs and NFs. (**A**) The differentially expressed cytokine genes identified by microarray analysis of CAFs and NFs. Data are shown as relative fold changes (> 1.2) in CAFs vs NFs. (**B**) Expression of 10 randomly selected cytokine genes was verified by qRT-PCR in CAFs and NFs. (**C**) Three dysregulated cytokines containing the RGD motif in CAFs were identified by a bioinformatic analysis. (**D**) mRNA levels of IL32, CD70, and BMP1 were determined by qRT-PCR in CAFs and NFs (**$P < 0.01$). (**E**) IL32 protein levels in the culture supernatant of CAFs and NFs were measured by an ELISA (**$P < 0.01$). (**F**) IL32 mRNA expression levels were detected in CAFs and their paired NFs derived from breast tumour tissues (**$P < 0.01$, n = 18). (**G**) IL32 protein levels in the culture supernatant of primary CAFs and their paired NFs were measured by an ELISA (*$P < 0.05$). (**H**) The dose effects of rIL32 (0–40 ng/ml) on the invasiveness of BT549 cells (*$P < 0.05$, **$P < 0.01$). The vehicle (PBS) served as a control for rIL32.

Thus, these data indicated that CAFs can promote breast cancer cell invasion via integrin β3.

3.3. CAF-secreted IL32 serves as a mediator between CAFs and breast cancer cells

The interactions between tumour cells and the tumour microenvironment are mediated via direct (stromal cells or deposited substances) or indirect (secretion of proteins) mechanisms [1]. The CM derived from CAFs promoted breast cancer cell invasion, suggesting that some of the secreted factors may play a role in the cross-talk between breast tumour cells and CAFs. Thus, we analysed the mRNA expression profiles of CAFs and NFs derived from breast tumour tissues. A set of cytokine genes was found to be dysregulated in CAFs. Among these, 41 cytokines were up-regulated, while 58 cytokines were down-regulated (Fig. 3A), as confirmed by qRT-PCR analysis of 10 randomly chosen dysregulated mRNAs (Fig. 3B). Integrin β3 is a cell surface receptor that recognises its ligand through the RGD motif. To identify the potential ligands of integrin β3 among these dysregulated cytokines, we compared these cytokines with all RGD motif–containing proteins in a

