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Gene expression profile analysis of an isogenic tumour metastasis model reveals a functional role for oncogene AF1Q in breast cancer metastasis

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ABSTRACT

To study the molecular mechanisms underlying breast cancer metastasis, gene expression profile analysis was performed on two well-established breast cancer cell lines with high and low metastatic potentials: MDA-MB-435HM and MDA-MB-435LM. The analysis was conducted using cDNA microarrays containing 8000 genes. Of 60 differentially expressed genes, ALL1-fused gene from chromosome 1q (AF1Q), a putative oncogene not described previously in breast cancer, was identified and found to be over-expressed in MDA-MB-435HM cells compared with MDA-MB-435LM cells. The results indicate that AF1Q may play an important role in breast cancer metastasis. To test this hypothesis, we generated an AF1Q high-expression cell line by stable transfection of AF1Q cDNA into MDA-MB-435LM cells. Results showed that over-expression of AF1Q led to a marked increase in the invasive and metastatic potential of MDA-MB-435LM cells *in vitro* and *in vivo*, accompanied by the up-regulation of matrix metalloproteinase-2 (MMP-2), MMP-9, transcription factor Ets-1, and RhoC expression in both mRNA and protein levels. Consistent with this observation, reduced AF1Q expression in MDA-MB-435HM cells by small interfering RNA (siRNA) resulted in a significant decrease in the invasive potential of MDA-MB-435HM cells *in vitro* and in the protein expression of MMP-2, MMP-9, Ets-1, and RhoC, compared with either parental or non-silencing control cells. These data provide functional evidence that oncogene AF1Q may be a novel mediator of metastasis promotion in human breast cancer through regulation of the MMP pathway and RhoC expression.

1. Introduction

Despite significant advances in the treatment of primary tumours, the development of metastasis presents a continuing therapeutic challenge and is the common cause of death for patients with breast cancer.[1] Thus, an improved understanding of the molecular mechanisms underlying breast cancer metastasis is essential to develop novel and more effective molecular targets for therapy, and identify patients with the highest risk for disease relapse. Although a number of molecules have been implicated in the complex process, the precise mechanisms promoting breast cancer metastasis remain unclear.[2,3]

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The poor understanding of the molecular mechanisms involved in breast cancer metastasis is due, in part, to the lack of ideal models for study. Recently, we developed a spontaneous model of breast cancer metastasis through *in vivo* stepwise selection of pulmonary metastatic cells by human breast cancer MDA-MB-435 cells in athymic mice according to the published methods with some modifications.[4–7] A pair of breast cancer cell lines with low and high metastatic potential, termed MDA-MB-435LM and MDA-MB-435HM, was derived within the presented model system as described previously.[8] The well-defined differences in metastatic potential of the genetically related cell lines make it a valuable model system for the comparative study of the molecular events involved in breast cancer metastasis.

The development of cDNA microarray technology has provided new insight into the genetic basis of tumour metastasis.[9–12] To define genetic determinants of breast cancer metastasis, we applied cDNA microarrays to compare gene expression profile of the two genetically related breast cancer cell lines with different metastatic potentials. Array analyses have defined 60 of 8000 unique printed cDNA probes (genes) that are differentially expressed. Within the set of differentially expressed genes, of particular interest was the overexpression of a putative oncogene not described previously in breast cancer; ALL1-fused gene from chromosome 1q (AF1Q), which encodes a 9 kDa transmembrane protein without apparent similarity to any other protein.[13]

