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Original Articles

Silencing Y-box binding protein-1 inhibits triple-negative breast cancer cell invasiveness *via* regulation of MMP1 and beta-catenin expression

Jia Pei Lim[a,b], Sunitha Nair[a,b], Sukanya Shyamasundar[a], Pei Jou Chua[a], Umamaheswari Muniasamy[a], Ken Matsumoto[c], Jayantha Gunaratne[a,b,**], Boon Huat Bay[a,*]

[a] Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore, 117594, Singapore
[b] Translational Biomedical Proteomics Group, Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, 138673, Singapore
[c] Chemical Genomics Research Group, RIKEN Center for Sustainable Resource Science, RIKEN, Saitama, Japan

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ABSTRACT

Y-box binding protein-1 (YB-1), an important transcription and translation regulator protein, is known to increase cancer cell invasiveness and spreading. Here, we report its role in breast cancer, particularly in mediating cell invasion in triple-negative breast cancer (TNBC). YB-1 stable knockdown (*sh*YB-1) significantly reduced the invasive potential of MDA-MB-231 TNBC cells in 2D and 3D (spheroid) cultures. Whole proteome mass spectrometry analysis showed an enrichment of cell adhesion and cell to matrix interaction proteins, notably, matrix metalloproteinase-1 (MMP1) and beta-catenin (CTNNB1), which are known to play critical roles in cancer metastasis. *sh*YB-1 cells exhibited substantial downregulation of *MMP1* and *CTNNB1* mRNA and protein expression, with reduced MMP1 enzyme activity. YB-1 was also observed to bind to the promoter of *MMP1* and overexpression of MMP1 plasmid in *sh*YB-1 cells increased cell invasion. Finally, analysis of tumour samples from the Gene Expression-Based Outcome for Breast Cancer Online (GOBO) database revealed that high gene expressions of *YBX1*, *MMP1* and *CTNNB1* predict for a significantly lower 10-year distant metastasis free survival. Altogether, this study shows that YB-1 mediates breast cancer invasion and metastasis *via* regulation of MMP1 and beta-catenin.

1. Introduction

Y-box binding protein-1 (YB-1) is an evolutionary conserved master regulator of transcription and translation [1]. It is known to be engaged in pleotropic cellular processes, including DNA repair, cell proliferation and pre-mRNA splicing [1]. YB-1 is often found to be dysregulated in human malignancies, with overexpression in several cancers, including breast [2], gastric [3] and prostate [4] cancers. Nuclear expression of YB-1 is known to correlate with an increase in P-glycoprotein, a multidrug resistance gene 1 (*MDR1*) product [5], leading to enhanced chemoresistance to anticancer drugs such as cisplatin [6,7], mitomycin C [7] and paclitaxel [8,9]. High expression of YB-1 in triple-negative breast cancer (TNBC) cell lines is known to confer resistance to paclitaxel, a drug commonly used in the treatment of advanced breast cancer stage *via* reduced level of early growth response 1 (*EGR1*) gene [9]. YB-1 also regulates transcription of cell cycle- related genes, such as *cyclin A*, *cyclin B1* [10], *cell division control protein 6* (*CDC6*) [11] and *cyclin D1* [12]. In a transgenic mouse model, elevated YB-1 enhanced breast cancer progression through chromosomal instability, centrosomal abnormalities and mitotic failure [13].

Notably, reports have linked YB-1 with cancer cell invasiveness, promoting metastasis of liver cancer [14], and relapse of gastric [3,14] and breast [15] cancers. Metastasis which involves dissemination of cancer cells from the primary tumours to distant sites is responsible for the majority of cancer-related deaths [16]. YB-1 is associated with epithelial-mesenchymal-transition (EMT), which is one of the initial steps of the “invasion-metastatic” cascade, through enhancing the translation of Snail1 [17]. Orthotopic injection of YB-1 overexpressing MCF10AT breast cells in the mammary fat pads of mice led to generation of spontaneous metastatic tumours in the lungs and thoracic cavities [17]. YB-1 has also been shown to induce Twist expression, an important EMT mediator [18], and activate EMT phenotype such as reduced cell to cell contact and increased cell elongation in prostate cancer [19]. Whereas in pancreatic ductal adenocarcinoma, silencing of YB-1 has been observed to reduce the generation of liver metastasis and suppress the progression of tumour [20].

* Corresponding author.
** Corresponding author. Department of Anatomy, Yong Loo Lin School of Medicine, National University of Singapore, 117594, Singapore.
E-mail addresses: jayanthag@imcb.a-star.edu.sg (J. Gunaratne), boon_huat_bay@nuhs.edu.sg (B.H. Bay).

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Moreover, YB-1 is reported to stimulate the transcription of *matrix metalloproteinase 2* (*MMP2*) gene which plays a role in the degradation of extracellular matrix, aiding in tumour metastasis [21]. In MCF7 breast cancer cells transfected with YB-1/EGFP plasmid, YB-1 was observed to be concentrated at the invadopodia of motile cells [22]. Higher expression of YB-1 levels could elevate the endocytosis of MT1-MMP (another important matrix metalloproteinase implicated in metastasis) and recycling to the cell surface. We have recently demonstrated that YB-1 facilitates cell migration and invasion of TNBCs *via* coronin-1C (*CORO1C*), an actin binding protein, thus suggesting the involvement of YB-1 in cytoskeletal remodelling [23]. The diverse roles of YB-1 in oncogenic cell progression requires more in-depth investigation.

This study explores the functional role of YB-1 particularly in breast cancer invasion mediated *via* stable knockdown of YB-1 in the aggressive MDA-MB-231 TNBC cell line. We show that stable knockdown of YB-1 led to a reduction of cell invasion. Subsequent quantitative proteomics analysis and downstream assays demonstrate that YB-1 regulates both MMP1 and beta-catenin, which are involved in cell adhesion and cell-matrix interactions. Moreover, a combined set of YB-1, MMP1 and beta-catenin genes appear to have prognostic value in determining distant metastasis free survival.

2. Materials and methods

2.1. Bioinformatics analysis

Evaluation of *YBX1* gene expression in 51 different breast cancer cell lines was conducted with GOBO Gene Set Analysis (GSA)-cell line (http://co.bmc.lu.se/dobo), an online database which provides assessment of expression of a single gene or sets of genes in commonly used breast cancer cell lines [24]. An evaluation of a combination gene set comprising *YBX1, MMP1* and *CTNNB1* genes analysed by Affymetrix U133A arrays and correlation with 10-year distant metastasis free survival (DMFS) across 1881 cases of breast cancers, was conducted with the GOBO GSA-tumour database.

2.2. Short-hairpin RNA (shRNA)-mediated silencing of YB-1 in MDA-MB-231 breast cancer cells

Parental MDA-MB-231 cell line was cultured in RPMI 1640 complete medium, consisting of 10% fetal bovine serum (FBS). 3×10^5 parental MDA-MB-231 cells per well were seeded into 6-well plate, and were then transfected with four different 29mer shRNA constructs in retroviral pGFP-V-RS vector (Origene, Rockville, MD, USA) which specifically targets YB-1 protein or scrambled vector control (Origene) with Turbofectin 8.0 (Origene), following the guidelines by the manufacturer. The cells were maintained for an additional 48 h before being split at 1:10 ratio into new 6 well plates and single cell colonies were selected using Puromycin (Sigma Aldrich, St. Louis, MO, USA) at a concentration of 1 µg/ml, which was determined from a killing curve prior to transfection of the shRNA plasmids. Silencing efficiency of the cell colonies were determined by quantitative polymerase chain reaction (qPCR) and Western blot analysis as described below. Positive colonies were subsequently maintained with RPMI 1640 medium, containing 1 µg/ml of Puromycin and 10% FBS in a 5% CO_2 incubator with temperature set at 37 °C. Sequences of shRNA plasmids used are listed in Supplementary Table 1.

2.3. Cell invasion assay

Cell invasion assay was done by seeding 3×10^4 cells/well in BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences, San Jose, CA, USA) in triplicates, and the chambers were incubated for 21 h. The procedure was carried out as described previously [23]. Stained cells were visualised and captured at 100X magnification for five different fields with the Nikon SMZ 1500 stereo microscope, followed by quantification of the average number of cells per insert and calculating the mean of the invading cells in the triplicate inserts.