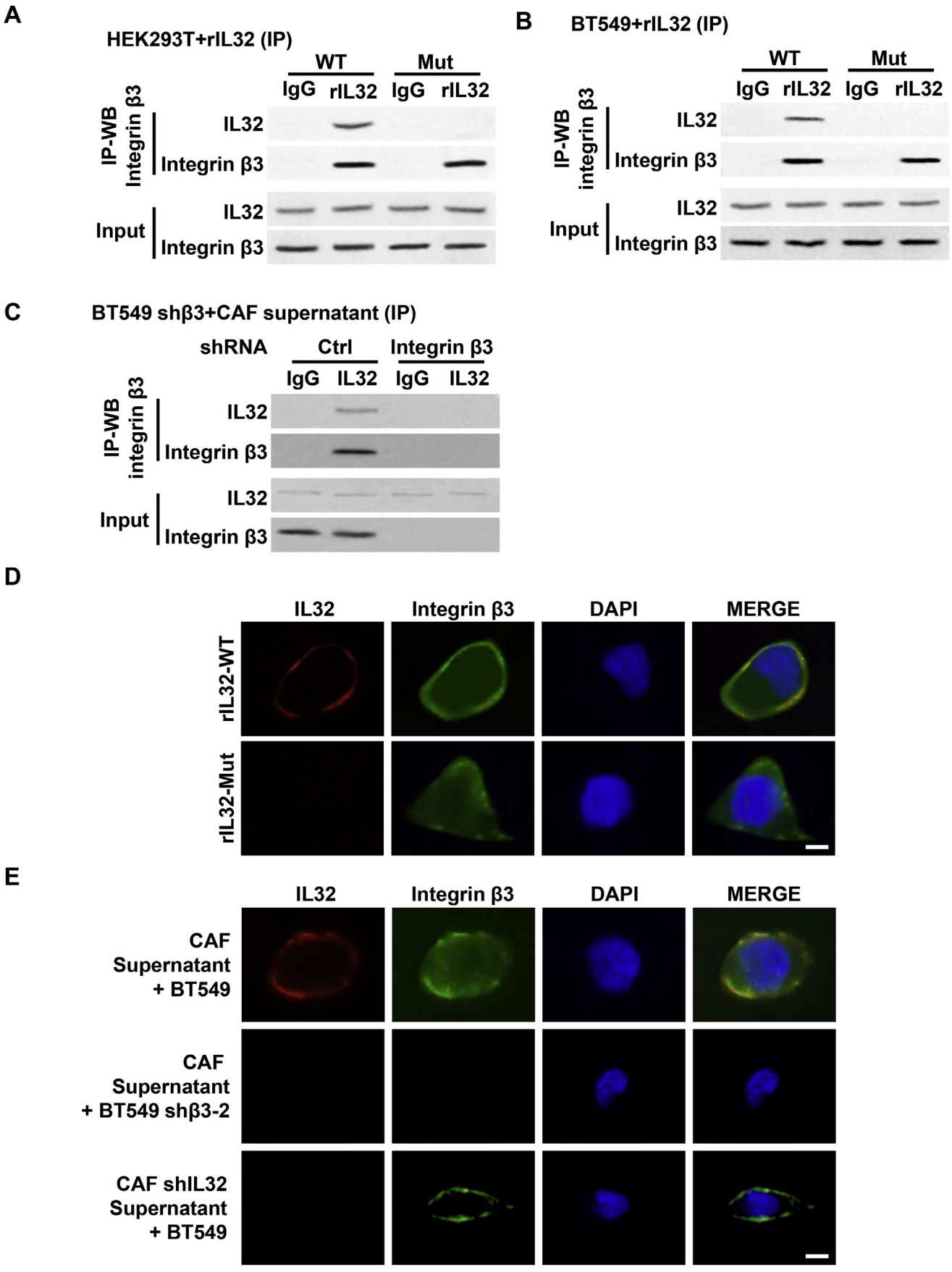

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Fig. 4. IL32 associates with transmembrane integrin β3 of breast cancer cells. (**A**) HEK293T cells transfected with an expression vector encoding integrin β3 were cultured in the FBS-free medium with recombinant wild-type IL32 (WT) or mutant IL32 (RGD motif mutated into RGE, Mut) for 3 h. Immunoprecipitation with western blotting was conducted with an antibody against IL32 or integrin β3. (**B**) BT549 cells were cultured in the FBS-free medium with recombinant wild-type IL32 (WT) or mutant IL32 (RGD motif mutated into RGE, Mut) for 3 h. Immunoprecipitation with western blotting was conducted as in (A). (**C**) BT549 or BT549/sh-β3 (shRNA-2) cells were cultured with the CM derived from CAFs for 3 h, and the binding of IL32 and integrin β3 was confirmed by immunoprecipitation with western blotting analysis. (**D**). BT549 cells were treated with recombinant wild-type IL32 (rIL32-WT) or mutant IL32 (rIL32-Mut) as in (A). Confocal microscopy showing that integrin β3 and IL32 co-localised at the membrane of BT549 cells stained with a Cy3-labelled anti-IL32 antibody and FITC-labelled anti–integrin β3 antibody. (**E**) BT549 cells (upper panel) or BT549/sh-β3 (shRNA-2) cells (middle panel) were treated with CM derived from CAFs or BT549 cells treated with CM from CAFs/sh-IL32 cells (lower panel) and stained with the antibodies described in (D). Co-localisation of transmembrane integrin β3 and IL32 was visualised by confocal microscopy. Cell nuclei were counterstained with DAPI (scale bar, 5 μm).

public database (http://prosite.expasy.org/). Three candidate cytokines including IL32, CD70, and BMP1 were identified (Fig. 3C). After checking their expression by qRT-PCR (Fig. 3D), IL32 was found to be an abundant signalling protein with the RGD motif in CAFs, as also proved by an ELISA (Fig. 3E) and Chen's findings [45]. Besides, the enhanced IL32 production in CAFs was next corroborated by measuring its expression in another 18 paired samples of primary CAFs and NFs from patients with breast cancer (Fig. 3F and G). Furthermore, treatment of BT549 cells with recombinant human IL32 (rIL32) enhanced the cell invasion ability in a dose-dependent manner (Fig. 3H).