The AF1Q gene, located on chromosome band 1q21, was initially identified as a mixed lineage leukaemia (MLL) fusion partner in an infant with acute myeloid leukaemia (AML) carrying the t (1; 11) (q21; q23) translocation.[13] Previous studies have demonstrated that AF1Q expression is up-regulated in lymphoid and myeloid malignancies as well as thyroid oncocytic tumours.[14–17] In children with AML, high AF1Q expression indicates a poor prognosis.[18]. More recently, Keshava and colleagues have demonstrated that expression of AF1Q was induced in primary normal mammary epithelial cells when exposed to chemical carcinogen benzo[a]pyrene.[19] Based on these known functions of the AF1Q protein, we hypothesised that increased AF1Q expression may be involved in breast cancer development and metastasis. To test this hypothesis, we generated an AF1Q high-expression cell line by stable transfection of AF1Q cDNA into MDA-MB-435LM cells. Results showed that induced expression of AF1Q in MDA-MB-435LM cells enhances the invasive and metastatic potential of MDA-MB-435LM cells *in vitro* and *in vivo*, accompanied by up-regulation of matrix metalloproteinase-2 (MMP-2), MMP-9, transcriptional factor Ets-1, and RhoC expression. These results were further supported by the data obtained from small interfering RNA (siRNA) experiments *in vitro*. These data provide functional evidence that AF1Q may be a novel mediator of metastasis promotion in human breast cancer.

2. Materials and methods

2.1. Cell lines and animals

MDA-MB-435LM and MDA-MB-435HM cell lines were established from the same parent cell line, MDA-MB-435, as described previously,[8] and maintained in a 1:1 (v/v) mixture of Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 containing 10% foetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. The medium was changed every 2–3 d, and cells were harvested by treatment with 0.25% trypsin/0.53 mM EDTA solution. All culture medium components were obtained from Gibco BRL (Grand Island, NY, United States of America (USA)). Female BALB/c-*nu*/*nu* nude mice, 4–6 weeks old, were obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences (Shanghai, China), and housed in laminar-flow cabinets under specific pathogen-free conditions with food and water *ad libitum*. All experiments on mice were conducted in accordance with the guidelines of National Institutes of Health for the Care and Use of Laboratory Animals. The study protocol was also approved by the Institutional Animal Care and Use Committee of Fudan University (Shanghai, China).

2.2. Microarray assay

To gain insights into the molecular events involved in breast cancer metastasis, we compared the gene expression profile of two well-established breast cancer cell lines with high and low metastatic potentials, MDA-MB-435HM and MDA-MB-435LM. Analyses were conducted on a BiostarH-80s cDNA chip (Biostar Genechip, Inc., Shanghai, China) containing 8000 target cDNA clones, as described previously.[20] Briefly, MDA-MB-435HM and MDA-MB-435LM cells were used for RNA extraction and reverse transcription preparation of fluorescent cDNA probes labelled with Cy3- or Cy5-deoxy UTP (Amersham Pharmacia Biotech, Piscataway, NJ, USA), respectively. Quantified probes were applied to pre-hybridised BiostarH-80s chips under a cover glass. After hybridisation, the chips were scanned with a ScanArray 4000 (GSI Lumonics, Billerica, MA, USA) at two wavelengths (532 and 653 nm for Cy3 and Cy5, respectively) to detect emission from both Cy3 and Cy5. The resulting images were analysed using GenePix Pro 3 software (Axon Instruments, Foster City, CA, USA). Overall intensities were normalised using the corresponding GenePix default normalisation factor. Regulated genes were identified by ratio more than 2.0 or less than 0.5, which indicates at least two-fold up-regulation or 50% reduction. Experiments were performed in duplicate, and clones that were differentially expressed in both hybridisations were selected for further analysis.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

To validate results obtained from microarray analysis, RT-PCR analysis of selected genes was performed. Total RNA was isolated from cultured MDA-MB-435LM and MDA-MB-435HM cells using Trizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's instructions, and RT-PCR was performed as described previously.[21] Gene-specific primers for human AF1Q, nucleosomal binding protein 1 (NSBP1), interleukin 13 receptor, alpha 2 (IL13 RA2), carbonic anhydrase VIII (CA8), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and their annealing temperatures are listed in Table 1. All experiments were repeated three times, and

mRNA level of each gene was normalised to that of GAPDH as an internal control.