2.4. Spheroid invasion assay

5000 scrambled or *sh*YB-1 cells were seeded in 100 µl cell suspensions into the wells of 96-well Corning spheroid microplates (Corning, NY, USA) and incubated at 37 °C and 5% CO_2 for 72 h for spheroid formation. Subsequently, 100 µl of a mixture consisting of cold 7.5 mg/ml matrigel basement membrane matrix (Corning) and 250µg/ml of rat tail collagen I (Life Technologies, Carlsbad, CA, USA) was loaded carefully into each well containing the spheroids with the plate placed on ice. The plate was then centrifuged at 300 × *g* for 3 min and re-incubated at 37 °C and 5% CO_2 for one hour to harden the matrigel mixture, followed by addition of 100 µl of RPM1 complete media on top of the matrigel mixture. The plate was incubated up to 96 h and the images of the spheroids were taken with an Olympus CKX53 inverted microscope with 40X magnification every 24 h to monitor the extent of spheroid invasion.

2.5. Collagen-based cell invasion assay

Cells were serum starved overnight prior to seeding. A total of 6×10^4 cells were seeded in triplicates in QCM 24-well collagen-based invasion assay inserts (Chemicon, Merck Milipore). Briefly, the interior of the inserts were added with 300 µl of pre-warmed serum free RPMI and incubated for 15–30 min to rehydrate the collagen layer. Next, 250 µl of the media was removed and 250 µl of the cell suspension in serum free RPMI was added to the interior of the inserts. RPMI with 10% FBS (500 µl) which acted as chemoattractant was then pipetted to the lower chamber of each insert. The plate was re-incubated at 37 °C and 5% CO_2 for 24 h for cell invasion. Subsequently, cells were stained with 400 µl of cell stain from the kit and incubated at room temperature for 20 min. The inserts were then rinsed in water, cleaned and invaded cells were counted similar to the cell invasion assay as described previously [23].

2.6. SILAC labeling

MDA-MB-231 scrambled control cells (Scr 2B) or YB-1 stable knockdown cells (Sq 4(4A)) which has the highest knockdown efficiency were used for the experiments. SILAC labelling was carried out as described previously [25]. Both Scr 2B and Sq 4(4A) *sh*YB-1 MDA-MB-231 cells were adapted to SILAC RPMI (Thermo Scientific, Waltham, MA, USA) media containing either ^{13}C and ^{15}N labelled Lysine and Arginine (Sigma-Aldrich) ("K8R10 - heavy") or ^{12}C and ^{14}N labelled Lysine and Arginine (Sigma Aldrich) ("K0R0 – light") with 10% dialysed FBS (Thermo Scientific).

2.7. Cell lysate preparation for whole proteome analysis

Cell cultures were harvested once sufficient incorporation of heavy labels were determined. Cells were then lysed in a buffer consisting of 8M urea (Sigma-Aldrich) and 100 mM ammonium bicarbonate (Sigma-Aldrich). Protein quantification was carried out using the Pierce™ 660 nm protein assay (Thermo Scientific). The cells used for forward and reverse experiments are explained in Fig. 2A. 500 µg of protein lysates from cells cultured in "light" and "heavy" SILAC media respectively were mixed for the forward and reverse experiments. A total of 1 mg of protein lysate each for the forward and reverse experiments were used. The samples were then subjected to in-solution digestion to obtain peptides. 5 mM DL-Dithiothreitol (DTT) (Sigma-Aldrich) was used for reduction for 30 min at room temperature, followed by 10 mM Iodoacetamide (IAA) (Sigma-Aldrich) for alkylation. After 30 min incubation in the dark, samples were subjected to digestion by Lys-C

(Wako, Tokyo, Japan) overnight and trypsin (Promega, Madison, WI, USA) digestion for another 8 h.

2.8. Liquid chromatography-mass spectrometry (LC-MS)

The resulting tryptic peptides were subjected to fractionation by isoelectric focusing (IEF) and LC-MS as previously described [25]. The harvested SILAC proteins were analysed on a Q Exactive Hybrid Quadrupole-Orbitrap (Thermo Fisher) coupled to a Proxeon Easy-nLC 1000.

2.9. Protein identification and analysis

MS analysis was performed using MaxQuant [26] version 1.5.0.30 against a 2016-06 human database (Uniprot). For assignment of upregulated proteins, a SILAC ratio cut off value of ≥2 and ≤ 0.5 was used in the forward and reverse experiments respectively and *vice versa* for the downregulated proteins. Scatterplot was generated using R. Downregulated proteins identified from both forward and reverse reactions were subjected to process networks enrichment using the MetaCore software version 6.33.

2.10. Isolation of proteins from cell supernatant for Western blot

Cells were first seeded in 6-well plates. The next day, the media was replaced with serum-free media and the cells were incubated overnight. The supernatants were pooled from the triplicate wells and centrifuged at 500 × *g* for 5 min to remove cell debris. Next, the supernatant was removed carefully without disturbing the cell debris at the bottom and transferred to Amicon Ultra-4 Centrifugal Filter Unit with Ultracel-10 membrane (Merck Milipore, Burlington, MA, USA) and centrifuged at 7500 × *g* for 15 min twice to concentrate proteins with a protein size of more than 10 kDa in the supernatant. The concentrated supernatant containing the proteins of interest was subsequently quantified using microtiter Bio-Rad Protein Assay (Bio-Rad, Hercules, CA, USA) and denatured to the desired amount.

2.11. Western blot

Samples were lysed with radio-immunoprecipitation (RIPA) lysis buffer (Pierce, Waltham, MA, USA), quantitated and denatured as described previously [23]. The same amount of protein lysates were subjected to protein separation in 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) or 4–20% mini-PROTEAN TGX Precast protein gels (Bio-Rad), transferred to polyvinyl difluoride (PVDF) membranes or Trans-Blot Turbo Midi PVDF Transfer Packs (Bio-Rad). The membranes were blocked using 5% skim milk or 5% BSA, followed by primary antibodies incubation which include anti-YB-1 (1:1000 dilution) [23,27], anti-beta-catenin (1:1000) (Cell Signalling Technology, Danvers, MA, USA), anti-MMP1 (1:1000 dilution) (Proteintech, Rosemont, IL, USA) and anti-β-actin (1:6000 dilution) (Sigma-Aldrich) at 4 °C overnight. Next, membranes were incubated with the corresponding HRP-conjugated secondary antibodies (Sigma-Aldrich). The blot signals were detected with SuperSignal West Pico Chemiluminescent ECL substrate (Pierce) and quantitated using the GS-800 densitometer (Bio-Rad). For Western blots of supernatant proteins, Ponceau staining was used to confirm equal loading and membranes were incubated in Ponceau S stain [0.1% (w/v) in 5% (v/v) acetic acid] on a shaker for 5 min at room temperature following Western blot gel transfer, before performing the blocking step.

2.12. Nuclear fraction protein extraction

A total of 2.5 × 10^6 cells were seeded in two 10 cm plates each for scrambled control cells Scr 2B and *shYB-1* Sq 4(4A) MDA-MB-231 cells. Two days later, cells were harvested and washed with 1X PBS, before centrifugation at 500 × *g* for 3 min in 1.5 ml tubes. After removal of supernatant from the cell pellets, nuclear fraction of the cells were isolated using the NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Scientific). Briefly, ice cold CER I was added to the cell pellets and then vortexed at the highest speed for 15s. The tubes were then incubated on ice for 10 min, followed by addition of ice cold CER II to the cell pellets. Next, the tubes were then vortexed before subjecting to micro-centrifugation at 16,000 × *g* for 5 min. The supernatant was removed and the insoluble fractions which contained the nuclear fractions were then added with ice-cold NER, and sonicated briefly at 4 °C until the pellets were dissolved. Next, the suspension was centrifuged at 16,000 × *g* for 10 min. Finally, the resulting supernatant containing the nuclear extract proteins were transferred to clean tubes and stored at −80 °C until use.

2.13. Total RNA extraction, cDNA conversion and qPCR

Total RNA was extracted with the RNeasy Mini extraction kit (Qiagen, Hilden, Germany). First strand cDNA synthesis with 1 μg of total RNAs was conducted as previously described [23]. The gene expression changes were quantitated using Applied Biosystems 7900HT Fast Real-Time PCR system. Samples were run in triplicates and with *GAPDH* as the housekeeping gene for normalisation. Gene expressions were expressed as fold change using the $2^{-\Delta\Delta CT}$ method [28]. Primer sequences used are listed in Supplementary Table 2.