Subsequently, we wanted to know whether IL32 interacts with integrin β3 at the plasma membrane of breast cancer cells. Herein, the binding of IL32 to integrin β3 was firstly proved by immunoprecipitation with western blotting. HEK293T cells transfected with integrin β3 were subjected to immunoprecipitation with wild-type rIL32 or mutant rIL32 (RGD mutated to RGE). As depicted in Fig. 4A and B, wild-type rIL32 bound to ectopic integrin β3 in HEK293T cells (Fig. 4A) and to endogenous integrin β3 in BT549 cells (Fig. 4B). Substitution of the RGD motif with an inactive RGE motif in IL32 abrogated the ability of IL32 to bind to integrin β3, thereby confirming the RGD-dependent interaction of IL32 with integrin β3. Consistently with these findings, the IL32 secreted from CAFs also bound to integrin β3 in BT549 cells (Fig. 4C). By contrast, after the knockdown of integrin β3 in BT549 cells, the interaction between IL32 and integrin β3 was not detectable (Fig. 4C). In addition, immunofluorescent staining clearly indicated that wild-type but not mutant IL32 can co-localise with integrin β3 at the plasma membrane of HEK293T cells (Fig. 4D). Moreover, CAF-derived IL32 co-localised with integrin β3 at the plasma membrane of BT549 cells. shRNA-mediated silencing of integrin β3 in BT549 cells or silencing of IL32 in CAFs (Fig S2A and S2B) efficiently abrogated IL32 localisation at the tumour cell membrane (Fig. 4E). Accordingly, these data showed that IL32 specifically binds to integrin β3 at the breast cancer cell membrane, suggesting that IL32 serves as a mediator of the cross-talk between CAFs and breast cancer cells.

αvβ3 and α5β1, both RGD-binding integrins, were detected in breast cancer cells [46] (Fig. 1B). To assess their affinity for IL32, saturation binding assays were conducted. As illustrated in Figure S2C, the αvβ3 integrin has stronger affinity for IL32 than α5β1 does. Furthermore, a loss of integrin β3 notably abrogated rIL32-stimulated cell invasion, suggesting that integrin β3 is essential for IL32-mediated cross-talk between CAFs and tumour cells (Fig S2D).

3.4. The interaction between IL32 and integrin β3 activates p38 MAPK signalling in breast cancer cells

p38 MAPK, PI3K–AKT, and JAK–STAT cascades have been reported to be the canonical downstream pathways of integrin β3 in various cells [19,21,22]. To elucidate which pathway(s) is/are stimulated by the IL32–integrin β3 axis in breast cancer cells, activation of relevant potential signalling molecules was determined by western blotting analysis. During co-culture with CAFs but not NFs, the p38 MAPK pathway, but not PI3K–AKT or JAK–STAT pathway, was activated in BT549 and Hs578T cells (Fig. 5A). Moreover, treatment of BT549 cells with rIL32 raised the phosphorylated-p38 levels in a dose-dependent manner (Fig. 5B). rIL32 (20 ng/ml) stimulated phosphorylation of p38, which was obviously decreased in integrin β3 knockdown breast cancer BT549 and Hs578T cells (Fig. 5C). In addition, the CAF CM neutralised with an anti-IL32 antibody remarkably attenuated the phosphorylation of the p38 protein in BT549 and Hs578T cells (Fig. 5D). Collectively, these data suggested that the p38 MAPK pathway is downstream of the IL32–integrin β3 axis in BT549 and Hs578T breast cancer cells.

3.5. The IL32–integrin β3–p38 MAPK signalling axis promotes breast cancer cell invasion and EMT

To understand the biological significance of the IL32–integrin β3–p38 MAPK axis, we evaluated the EMT biomarker expression levels during breast cancer cell invasion. Compared with the CM from NFs, CAFs' CM significantly increased the expression of mesenchymal biomarkers – fibronectin, N-cadherin, and vimentin – in BT549 and Hs578T cells (Fig. 6A). Adding rIL32 into the CM of NFs notably up-regulated mesenchymal proteins in BT549 and Hs578T cells (Fig. 6B). Nonetheless, CAF CM neutralised with the anti–IL-32 antibody blunted the expression of mesenchymal biomarkers (Fig. 6C). Similarly, after the knockdown of integrin β3 in BT549 and Hs578T cells, CAF CM could not stimulate fibronectin, N-cadherin, and vimentin expression, in contrast to the control tumour cells (Fig. 6D). In line with these findings, CM from IL32-deficient CAFs did not promote EMT marker expression (Fig. 6D). Furthermore, SB203580 (a p38 MAPK inhibitor) abrogated the rIL32-stimulated upregulation of mesenchymal markers (Fig. 6E). In agreement with these findings, CAF CM neutralised with the anti–IL-32 antibody significantly decreased invasion abilities of BT549 and Hs578T cells (Fig. 6F). Besides, adding rIL32 to CM from NFs notably increased BT549 and Hs578T cell invasion (Fig. 6G). SB203580 apparently abrogated the rIL32-stimulated invasive abilities of BT549 and Hs578 cells (Fig. 6H). These data indicated that the IL32–integrin β3–p38 MAPK axis facilitates breast cancer cell invasion and EMT.