2.4. Construction of human AF1Q expression vector and stable transfections

To construct the human AF1Q expression vector, the entire open reading frame of human AF1Q gene (GenBank Accession No. NM_006818) was amplified from MDA-MB-435HM cells by RT-PCR using specific primers (upstream primer: 5′-TTTGGATCCATGAGGGACCCTGTGAGTA-3′, with an added *Bam*HI site underlined; downstream primer: 5′-AACGAATTCTTAGAGCAAGTCCATTCGA-3′, with an added *Eco*RI site underlined), and then subcloned in-frame into the upstream region of a sequence encoding V5- and $(His)_6$-epitope tags in the mammalian expression vector pcDNA3.1/V5-His, creating a fusion gene for a carboxyl-terminally tagged AF1Q. The recombinant vector was identified by restriction digestion and automated sequencing analysis (ABI 377; Applied Biosystems, Shanghai, China). To determine the effects of AF1Q on the invasive and metastatic potential of MDA-MB-435LM cells, cells were stably transfected with either resultant constructs or empty vector by using Lipofectamine™ 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, and selected in the presence of 800 μg/ml Geneticin (G418 sulphate; Invitrogen) for 4 weeks. The AF1Q-positive colonies were identified by RT-PCR and Western blotting analysis. In this study, the clone in which AF1Q gene was successfully transfected was named as MDA-MB-435LM/AF1Q clones (two positive clones were selected, named AF1Q1 and AF1Q2, respectively). The one only transfected with empty

Table 1 – Primers for RT-PCR and their annealing temperatures

Genes		Primer sequences	Product size (bp)	Annealing temperature (°C)
AF1Q	Up	5′-GCGGGATTACTGGGTGTATG-3′		
	Down	5′-GGTGCAGCTCAAAAGTTTCC-3′	333	55
NSBP1	Up	5′-AAAGAAAGGCTGCAGGTCAA-3′		
	Down	5′-CCCTTTTCTGTGGCATCTTC-3′	328	57
IL13RA2	Up	5′-CCCCACTGTCTCTGGATCAT-3′		
	Down	5′-TCTGATGCCTCCAAATAGGG-3′	454	57
CA8	Up	5′-CACTGGGGAAGAGAAAACCA-3′		
	Down	5′-CCGAAAGTTGTCTCCCAAAA-3′	474	58
MMP1	Up	5′-TTCATTTCTGTTTTCTGGCC-3′		
	Down	5′-ATTTTTCCTGCAGTTGAACC-3′	462	52
MMP2	Up	5′-CAGGCTCTTCTCCTTTCACAAC-3′		
	Down	5′-AAGCCACGGCTTGGTTTTCCTC-3′	398	55
MMP7	Up	5′-GTTTAG,AAGCCAAACTCAAGG–3′		
	Down	5′-CTTTGACACTAATCGATCCAC-3′	232	55
MMP9	Up	5′-TGGGCTACGTGACCTATGACAT-3′		
	Down	5′-GCCCAGCCCACCTCCACTCCTC-3′	150	60
Ets-1	Up	5′-GGGTGACGACTTCTTGTTTG-3′		
	Down	5′-GTTAATGGAGTCAACCCAGC-3′	274	57
uPA	Up	5′-GTGGCCAAAAGACTCTGAGG-3′		
	Down	5′-GGCAGGCAGATGGTCTGTAT-3′	400	58
uPAR	Up	5′-AGCTATCGGACTGGCTTGAA-3′		
	Down	5′-TGTTGCAGCATTTCAGGAAG-3′	352	55
Cathe D	Up	5′-GACACAGGCACTTCCCTCAT-3′		
	Down	5′-GTAGTAGCGGCCGATGAAGA-3′	300	55
Maspin	Up	5′-CCCTATGCAAAGGAATTGGA-3′		
	Down	5′-CAAGCCTGTGGACTCATCCT-3′	399	57
cystatin C	Up	5′-CCAGCAACGACATGTACCAC-3′		
	Down	5′-AAGGCACAGCGTAGATCTGG-3′	207	57
VEGF	Up	5′-CTACCTCCACCATGCCAAGT-3′		
	Down	5′-TCTCTCCTATGTGCTGGCCT-3′	311	60
bFGF	Up	5′-AGAGCGACCCTCACATCAAG-3′		
	Down	5′-ACTGCCCAGTTCGTTTCAGT-3′	234	60
Rho C	Up	5′-ATGGCTGCAATCCGAAAGAAAG-3′		
	Down	5′-TCAGAGAATGGGACAGCCCCT-3	582	55
GADPH	Up	5′-GGGAGCCAAAAGGGTCATCATCTC-3′		
	Down	5′-CCATGCCAGTGAGCTTCCCGTTC-3′	353	60

vector was named as MDA-MB-435LM/vector. For all functional and biological assays, cells at 70–90% confluence were used with viability >95%.