2.14. Transfection of MMP1 overexpression plasmid in the YB-1 stable knockdown MDA-MB-231 cells

6.75 × 10^5 *sh*YB-1 Sq 4(4A) or scrambled control cells Scr 2B MDA-MB-231 stable cell colonies were seeded per well in a 6-well plate in triplicates. Next day, the cells were transfected with 3.5 μg of pReceiver-M56 control vector (EV-mCherry) (Genecopeia, Rockville, MD, USA) or *MMP1* ORF construct with C-terminal mCherry tag (MMP1-mCherry) (Genecopeia) with Lipofectamine 3000 (Invitrogen) using the guidelines from the manufacturer and the transfected cells were incubated overnight. The cells were replenished with fresh media the following day, and incubated for another 24 h (for cell invasion) or 48 h (for Western blot).

2.15. MMP1 promoter reporter luciferase assay

1.5 × 10^5 wild type MDA-MB-231 cells were seeded in triplicates in a 24-well plate. The next day, cells were co-transfected with 250 ng of control vector pCMV-AC6-GFP (Origene) or YB-1 ORF plasmid (pCMV-AC6-YBX1-GFP) and 250 ng of negative luciferase construct pEZX-PG04 (Origene) or *MMP1* promoter luciferase construct (Origene) with Lipofectamine 3000 (Invitrogen). The transfected cells were incubated at 37 °C and 5% CO_2 overnight, following which the media was removed from each well and new complete media was added. 48 h post transfection, luciferase assay was carried out with the Secrete-pair dual luminescence assay kit (Genecopeia), following the protocol outlined by the manufacturer. Each sample was run in triplicate readings. The relative luminescence unit (RLU) for each sample was calculated by dividing the average triplicate readings from the gaussia luciferase signal to the secreted alkaline phosphatase (SEAP) signal. Subsequently, the average RLU readings were calculated from the triplicate samples for each of the co-transfection conditions.

2.16. Active MMP1 fluorokine assay

Supernatants were collected from Scr 2B and *sh*YB-1 Sq 4(4A) MDA-MB-231 cells cultured in T-25 flasks. The supernatant was subjected to 500 × *g* centrifugation for 5 min to remove cell debris. Fluorokine E human active MMP1 fluorescent assay kit (R&D System, MN, USA) was used to measure the amount of active MMP1 secreted into the cell supernatant. The working standards and samples were added in duplicates and the procedure was in accordance with guidelines provided by the manufacturer. Relative fluorescence units (RFU) per well was ascertained with a fluorescence plate reader set at 320 nm excitation wavelength and 405 nm emission wavelength with 20 ms integration time. The duplicate readings were averaged and the average zero standard RFU was subtracted from each of the average duplicate readings. A standard curve was generated using GraphPad Prism 5.0 with average RFU of the standards on the Y-axis against the Log_{10} of the human active MMP1 concentrations on the X-axis, followed by a best fit curve through the points on the graph. The corresponding active MMP1 concentration for each sample was interpolated from the standard curve with the average RFU for each of the samples using GraphPad Prism 5.0. Next, the relative fold change was determined by dividing the active MMP1 active concentrations of the samples over the concentration for the control sample.

2.17. Statistical analysis

The GraphPad Prism 5.0 was used for the analysis. Samples with two groups were analysed with Student's t-test and samples with more than two groups were analysed with One-way ANOVA. The values were represented as mean ± SEM. When p is < 0.05, the results were statistically significant.

3. Results

3.1. YBX1 gene is highly expressed in TNBC cell lines and stable knockdown of YB-1 protein decreases cell invasion

YBX1 gene expression levels were first determined in the different breast cancer cell lines, by using the GOBO-GSA cell line software, which enables evaluation of *YBX1* gene expression across 51 breast cancer cell lines [24]. *YBX1* gene expression was found to be highest in basal B cell lines, which is associated with stem-cell like features and mesenchymal morphology [29], followed by basal A and lowest in luminal cell lines (Fig. 1A). Furthermore, *YBX1* showed highest expression in the TNBC cell lines (Fig. 1A). TNBC is the most aggressive form of breast cancer which thus far lacks an effective therapeutic target [30].

As YB-1 showed the highest expression in TNBC cell lines, we decided to evaluate the role of YB-1, particularly in metastasis through shRNA-mediated stable silencing of *YBX1* in MDA-MB-231 TNBC cells. By introducing four different YB-1 shRNA plasmids into MDA-MB-231 cells, we obtained YB-1 stable knockdown clones and used two of them, Sq 2(2E) and Sq 4(4A), in the following experiments. YB-1 protein was knocked down by approximately 19% and 75% in the *shYB-1* Sq 2(2E) and Sq 4(4A) cells, respectively, which were selected from two different shRNA plasmids when compared to Scr 2B control cells which had a scrambled shRNA construct introduced (Fig. 1B).

A significant reduction in cell invasion was observed in the two YB-1 stable knockdown cells Sq 2(2E) and Sq 4(4A) when compared to Scr 2B control cells (Fig. 1C). Furthermore, 3D spheroid invasion assay showed that Scr 2B control spheroids were able to invade the surrounding basement membrane matrix more extensively, whereas it was also observed that Sq 4(4A) spheroids which showed the highest YB-1 protein knockdown efficiency were more compact than the Scr 2B and Sq 2(2E) spheroids (Fig. 1 D).

3.2. Whole proteome analysis shows that silencing YB-1 downregulates cell adhesion and cell to matrix interaction proteins

Both Scr 2B control cells and Sq 4(4A) *sh*YB-1 MDA-MB-231 cells were adapted to SILAC "heavy" and "light" media for the whole proteome analysis. Sq 4(4A) cells were selected for adaptation in SILAC media as they showed the greatest knockdown of YB-1 when compared to Scr 2B control cells (Fig. 1B). One set of forward experiment, in which cell lysates from "light"-labeled Scr 2B and "heavy"-labeled Sq 4(4A) were mixed, and one set of reverse experiment, in which cell lysates from "heavy"-labeled Scr 2B and "light"-labeled Sq 4(4A) were mixed, were performed (Fig. 2A). A total of 8309 proteins were identified (Fig. 2B). Using 2 fold as the cut-off value, a total of 136 proteins were found to be downregulated (Supplementary Table 3) while 62 proteins were found to be upregulated (Supplementary Table 4).

To understand the processes involved in *sh*YB-1 MDA-MB-231 cells, down-regulated proteins were subjected to enrichment analysis using the MetaCore process networks function. The list of proteins in the enrichment analysis are listed in Supplementary Table 5. The process networks enrichment analysis revealed that these downregulated proteins were mainly involved in cell adhesion and cell to matrix interactions, proteolysis of connective tissues and extracellular matrix (ECM), reproduction and development processes (Fig. 2C). Of particular interest is MMP1, a well known secreted protein involved in ECM remodelling and cancer cell invasiveness. The list of downregulated proteins that are involved in cell adhesion and cell to matrix interactions are listed in Table 1.