To determine whether IL32 affects integrin β3 expression, mRNA and protein expression levels of integrin β3 in BT549 cells were measured during rIL32 treatment. Indeed, rIL32 up-regulated mRNA and protein expression of integrin β3 (Fig S3A–S3B). TNF-α, MIP2, and IL8 are the main pro-inflammatory cytokines induced by IL32 [23]; however, only IL8 (not TNF-α or MIP2) stimulated integrin β3 mRNA and protein expression (Fig S3C and S3D) and BT549 cell invasiveness (Fig S3E). These data indicated that IL32 per se and its target cytokine IL8 also promoted breast cancer cell invasion by up-regulating integrin β3.

3.6. IL32 enhances metastasis of breast cancers in vivo

To evaluate the pro-metastatic effect of the IL32-integrin β3-p38 MAPK signalling axis in vivo, BT549 cells (clones BT549-shNC and BT549-shβ3 (shRNA-2)) mixed with NFs or CAFs (clones CAF-shNC or CAF-shIL32) were subcutaneously injected into nude mice. The mice injected with the mixture of BT549 and CAFs developed the largest tumour; NFs slowed the tumour growth at an early stage but promoted the tumour growth at a late stage. The knockdown of IL32 in CAFs slightly enhanced the tumour growth; the knockdown of integrin β3 in cancer cells decreased the tumour growth (Fig. 7A and B). Ki67 staining revealed similar results in tumour tissue samples (Fig S4A). In line with

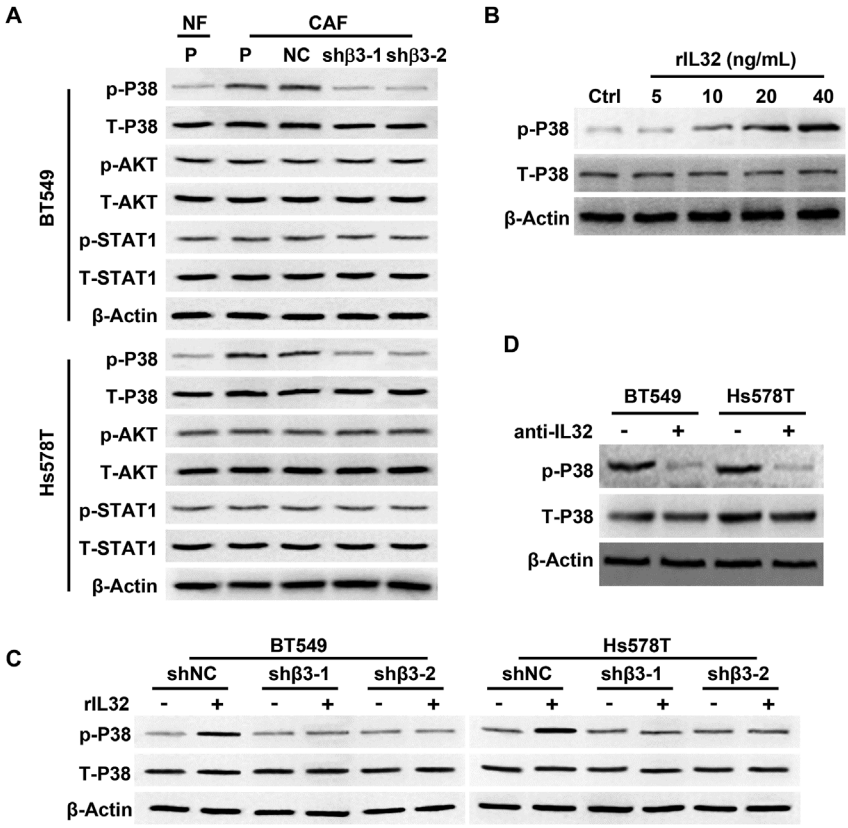

Fig. 5. IL32 activates the p38 MAPK pathway by interacting with integrin β3. (**A**) Western blotting to detect the activation of p38 MAPK, PI3K–AKT, and JAK–STAT signalling pathways in BT549 and Hs578T with integrin β3 and shNC or parental cells in the presence of the CM from CAFs or NFs. (**B**) The dose effects of rIL32 (0–40 ng/ml) on activation of p38 MAPK signalling in BT549 cells according to western blotting. (**C**) rIL32 (20 ng/ml) was added to cultured BT549 and Hs578T cells transfected with shβ3 and to control cells. Activation of p38 MAPK signalling was detected by western blotting. (**D**) CM from CAFs neutralised with the specific antibody against IL32 (10 μg/ml) was used to culture BT549 and Hs578T cells for 3 h. The activation of p38 MAPK signalling was determined by western blotting. β-Actin served as a loading control in all the western blot analyses. (*Legend.* P: parental cells; NC: control shRNA; shβ3: shRNA against integrin β3).