2.5. Expression analysis of AF1Q transfectants

For RT-PCR analysis, total RNA isolated from control and AF1Q-transfected cells was reverse-transcribed, and a 273-base pair human AF1Q product was amplified by PCR using ATGAGGGACCCTGTGAGTA and TTAGAGCAAGTCCATTCGA as 5′ and 3′ primers, respectively, and *Taq* DNA polymerase (Invitrogen). Total mRNA samples without reverse transcription were used as negative control. Western blotting analysis was performed using a monoclonal mouse anti-human V5 antibody (Invitrogen) according to the published method (21, 22). Normalisation of protein loading was performed with β-actin.

2.6. In vitro invasion assays

In vitro invasion assays were performed to analyse the invasive potential of MDA-MB-435LM, MDA-MB-435LM/vector, and MDA-MB-435LM/AF1Q transfectants, as described previously.[21]

2.7. RT-PCR and Western blotting analysis

RT-PCR and Western blotting analyses were performed to determine the mRNA and protein expression of matrix metalloproteinase-1 (MMP-1), MMP-2, MMP-7, MMP-9, transcription factor Ets-1, tissue inhibitor of MMP (TIMP)-1, TIMP-2, urokinase-type plasminogen activator (uPA), uPA receptor (uPAR), cathepsin D, maspin, cystatin C, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and RhoC in the AF1Q transfectants and vector control cells, as described previously.[21,22] Gene-specific primers and their annealing temperatures for RT-PCR are listed in Table 1. All primary antibodies and horseradish peroxidase-conjugated secondary antibodies for Western blotting analysis were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Amersham Pharmacia Biotech (Piscataway, NJ, USA), respectively.

2.8. Gelatin zymography

Gelatin zymography was used to evaluate MMP-2 and MMP-9 activities in tissue culture supernatant, as described previously.[23] Briefly, serum-free medium was placed on confluent cultures of MDA-MB-435LM and AF1Q transfectants for 24 h. Protein concentration in culture supernatants was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, USA). Samples of conditioned medium were fractionated on 10% SDS-PAGE containing 1 mg/ml gelatin (Sigma Chemical Co., St Louis, MO, USA). The gels were washed for 30 min in 50 mM Tris, 0.02% NaN_3 and 2.5% Triton X-100, pH7.5, and for another 30 min in the same buffer supplemented with 5 mM $CaCl_2$ and 1 mM $ZnCl_2$. Then, the gels were incubated in 50 mM Tris, 0.02% NaN_3, 5 mM $CaCl_2$, and 1 mM $ZnCl_2$ for 24 h at 37°C, fixed in 50% methanol and 7% acetic acid, and stained with 0.2% Coomassie Blue R250. Densitometry was used to analyse relative MMP-2 and MMP-9 activities with NIH Image version 1.62.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The media were collected after 48 h for MMP-2 and MMP-9 ELISA determinations, as described below. The cells were taken through three freeze-thaw cycles, centrifuged and supernatant was collected for determination of protein concentration, as described above. MMP-2 and MMP-9 levels in the cell culture media were measured using a Quantikine kit from R & D Diagnostics (Minneapolis, MN, USA) using the procedure provided by the supplier. Human recombinant MMP-2 and MMP-9 included in the kit was used to construct a standard curve and obtain absolute values of MMP-2 and MMP-9 protein content. The values were then normalised to the total protein concentration in each dish.