3.3. Silencing YB-1 downregulates MMP1 and beta-catenin expression

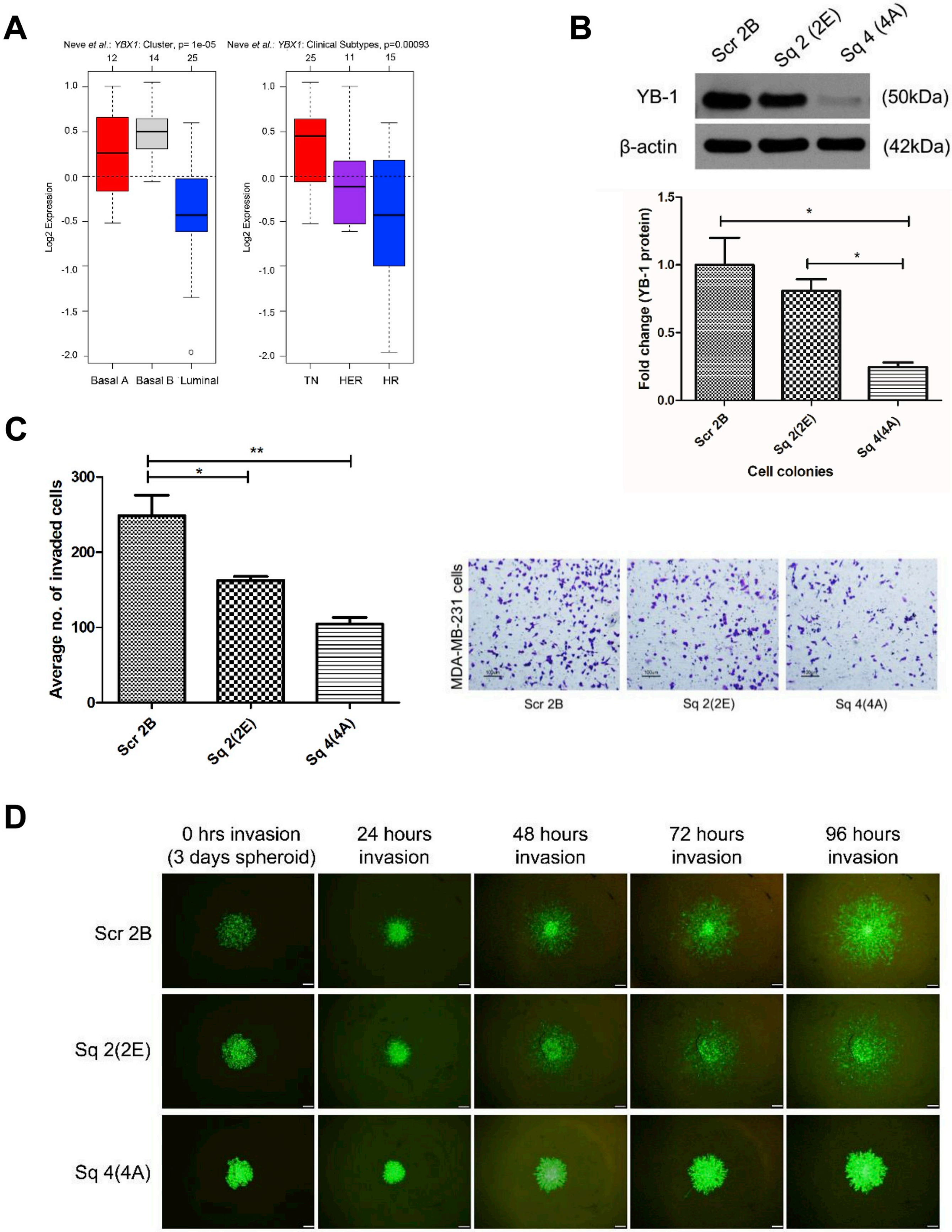
Our whole proteome analysis revealed that MMP1 was downregulated following YB-1 reduction in MDA-MB-231 cells (Table 1). To validate the results, Western blot was carried out to evaluate the protein expression of MMP1 in both whole cell lysates and in the culture supernatant of *sh*YB-1 knockdown cells. It was observed that YB-1 knockdown resulted in a decrease in MMP1 protein expression in the cell lysates (Fig. 3A) as well as in the secretion of MMP1 into the culture supernatant (Fig. 3B and Supplementary Fig. 1). Active MMP1 fluorokine assay further showed that the MMP1 enzyme activity was decreased in the *sh*YB-1 MDA-MB-231 cells (Fig. 3C).

From the process network analysis, we found that Wnt/beta-catenin signalling was among the top hit lists generated with beta-catenin being one of the downregulated proteins involved (Fig. 2C). As beta-catenin (CTNNB1) has been reported to transcriptionally activate the expression of MMP1 [31], and that overexpression of YB-1 has been reported to enhance beta-catenin transcriptional activity using the TOP-flash luciferase assay [32], we therefore evaluated beta-catenin protein expression in *sh*YB-1 MDA-MB-231 cells. The beta-catenin protein was indeed decreased in the YB-1 knocked down cells although not statistically significant (Fig. 3D). To further verify this finding, we examined the nuclear fraction of beta-catenin in *shYB-1* knockdown MDA-MB-231 cells, which showed a concomitant downregulation of nuclear beta-catenin protein. (Supplementary Fig. 2).

3.4. YB-1 regulates cell invasion via MMP1 by direct binding to the MMP1 promoter

Next, qPCR was carried out to determine if YB-1 regulates the expression of both *MMP1* and *CTNNB1* at the transcriptional level. We observed that both *MMP1* (Fig. 4B) and *CTNNB1* (Fig. 4C) gene expression were downregulated in *sh*YB-1 cells, correlating with the gene expression levels of YB-1 in the cells (Sq 2(2E) and Sq 4(4A)) (Fig. 4A). This suggests that YB-1 regulates transcription of both genes (*MMP1* and *CTNNB1*) either directly or indirectly.

To further determine if YB-1 regulates *MMP1* gene transcription directly, luciferase assays with a *MMP1* promoter reporter were carried out. The promoter construct contains 1310 bp upstream and 186 bp downstream of *MMP1* gene transcription start site (Fig. 4D). A significant increase in the relative luminescence unit (RLU) signal was observed in MDA-MB-231 cells transfected with both YB-1 plasmid and *MMP1* promoter luciferase plasmid, thereby suggesting that YB-1 protein binds to the *MMP1* promoter region and regulates *MMP1* gene transcription directly (Fig. 4E).



(caption on next page)

Fig. 1. YB-1 is upregulated in basal and triple-negative cell lines, affecting the cell invasion activity. (A) Box plots of *YBX1* gene expression across cell lines that were grouped as basal A, basal B and luminal based on the data from Neve et al. [29] (left). The box plot on the right shows YB-1 protein expression across cell lines that were grouped into triple-negative (TN, red), HER2-positive (HER2, purple) and hormone receptor positive (HR, blue). (B) YB-1 protein expression in the scrambled control Scr 2B and stable *sh*YB-1 cells (Sq 2(2E) and Sq 4(4A)) cells, which were selected from two different shRNA plasmid sequences in MDA-MB-231 cells. (C) Boyden chamber invasion assays revealed a significant reduction in cell invasion for the YB-1 knockdown cells (Sq 2(2E) and Sq 4(4A)), as compared to Scr 2B control cells. (D) Spheroid invasion assays show reduced cell invasion for the *sh*YB-1 knockdown MDA-MB-231 cells. Each assays were repeated at least twice with triplicates. Scale bar = 100 μm. Error bar = SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