tumour growth, IL32 treatment or integrin β3 silencing in BT549 cells slightly slowed down the proliferation of BT549 cells in contrast with control BT549 cells (Fig S4B).

As expected, the mice injected with BT549 cells mixed with CAFs had more metastatic nodules in the lungs than the other mice did. The loss of IL32 in CAFs or silencing of integrin β3 in cancer cells reduced the metastases in mouse lungs. Compared with the mice injected with BT549 alone, exogenous addition of IL32 remarkably promoted cancer cell metastasis; however, the loss of integrin β3 in cancer cells efficiently attenuated the IL32-stimulated metastasis in mouse lungs (Fig. 7C). Consistently with tumour metastasis, the phosphorylated-p38 levels were much higher in the tumour of mice injected with the mixture of BT549 cells and CAFs than in tumours of other groups of mice. The knockdown of IL32 in CAFs or silencing of integrin β3 in cancer cells blunted the phosphorylation of p38; IL32 treatment of the tumour-bearing mice injected with BT549 cells alone stimulated p38 signalling in the tumour; however, silencing of integrin β3 in cancer cells almost eliminated the response to IL32 stimulation in tumours (Fig. 7D). The status of p38 signalling in tumour tissues was next confirmed by western blotting (Fig. 7E). These data suggested that IL32 secreted from CAFs contributed to breast cancer metastasis in vivo. Thus, our data provided evidence that CAFs secrete IL32, which binds to integrin β3 at the tumour cell membrane and activates p38 MAPK signalling to promote EMT and invasiveness of breast cancer cells (Fig. 7F).

4. Discussion

The tumour microenvironment is well known to facilitate tumour progression. The coordination between CAFs – as the major type of the stromal cells in a tumour microenvironment – and cancer cells is pivotal for tumour initiation, invasion, metastasis, and drug resistance [47]. Nonetheless, the underlying mechanisms are not well understood. Our previous study has shown that a set of integrins is dysregulated during EMT [39]. In the current work, we found that CAFs (in contrast to NFs) can promote invasion of integrin β3–positive breast cancer cells. IL32, an RDG domain–containing secreted protein, is highly expressed in CAFs and serves as a specific ligand of integrin β3. Of note, IL32 binding to integrin β3 at the tumour cell membrane in vitro and in vivo activates downstream p38 MAPK signalling to up-regulate EMT markers and enhance cancer cell invasion. These biological phenotypes and functions are blunted after either IL32 is suppressed in CAFs or integrin β3 is knocked down in cancer cells. Our findings suggest that the interaction of CAF-derived IL32 and integrin β3 at the tumour cell membrane mediates the cross-talk between CAFs and breast tumour cells thereby promoting breast cancer invasion.

The aberrant expression of integrin β3 is seen in some invasive and/or metastatic carcinomas such as glioblastoma, melanoma, colorectal cancer, and lung cancer [12,14,16,17]. Integrin β3 recognises ligands containing the RGD motif, a tripeptide which endows the ligand with an ability to interact with specific integrins. In accordance with the synergistic domains that confer integrin specificity and conformation of the RGD motif, the RGD-recognizing integrins have been described to bind to other ECM ligands with various degrees of affinity [46]. Some studies indicate that cytokines such as VEGF and tenascin C (TN-c) have a synergistic effect with integrin β3 in terms of promotion of breast cancer metastasis [21,48]. IL8 and TGF-β can induce cell invasion by up-regulating integrin β3 in lung cancer [49,50] although the mechanisms have yet to be elucidated. Our work reveals that integrin β3 acts as a receptor directly interacting with IL32 derived from CAFs to enhance breast cancer cell invasion.