2.10. Small interfering RNA (siRNA)

To further demonstrate the role of the AF1Q gene in the progression of human breast cancer, we used the siRNA to down-regulate the expression of AF1Q gene in MDA-MB-435HM cells. Double-stranded 19-nucleotide RNAs were synthesised by Shanghai GeneChem Co., Ltd (Shanghai, China). The targeting sequence of human AF1Q was 5′-GCAGCAGACCAGGAGAAAA-3′, corresponding to the coding region 1045–1064 relative to the first nucleotide of the start codon. The sequence was submitted to a BLAST search against the human genome sequence to ensure that only the AF1Q gene was targeted. Oligonucleotides were annealed by incubation in a 1 × universal buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, and 2 mM magnesium acetate) for 2 min at 90°C, followed by overnight at 4°C. The AF1Q siRNA duplex and a control scramble siRNA duplex (Shanghai GeneChem Co., Ltd) were transfected into subconfluent MDA-MB-435HM cells using Lipofectamine™ 2000 reagent following the manufacturer's recommended protocol. The knock-down level of AF1Q gene was analysed by RT-PCR methods, as described above. Forty-eight hours after transfection, the effects of reduced AF1Q expression on the invasion capacity and the protein expression of MMP-2, MMP-9, Ets-1, and RhoC in MDA-MB-435HM cells were determined by *in vitro* invasion assay and Western blotting analysis, respectively, as described previously. All experiments were performed in duplicate.

2.11. Tumourigenicity and metastasis assays in nude mice

The tumourigenicity and spontaneous metastatic capability of the cell lines were determined by injection into the mammary fat pad, as described previously.[21,22] Animals were divided into four groups, including MDA-MB-435LM, MDA-MB-435LM/vector, MDA-MB-435LM/AF1Q1, and MDA-MB-435LM/AF1Q2 groups, and each group had 8 mice.

2.12. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) software, version 11.5 for

Windows (Chicago, IL, USA), with $P < 0.05$ as the statistically significant level.

3. Results

3.1. Molecular characterisation of MDA-MB-435HM and MDA-MB-435LM

To gain insight into the molecular changes associated with the enhanced pulmonary metastatic potential of MDA-MB-435HM cells, microarray analyses were performed to determine the differences in gene expression profile between MDA-MB-435HM and MDA-MB-435LM cells. Results showed that 60 of the 8000 genes were differentially expressed in replicate microarray experiments (Table 2). To confirm the results of cDNA microarray analysis, four genes identified by microarray analysis were chosen randomly for further validation by RT-PCR in this model. As shown in Fig. 1, analysis of replicate samples from MDA-MB-435HM and MDA-MB-435LM cells confirmed a close correlation with the level of expression as revealed by microarray analysis. One of these genes, AF1Q, a putative oncogene not described previously in breast cancer, was further examined for functional relevance in breast cancer development and metastasis.

3.2. Construct of human AF1Q expression vector and stable transfections

The entire open reading frame of human AF1Q gene was amplified by RT-PCR from MDA-MB-435HM RNA, and then cloned into the pcDNA3.1/V5-His expression vector. The resultant construct was confirmed by restriction enzyme analysis and DNA sequencing. To investigate the effects of AF1Q over-expression on the metastatic phenotype of MDA-MB-435LM cells, stable AF1Q transfectants were established. As shown in Fig. 2(A), RT-PCR analysis revealed that levels of AF1Q expression were found to be 5.1- and 5.2-fold higher in two AF1Q transfectants (AF1Q1 and AF1Q2), respectively, compared with either vector control cells or its parental counterpart. In addition, the small, highly immunoreactive V5 epitope tag (GKPIPNPLLGLDST) contained in the pcDNA3.1/V5-His expression vector allowed us to screen colonies directly for protein expression by Western blotting analysis using a monoclonal V5 tag antibody. Results showed that two AF1Q transfectants (AF1Q1 and AF1Q2) highly express V5-tagged AF1Q protein (Fig. 2(B)). In this study, both clones were selected for the subsequent experiments.

3.3. Over-expression of AF1Q enhances the invasive potential of MDA-MB-435LM cells in vitro through regulation of MMP-2, MMP-9, Ets-1 and RhoC expression