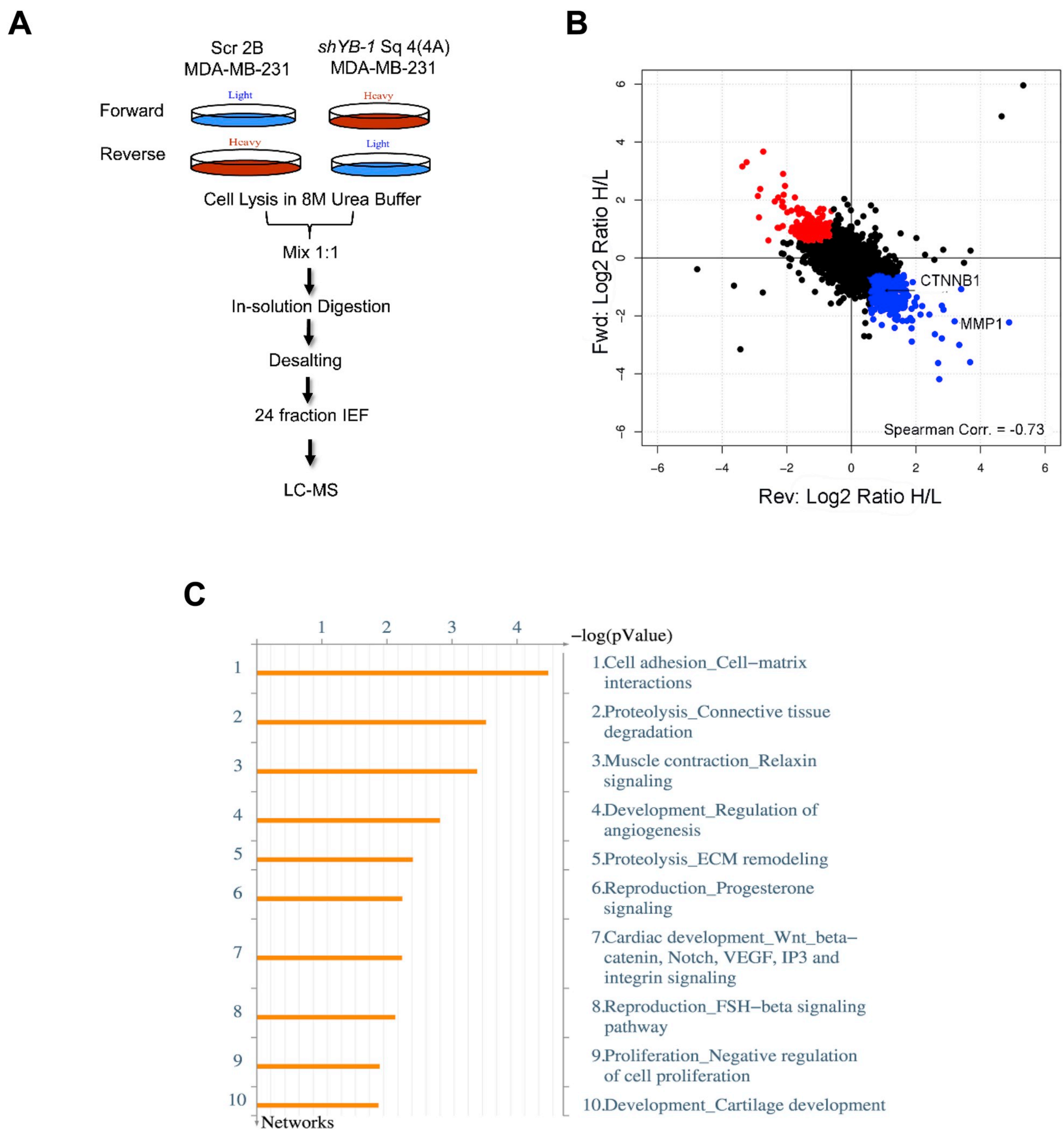


Fig. 2. Whole proteome analysis reveals an enrichment of proteins involved in cell adhesion and proteolysis of connective tissues processes. (A) Schematic diagram showing the workflow and adaptation of cells to SILAC "light" and "heavy" media for the Scr 2B and *shYB-1* Sq 4(4A) MDA-MB-231 cells. (B) Correlation plot for forward and reverse SILAC experiments. MMP1 and beta-catenin (CTNNB1) were identified to be down-regulated in the *sh*YB-1 MDA-MB-231 cells. (C) MetaCore process networks analysis revealed that the differentially down-regulated proteins were enriched for cell adhesion and cell to matrix interactions, proteolysis of connective tissues and ECM, reproduction and development processes.

Table 1
List of differentially down-regulated proteins which were involved in cell adhesion and cell to matrix interactions processes.

Protein name	Gene name	Ratio H/L normalised (Forward)	Ratio H/L normalised (Reverse)
Matrix metalloproteinase 1	*MMP1*	0.21916	9.1605
Chondroitin sulfate proteoglycan 4	*CSPG4*	0.23176	2.5553
Fibrillin-1	*FBN1*	0.25803	4.4039
Collagen alpha-1(IV) chain	*COL4A1*	0.30425	3.0861
A disintegrin and metalloproteinase with thrombospondin motifs 1	*ADAMTS1*	0.39135	2.8464

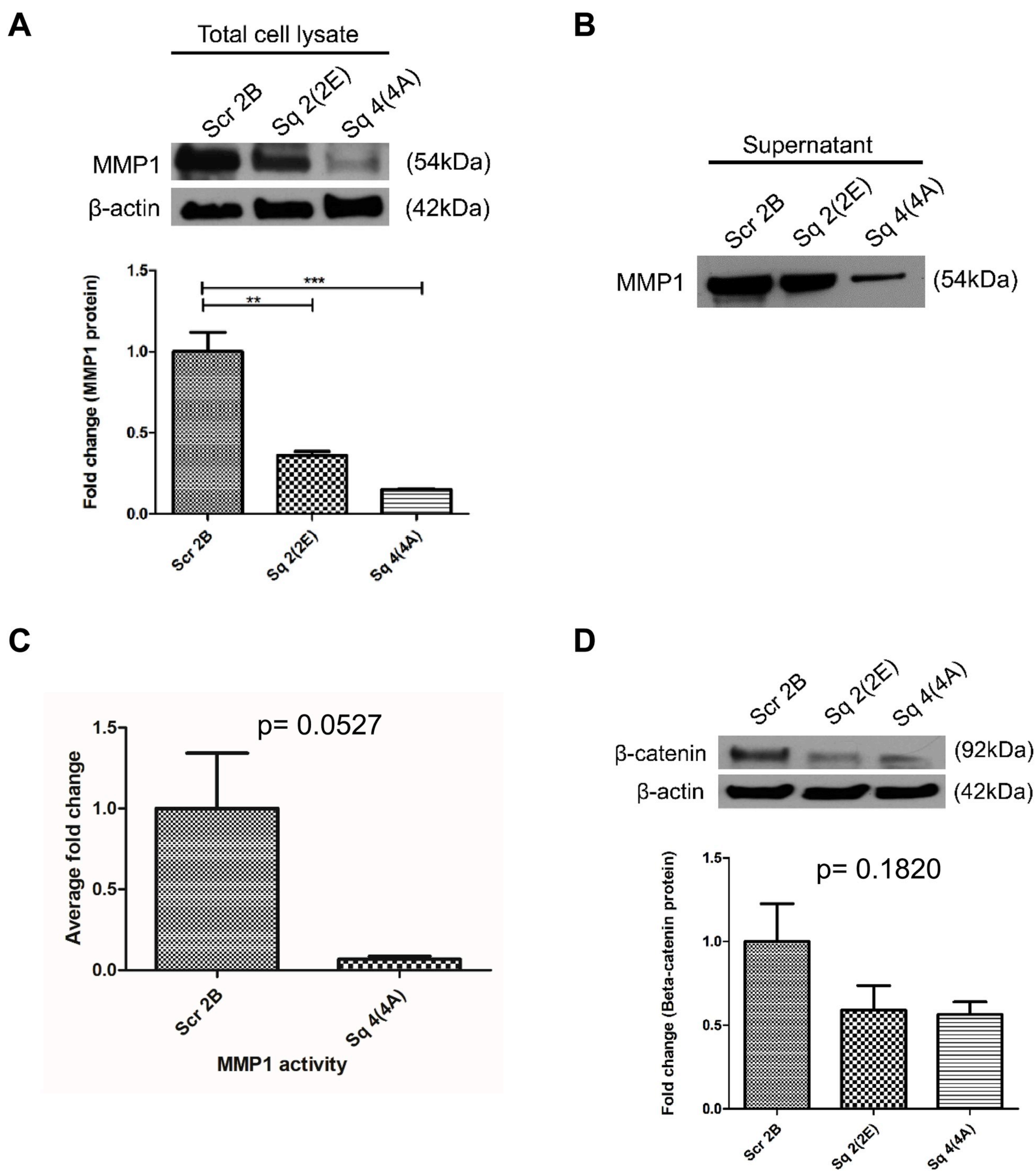


Fig. 3. MMP-1 and beta-catenin are downregulated after stable YB-1 knockdown. (A) MMP1 protein was decreased in the total cell lysates and (B) culture supernatant upon stable YB-1 knockdown in MDA-MB-231 cells. The culture supernatant with the same amount of proteins were analysed. (C) Active MMP1 fluorescence assay revealed a decrease in MMP1 enzyme activity in the stable YB-1 knockdown cells. (D) Beta-catenin protein was also reduced in the *shYB-1* MDA-MB-231 cells. Representative Western blots were shown. Error bar = SEM, $^{**}p < 0.01$, $^{***}p < 0.001$.