Interleukins belong to a large family of proteins, which was firstly described as originating from leukocytes, and often act as pro-inflammatory factors regulating an immune response and angiogenesis [32,33]. Besides, some interleukins such as IL8 and IL6 can promote cancer progression as well [51]. IL32, a newly identified signalling protein, plays a crucial role in autoimmune disease responses (e.g. allergic rhinitis and rheumatoid arthritis). It has been reported that IL32 can suppress proliferation of vascular smooth muscle cells and

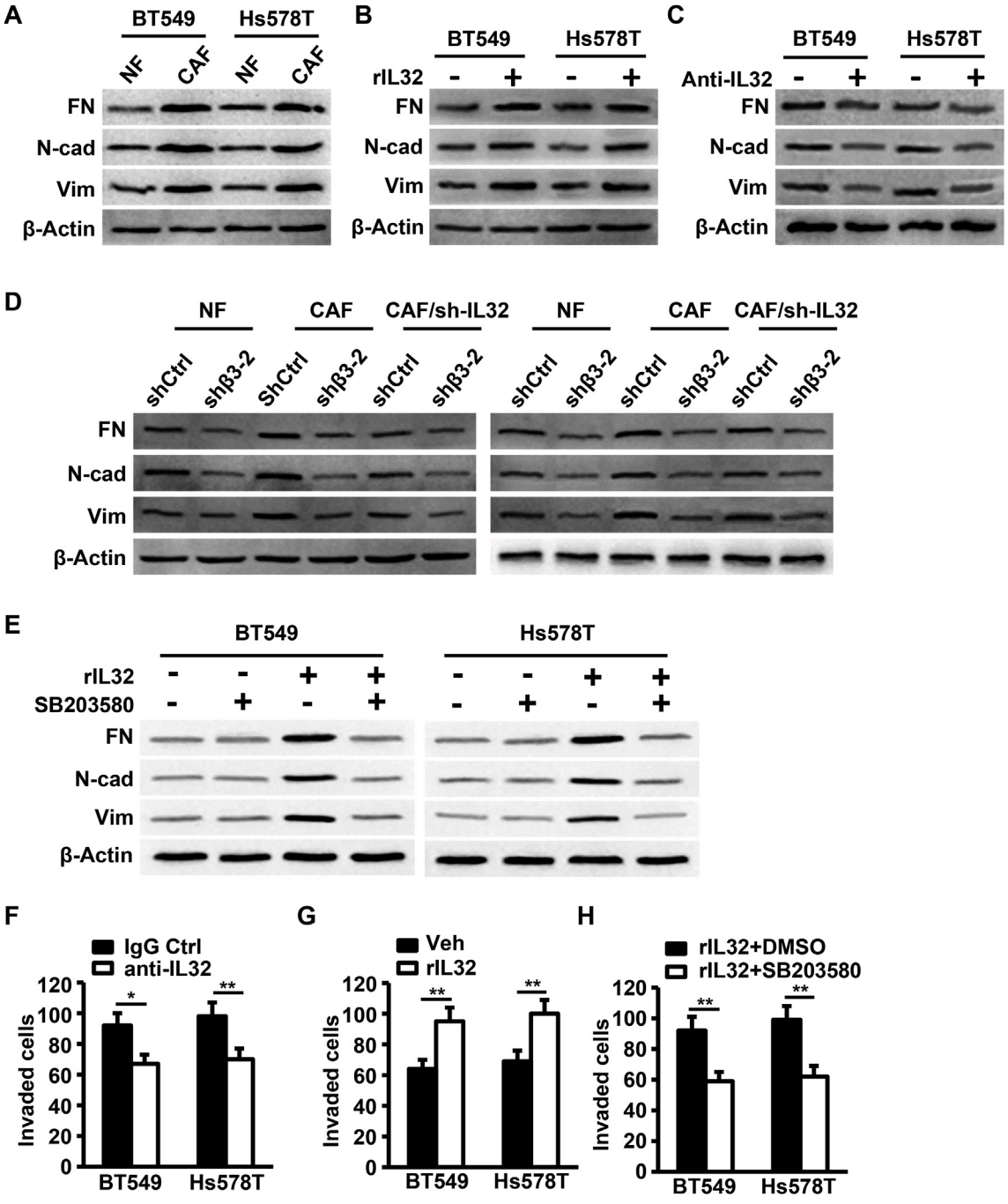

Fig. 6. IL32 promotes breast cancer cell invasion by stimulating p38 MAPK and EMT. (**A**) A western blotting assay to quantify the EMT markers in BT549 and Hs578T cells co-cultured with CM from CAFs or NFs. (**B**) CM from NFs with rIL32 (20 ng/ml) was employed to culture BT549 and Hs578T cells for 24 h, the expression of EMT markers was detected by western blotting. (**C**) CM from CAFs neutralised with the specific antibody against IL32 (10 μg/ml) was used to culture BT549 and Hs578T cells for 24 h, and western blotting was performed to determine the expression levels of EMT markers. (**D**) BT549/sh-β3 (shRNA-2) cells (left panel) and Hs578T/sh-β3 (shRNA-2) cells (right panel) were co-cultured with CM derived from NFs, CAFs, or CAFs/sh-IL32, and a western blotting assay was conducted to quantify the EMT markers. (**E**) The BT549 and Hs578T cells were treated with rIL32 together with or without SB203580 (p38 MAPK inhibitor, 20 μM) for 24 h. Western blotting was performed to determine the levels of EMT markers. (**F**) Cell culture conditions were similar to those in (C), and the invasive ability was measured by a Transwell assay (*$P < 0.05$, **$P < 0.01$). (**G**) Cell culture conditions were similar to those in (B). Invasive abilities of BT549 and Hs578T cells were determined by a Transwell assay (**$P < 0.01$). (**H**) BT549 and Hs578T cells were treated the same as in (E), and the Transwell assay was carried out to evaluate the invasive potentials of tumour cells (**$P < 0.01$). *Legend.* FN: fibronectin, N-cad: N-cadherin, Vim: vimentin. An isotype-matched non-specific IgG served as an anti-IL32 antibody control. PBS or DMSO was used as a control of group rIL32 and group SB203580, respectively. β-Actin is the loading control in all the western blotting analyses.

melanoma cells [52,53]. Indeed, silencing of IL32 in CAFs partly increased tumour growth in mice in the present study. Nevertheless, some evidence indicates that IL32 closely correlates with tumour malignancy (e.g. oesophageal cancer) [54], but the underlining mechanism is unclear. In the present report, we show that CAF-secreted IL32 can promote breast cancer cell invasion through interaction with integrin β3 at the plasma membrane of breast cancer cells. Treatment of these cells with rIL32 caused marked invasion of breast cancer cells in vitro