We used an *in vitro* reconstituted basement membrane (Matrigel) invasion assay to determine the effect of AF1Q on cell invasion. In this assay, all cell lines tested were invasive through Matrigel after 24 h. However, the AF1Q transfectants showed, on average, two-fold increase in invasive capacity compared with either parental or vector control-transfected cells (Fig. 3(A)). To further investigate the molecular mechanisms underlying AF1Q-mediated invasion phenotype *in vitro*, we focused our attention on the several recognised invasion- and metastasis-associated genes. RT-PCR and Western blotting analysis showed that AF1Q transfectants consistently enhance the mRNA and protein expression of MMP-2, MMP-9, transcriptional factor Ets-1, and RhoC compared with parental or vector control-transfected cells (Fig. 3(B, C, D and E)). However, we did not find any significant differences in MMP-1, MMP-7, TIMP-1, TIMP-2, u-PA, u-PAR, cathepsin D, mapsin, cystatin C, bFGF, or VEGF expression in either mRNA or protein levels between AF1Q transfectants and control cells (data not shown). As the effects of AF1Q were pronounced on MMP-2 and MMP-9, the MMP-2 and MMP-9 proteins secreted by the AF1Q transfectants or by control cells were further determined by gelatin zymography and enzyme-linked immunosorbent assay (ELISA). By gelatin zymographic analysis, two AF1Q transfectants consistently secreted high levels of MMP-2 and MMP-9 (Fig. 3(F)). The MMP-2 and MMP-9 activities in AF1Q transfectants (AF1Q1 and AF1Q2) are, on average, 3.2- and 2.5-fold higher than those in MDA-MB-435LM/vector cells, respectively. Meanwhile, we also used ELISA assay to quantitate the amounts of secreted MMP-2 and MMP-9 proteins in the conditioned media. As shown in Fig. 3(G), the amounts of MMP-2 and MMP-9 proteins produced by AF1Q transfectants are, on average, 3.5- and 2.7-fold higher, respectively, than that secreted by the parental and vector-transfected MDA-MB-435LM cells.

3.4. Reduced AF1Q expression decreases tumour invasion in vitro and down-regulates MMP-2, MMP-9, Ets-1, and RhoC protein expression

We used the RNA interference technique to silence the AF1Q gene in MDA-MB-435HM cells to determine whether AF1Q expression is critical to the invasive phenotype of MDA-MB-435HM cells *in vitro*. Reduced AF1Q expression was detected by RT-PCR, which has been shown previously to be an accurate measure of gene knockdown.[24] As shown in Fig. 4(A and B), an approximate 65% reduction in AF1Q expression was observed in siRNA-targeted MDA-MB-435HM cells compared with parental and non-silencing control. As shown in Fig. 4(C and D), reduced AF1Q expression in MDA-MB-435HM cells resulted in a significant decrease in the invasive potential of MDA-MB-435HM cells *in vitro* and concomitant down-regulation of the MMP-2, MMP-9, Ets-1, and RhoC protein expression compared with either parental or non-silencing control cells.

3.5. Over-expression of AF1Q enhances tumour growth and pulmonary metastasis in the athymic mice

The effect of AF1Q expression on tumour growth and metastasis was further assayed using an orthotopic xenograft tumour model in the athymic mice. Results revealed that AF1Q transfectants grew much faster than either vector-transfected or parental cells in the nude mice (Fig. 5(A)). To study pulmonary metastasis, lungs were examined physically at autopsy and then subjected to microscopic examination for morphological evidence of tumour cells by light microscopy on haematoxylin and eosin (H& E)-stained paraffin sections. At the experimental endpoint, the number of overt surface

Table 2 – Differentially expressed genes in MDA-MB-435HM and MDA-MB-435LM cell lines revealed by cDNA microarray analysis