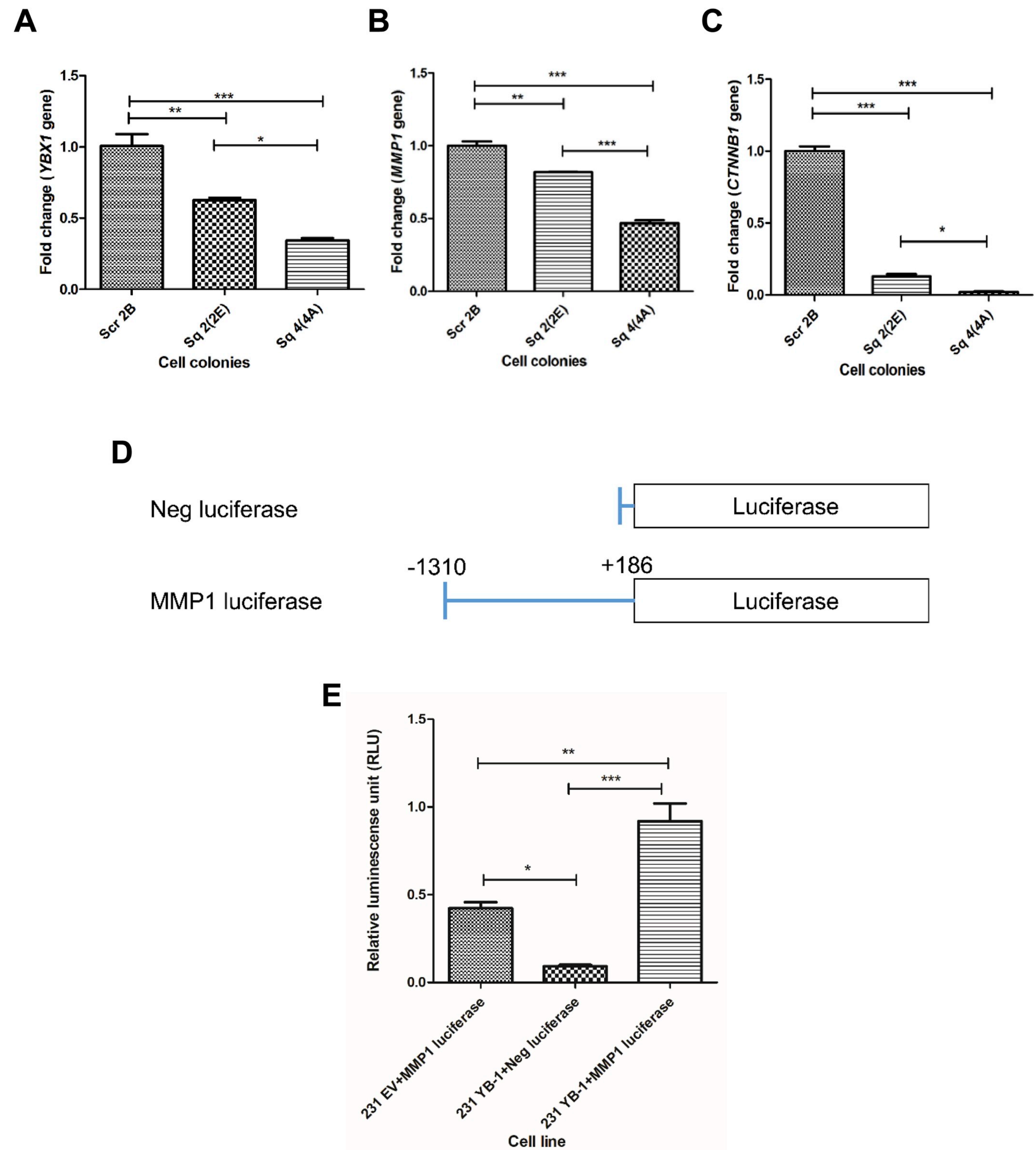


Fig. 4. MMP1 is a direct target of YB-1 protein. (A) *YBX1,* (B) *MMP1* and (C) *CTNNB1* genes were decreased in the YB-1 stable knockdown MDA-MB-231 cells. (D) MMP1 luciferase construct contains 1310 bp upstream and 186 bp downstream of *MMP1* gene transcription start site. (E) MMP1 promoter reporter luciferase assay in the transient YB-1 overexpressing cells (231 YB-1) showed that YB-1 binds to *MMP1* promoter. Error bar = SEM, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

In addition, a rescue experiment was designed to determine the role of MMP1 in triggering YB-1 induced cell invasion. To do this, MMP1 was overexpressed in the Sq 4(4A) *sh*YB-1 and Sq 2(2E) cells (Fig. 5A and B and Supplementary Fig. 3). An increase in cell invasion was seen upon overexpression of MMP1, albeit not statistically significant (Fig. 5C). Since collagen I is a substrate for MMP1, to further verify that YB-1 regulates cell invasion through MMP1, an invasion assay was conducted using inserts that were only layered with collagen I (Fig. 5D). It was observed that Sq 4(4A) *sh*YB-1 cells had reduced cell invasion for collagen I matrix as compared to Scr 2B control cells.

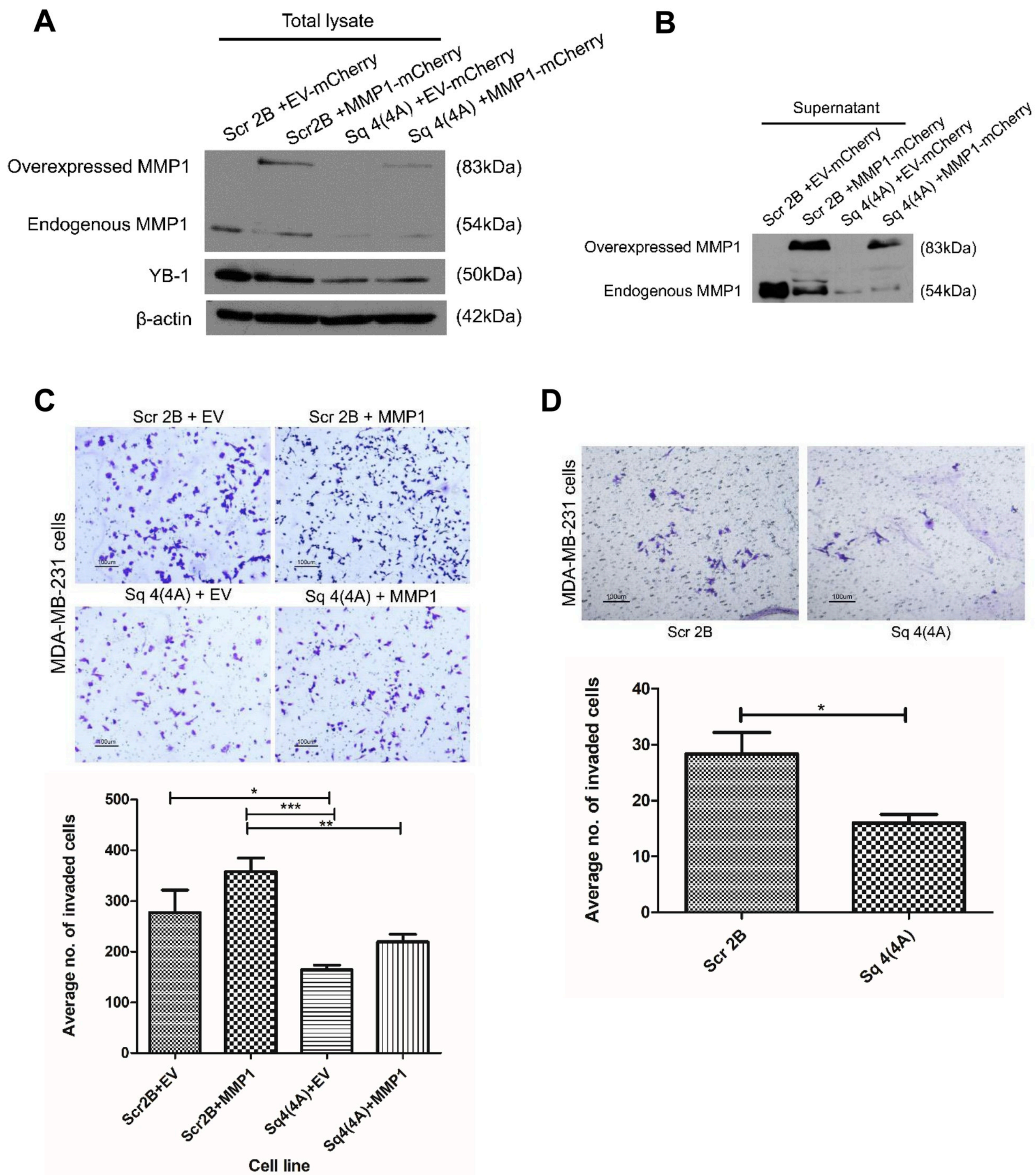


Fig. 5. YB-1 regulates cell invasion through MMP1. (A) Rescue experiment using MMP1 plasmid. Western blot confirmed the overexpression of MMP1-mCherry protein in the Scr 2B and Sq 4(4A) total cell lysates and (B) supernatant. (C) Overexpression of MMP1 protein in the Sq 4(4A) *shYB-1* knockdown MDA-MB-231 cells increased cell invasion, although not statistically significant. (D) Collagen-based invasion assay revealed a downregulation of cell invasion for Sq 4(4A) *sh*YB-1 MDA-MB-231 cells. Error bar = SEM, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