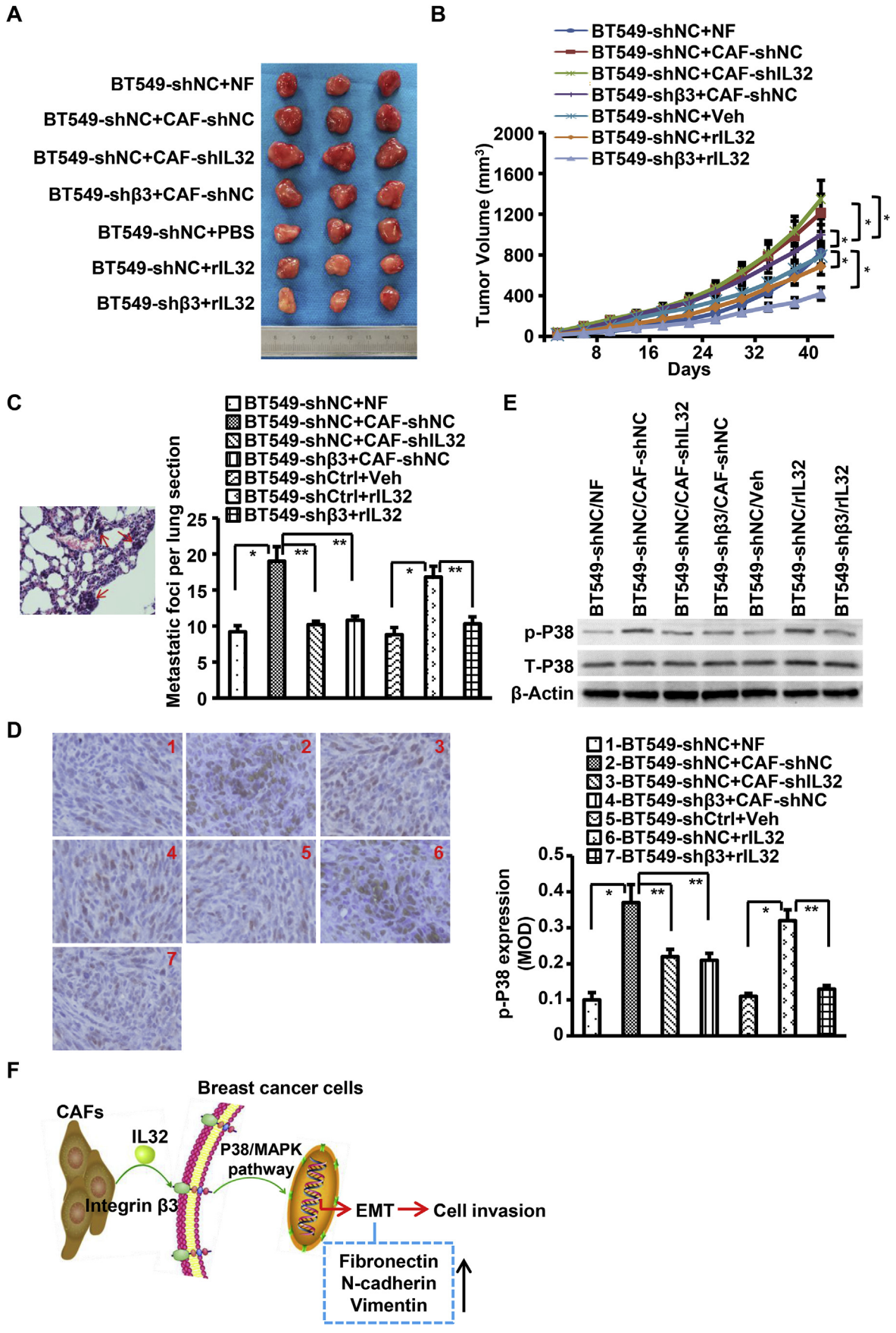

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Fig. 7. IL32 promotes lung metastasis of breast cancer xenografts via integrin β3. (**A**) The tumour sizes of each group. (**B**) The curves of tumour growth in mice (*$P < 0.05$). (**C**) Representative images of pulmonary metastases examined by H&E staining in lung sections. The arrows show metastatic foci; the histogram shows the metastatic foci per lung section from each mouse group (*$P < 0.05$; **$P < 0.01$). (**D**) Representative images (left panel) of IHC staining of p-p38 (left panel) and quantitation of p-p38 levels (right panel) in each group (*$P < 0.05$, **$P < 0.01$). (**E**) Protein levels of phosphorylated and total p38 in tumours were determined by western blotting. (**F**) A schematic model illustrating that the secreted protein IL32 derived from CAFs binds to integrin β3 at the breast cancer cell membrane to activate downstream p38 MAPK signal transduction, thus promoting breast cancer cell invasion. Magnification, × 200 in (C) and (D).

and yielded a greater number of metastatic nodules in mouse lungs; neutralisation of IL32 in the supernatant of CAFs by means of a specific antibody attenuated CAF-induced cancer aggressiveness. Moreover, suppression of the connection between IL32 and integrin β3 (such as a knockdown of IL32 in CAFs or silencing of integrin β3 in BT549 cancer cells) notably reduced the number of metastatic foci in mouse lungs. Thus, IL32, a new secreted protein, serves as an important mediator between stromal fibroblasts and cancer cells by contributing to tumour invasion and metastasis.

Our previous work suggests that the WNT-GSK3β, PI3K-AKT, and ERK-MAPK are the downstream networks of integrin β1 through FAK-ILK signalling [39]. Nonetheless, FAK-ILK signalling was unchanged during the interaction of integrin β3 with IL32 (data not shown). Moreover, in breast cancer cells, only p38 MAPK, a typical cytokine pathway, was activated after IL32 binding to integrin β3. This result is consistent with the finding that IL32 can activate p38 MAPK pathway in mouse macrophages and human oesophageal cancer cells [23,54], and that p38 MAPK is activated after integrin β3 interacts with an extracellular ligand (TN-c) [21]. Thus, it is worth noting that p38 MAPK may be a specific signalling protein that is stimulated by the interaction of integrin β3 with extracellular components (e.g. cytokines). Functionally, p38 MAPK signalling may contribute to EMT and cancer cell invasion. Indeed, our current work shows that fibronectin, N-cadherin, and vimentin are up-regulated in breast tumour cells, and tumour cell invasion and metastasis are strengthened only by p38 MAPK signalling.

In summary, our study shows that integrin β3 in breast tumour cells acts as a functional receptor of IL32, a crucial CAF-derived cytokine, by promoting breast cancer invasiveness. These findings have significant implications for the understanding of CAFs' molecular mechanisms of action regulating cancer behaviour. Our study offers a new possible therapeutic strategy for targeting stromal components of a breast tumour.

5. Conflicts of interest

The authors declare that no conflicts of interest exist.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.10.015.

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