3.5. High expressions of YBX1, MMP1 and CTNNB1 predict for a lower distant metastasis free survival (DMFS)

We further explored the predictive function of the three genes (*YBX1*, *MMP1* and *CTNNB1*) as biomarkers of metastasis for breast cancer in a clinical cohort. GOBO GSA-tumour analysis of 1881 breast tissue samples revealed that tumours with basal phenotype that were grouped according to HU gene sets and PAM50 gene sets have the highest gene expressions of all three (*YBX1*, *MMP1* and *CTNNB1)* genes (Fig. 6A and B). Tumours that were grouped as estrogen receptor negative also had higher gene expressions of all 3 genes (Fig. 6C). We further showed that the merged gene set (*YBX1*, *MMP1* and *CTNNB1*) was positively correlated with the grade of the tumours, with grade 3 tumours having the highest gene expression (Fig. 6D). Kaplan-Meier analysis of the 10-year distant metastasis free survival (DMFS) showed that high expression of *YBX1, MMP1* and *CTNNB1* was correlated with a lower survival rate (Fig. 6E). In addition, multivariate analysis revealed that grade 3 tumours and tumours with lymph node involvement

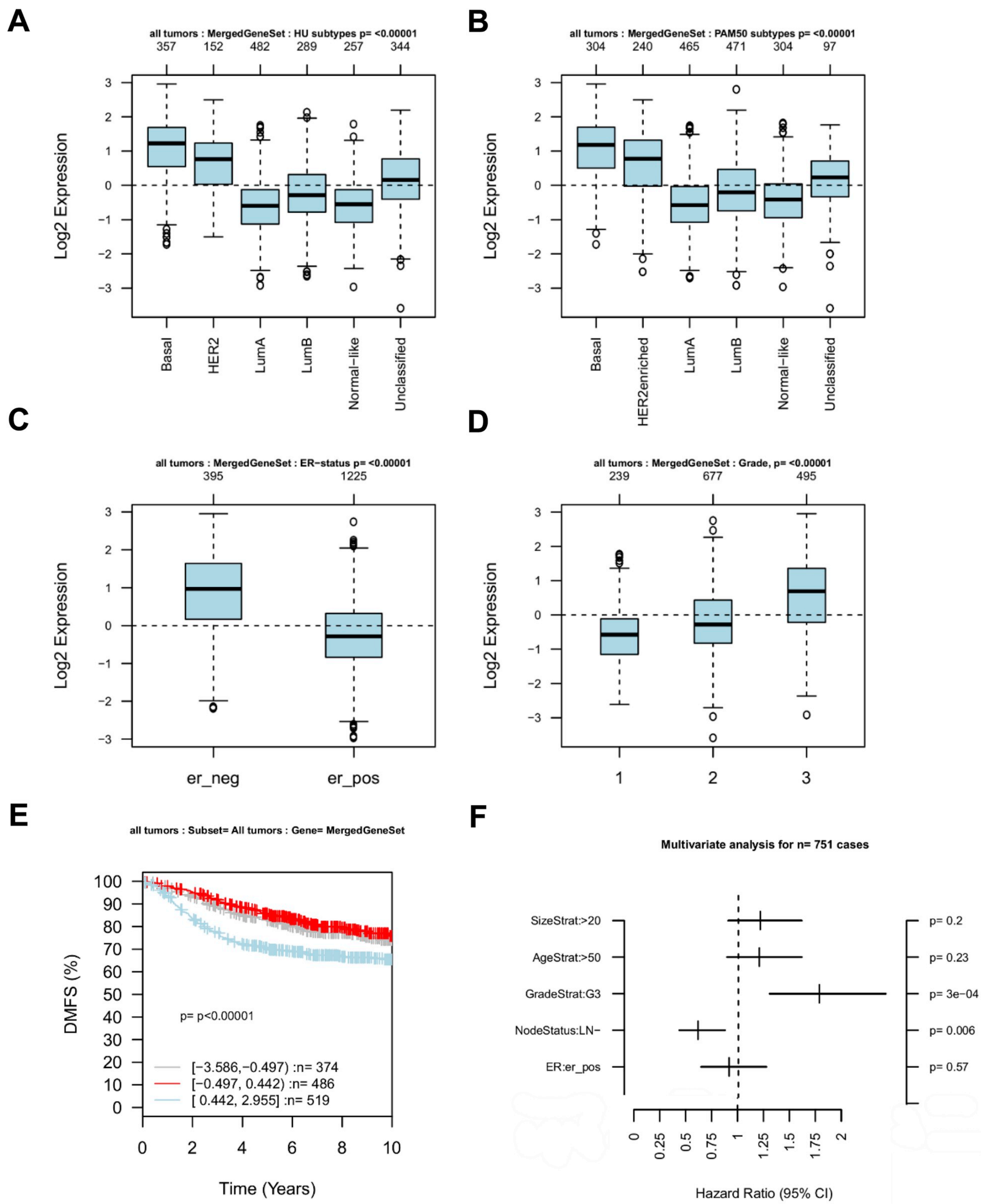


Fig. 6. GOBO gene set analysis for tumour samples using a combination of *YBX1*, *MMP1* and C*TNNB1* genes. (A–B) The combination of *YBX1*, *MMP1* and *CTNNB1* gene expression was higher in the basal-like tumours, stratified according to (A) HU subtypes and (B) PAM50 subtypes. (C) Estrogen Receptor negative (ER_neg) tumours have higher gene expressions of *YBX1*, *MMP1* and *CTNNB1* than estrogen positive (ER_pos) tumours. (D) Grade 3 tumours have higher gene expressions of YBX1, *MMP1* and *CTNNB1* genes. (E) Kaplan-Meier analysis showed that all tumour samples available in the GOBO database have a lower 10-year distant metastasis free survival (DMFS) when there were high gene expression of *YBX1*, *MMP1* and *CTNNB1* (n = 519). (F) Multivariate analysis revealed that tumours with positive lymph node infiltration and grade 3 histological grade have higher expression of all three genes.

had an overall higher expression of all three genes (Fig. 6F). Our analysis suggest that a combination of *YBX1*, *MMP1* and *CTNNB1* expression levels can serve as prognostic markers of breast cancer.

4. Discussion

YB-1 is a strong predictor of relapse in breast cancer and is upregulated in the highly malignant TNBC subtypes [15]. In this study, we determined that YB-1 is highly upregulated in breast cancer, particularly in the basal B and TNBC cell lines. YB-1 is also involved in EMT, a complex cellular process involving changes in epithelial cells morphology with acquisition of mesenchymal cell properties and enhancement of dissemination [33] in breast [17,34] and prostate cancers [19]. Furthermore, upregulation of YB-1 in MDA-MB-231 cells induced mammary tumour growth and cancer cells migration [35]. Our results showed that stable knockdown of YB-1 diminished cell invasion in the MDA-MB-231 cells, which are consistent with previous reports of YB-1 modulating cell invasion in breast cancer [23,34]. To shed light on the possible molecular mechanisms governing YB-1 actions using the *sh*YB-1 cells, we performed whole proteome profiling of these stable knockdown cells. Our results revealed that the downregulated proteins in the *sh*YB-1 cells were enriched in cell adhesion and proteolysis of connective tissues and extracellular remodelling, angiogenesis, Wnt/beta-catenin signalling and development processes. Here, we focused on the proteins involved in cell adhesion-related function, which are associated with metastasis.

Cell adhesion is often dysregulated during invasion-metastasis, that involves reorganisation of cytoskeletal structures and its interaction with extracellular matrix [36], thus affecting cellular movement. In this study, MMP1 was identified as one of the top downregulated proteins upon YB-1 reduction in MDA-MB-231 cells, which is involved in cell adhesion and cell to matrix interactions process. MMP1 is a member of the matrix metalloproteinases (MMPs) family, a group of endopeptidases or proteolytic enzymes which have various important functions in cancer biology, including cancer cells migration and invasion, angiogenesis, tissue remodelling and differentiation [37].

So far, 23 MMPs have been identified in humans, some of which are found to be dysregulated in several diseases including breast cancer [38]. The roles of different MMPs in breast cancer have been explored extensively and MMP1 in particular, is highly expressed by breast cancer tissues [39]. MMPs are grouped according to their structures [37] and MMP1 is a secreted form of interstitial collagenase which can exert effect on the tumour microenvironment [40]. The expression of MMP1 in both the stromal and tumour cells was reported to be significantly associated with tumour progression, with poorer prognosis and shorter survival in breast cancer [41]. Moreover, the expression of MMP-1 in cancer-associated stromal cells was found to be different in the breast cancer subtypes with its expression suggestive of breast cancer metastatic phenotype [41]. MMP1 is capable of enhancing melanoma cells invasion, acting as a downstream target of an EMT transcription factor, TWIST1 [42]. Taken together, these results indicate the significance of cellular and extracellular MMP1 levels in breast and other cancers such as melanoma [42], cervical [43] and head and neck cancer [44].

Notably, we observed that the secretion of MMP1 into the ECM was also affected by YB-1 protein expression, correlating with the amount of YB-1 protein in the whole cell lysate, suggesting that YB-1 could regulate MMP1 at the transcriptional level. Subsequent qPCR analysis revealed that YB-1 indeed regulates *MMP1* gene expression. As there is lack of understanding of the role of YB-1 in MMP1 regulation, particularly in breast cancer cells invasion, we focused on MMP1 protein for the downstream assays. We further showed that YB-1 could regulate the transcriptional activity dependent on the *MMP1* promoter region. Previous studies have reported that YB-1 could regulate the expression of MMP13 [45] and MMP12 [46] through binding of the AP-1 sequence in their promoter regions. Hence, it is plausible that YB-1 enhances *MMP1* transcription through binding of the AP-1 sequence in its promoter region. In addition, we found that MMP1 enzyme activity was decreased upon stable YB-1 reduction in the MDA-MB-231 cells, suggesting that YB-1 controls breast cancer cell invasion through regulation of *MMP1* gene transcription and enzyme activity. Overexpression of MMP1 in the *sh*YB-1 MDA-MB-231 cells could rescue the cell invasion (although not statistically significant), which suggest the possible involvement of other invasion enhancing proteins.

Interestingly, beta-catenin was also down-regulated following YB-1 knockdown. Beta-catenin is an important transcription factor of the Wingless-Int (Wnt) signalling pathway which contributes to cell development and stem cell renewal [47,48]. Abnormal expression and translocation of beta-catenin into the nucleus has been linked to malignant transformation of normal cells [49]. Moreover, nuclear beta-catenin accumulation is linked to tumours with triple-negative or basal-like phenotype and poorer clinical prognosis in breast cancer [49]. Some of the beta-catenin downstream targets which could promote tumourigenesis include cyclin D1 [50], MMP7 [51] and c-Myc [52]. Previous studies have identified that the beta-catenin/TCF4 complex binds to *MMP1* promoter, regulating its gene and protein expression, therefore affecting the ECM degradation [31]. YB-1 has been found to induce hepatocellular carcinoma (HCC) and maintain HCC initiating stem cell properties through regulation of Wnt/beta-catenin signalling pathway. Moreover, YB-1 knockdown was observed to reduce beta-catenin protein expression and its translocation into the nucleus [32]. Overexpression of YB-1 and beta-catenin lacking the GSK-3β phosphorylation domain led to an increase in transcriptional activity, suggesting that YB-1 could regulate beta-catenin in HCC [32]. In addition, it was reported that YB-1 forms a complex with Kindlin-2 and beta-catenin to activate transcription of epidermal growth factor receptor (*EGFR*), thus mediating cell proliferation, migration and invasion of glioma [53]. Our results are thus in agreement with the previous study that YB-1 could regulate beta-catenin gene and protein expression.

Using an *in vivo* metastasis model which involved injection of MDA-MB-231 breast cancer cells into the tail vein of Balbc/C nude mice, Minn et al. [54] demonstrated that *MMP1* belongs to a set of prometastatic genes that can mediate metastasis to the lungs. In another study using MDA-MB-231 variant cell lines with an enhanced ability to metastasize to the brain, Liu et al. [55] showed that upregulation of MMP1 expression in the breast cancer cells increased brain metastatic deposits. High *MMP1* gene expression has been reported to predict for a lower overall survival rate in invasive breast carcinoma [56] and poorer prognosis in patients treated with systemic therapy [57]. Recent studies have also found the association of beta-catenin expression with tumour promotion and poorer survival in breast cancer patients [58]. Furthermore, high expression of both c-Myc and beta-catenin predicts for a higher recurrence and lower survival rate in patients with TNBC [59]. To determine the clinical implications of a combination of *YBX1*, *MMP1* and *CTNNB1* in breast cancer patients, a GOBO GSA-tumour analysis was further conducted. High expression of all three genes (*YBX1*, *MMP1* and *CTNNB1*) was observed in higher grade tumours with basal-like phenotype and lymph node involvement, in which there is a lower 10-year DMFS rate in these breast cancer patients. These findings suggest that the combination of *YBX1*, *MMP1* and *CTNNB1* genes could serve as a reliable and valuable prognostic marker panel to identify TNBC patients with risk of developing metastasis recurrence.

Taken together, YB-1 is highly upregulated in TNBC, and plays a role in breast cancer cell invasion by regulating beta-catenin and MMP1 expression, and MMP1 enzymatic activity (Fig. 7). The novel association of YB-1 with MMP1 and beta-catenin offers a new biological insight into breast cancer invasion, and the fact that MMPs are widely explored as therapeutic targets in cancer progression, opens a new possibility to study MMP1 inhibition in YB-1 overexpressed cancers. The combination of YB-1, MMP1 and beta-catenin gene or protein expression could potentially serve as a reliable prognostic marker for breast cancer metastasis. Finally, elucidation of YB-1 and the regulatory mechanisms

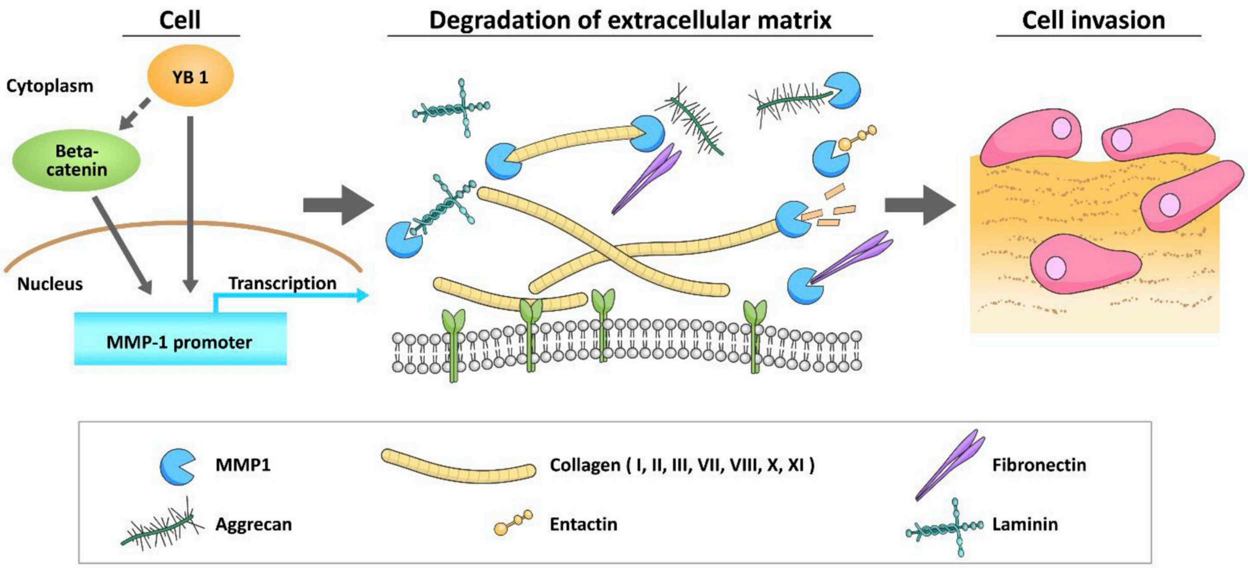


Fig. 7. Schematic diagram showing the role of YB-1, beta-catenin and MMP1 in the TNBC invasion. Both YB-1 and beta-catenin could translocate to the nucleus to activate the transcription of *MMP1* through binding to the *MMP1* promoter. MMP1 secretion into the extracellular matrix will lead to degradation of extracellular matrix proteins including collagen (I, II, III, VII, VIII, X, XI), entactin, fibronectin, aggrecan and laminin which are substrates of MMP1. Finally, degradation of these extracellular matrix proteins will eventually lead to enhanced cancer cell invasion.

governing its downstream targets could also provide novel therapeutic targets for the aggressive TNBC subtype.

Conflicts of interest

The authors have no competing financial interests to disclose.

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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.2019.03.014.

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