GenBank_ID	Definition	Ratios*
NM_004056	Homo sapiens carbonic anhydrase VIII (CA8), mRNA	0.130
NM_003507	Homo sapiens frizzled (Drosophila) homolog 7 (FZD7), mRNA	0.233
BC014890	Homo sapiens, slug (chicken homolog), zinc finger protein, clone MGC:10182 IMAGE:3908245, mRNA, complete cds	0.248
NM_006350	Homo sapiens follistatin (FST), transcript variant FST317, mRNA	0.261
NM_006209	Homo sapiens ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin) (ENPP2), mRNA	0.273
AL512688	Homo sapiens mRNA; cDNA DKFZp547J2313 (from clone DKFZp547J2313)	0.301
NM_012413	Homo sapiens glutaminyl-peptide cyclotransferase (glutaminyl cyclase) (QPCT), mRNA	0.311
AL133623	Homo sapiens mRNA; cDNA DKFZp434P0721 (from clone DKFZp434P0721); partial cds	0.340
NM_002765	Homo sapiens phosphoribosyl pyrophosphate synthetase 2 (PRPS2), mRNA	0.344
NM_015680	Homo sapiens hypothetical protein (CGI-57), mRNA	0.374
D86982	Human mRNA for KIAA0229 gene, partial cds	0.374
NM_005744	Homo sapiens ariadne (Drosophila) homolog, ubiquitin-conjugating enzyme E2-binding protein, 1 (ARIH1), mRNA	0.391
AL117657	Homo sapiens mRNA; cDNA DKFZp586F1924 (from clone DKFZp586F1924)	0.395
NM_004629	Homo sapiens Fanconi anaemia, complementation group G (FANCG), mRNA	0.404
NM_001693	Homo sapiens ATPase, H+ transporting, lysosomal (vacuolar proton pump), beta polypeptide, 56/58kD, isoform 2 (ATP6B2), mRNA	0.412
AB029551	Homo sapiens YEAF1 mRNA for YY1 and E4TF1 associated factor 1, complete cds	0.430
NM_001822	Homo sapiens chimerin (chimaerin) 1 (CHN1), mRNA	0.439
BC004538	Homo sapiens, clone IMAGE:3947276, mRNA, partial cds	0.457
NM_006392	Homo sapiens nucleolar protein (KKE/D repeat) (NOP56), mRNA	0.466
AB067489	Homo sapiens mRNA for KIAA1902 protein, partial cds	0.480
NM_001011	Homo sapiens ribosomal protein S7 (RPS7), mRNA	0.482
NM_004965	Homo sapiens high-mobility group (nonhistone chromosomal) protein 14 (HMG14), mRNA	0.489
NM_018457	Homo sapiens DKFZp564J157 protein (DKFZP564J157), mRNA	2.113
NM_014908	Homo sapiens KIAA1094 protein (KIAA1094), mRNA	2.116
NM_024056	Homo sapiens hypothetical protein MGC5576 (MGC5576), mRNA	2.117
NM_018046	Homo sapiens hypothetical protein FLJ10283 (FLJ10283), mRNA	2.135
AL110204	Homo sapiens mRNA; cDNA DKFZp586K1922 (from clone DKFZp586K1922)	2.178
NM_004598	Homo sapiens sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) (SPOCK), mRNA	2.233
NM_005816	Homo sapiens T cell activation, increased late expression (TACTILE), mRNA	2.245
NM_002039	Homo sapiens GRB2-associated binding protein 1 (GAB1), mRNA	2.253
L08895	Homo sapiens MADS/MEF2-family transcription factor (MEF2C) mRNA, complete cds	2.291
AB037746	Homo sapiens mRNA for KIAA1325 protein, partial cds	2.301
NM_001061	Homo sapiens thromboxane A synthase 1 (platelet, cytochrome P450, subfamily V) (TBXAS1), transcript variant TXS-I, mRNA	2.321
NM_017918	Homo sapiens hypothetical protein FLJ20647 (FLJ20647), mRNA	2.325
NM_016185	Homo sapiens haematological and neurological expressed 1 (HN1), mRNA	2.327
AL050107	Homo sapiens mRNA; cDNA DKFZp586I1419 (from clone DKFZp586I1419); partial cds	2.359
NM_001423	Homo sapiens epithelial membrane protein 1 (EMP1), mRNA	2.392
NM_003928	Homo sapiens CAAX box 1 (CXX1), mRNA	2.442
BC016962	Homo sapiens, clone IMAGE:4182947, mRNA	2.470
AL137311	Homo sapiens mRNA; cDNA DKFZp761G02121 (from clone DKFZp761G02121); partial cds	2.564
NM_021910	Homo sapiens FXYD domain-containing ion transport regulator 3 (FXYD3), transcript variant 2, mRNA	2.565
NM_018355	Homo sapiens hypothetical protein FLJ11191 (FLJ11191), mRNA	2.584
NM_002659	Homo sapiens plasminogen activator, urokinase receptor (PLAUR), mRNA	2.589
NM_002727	Homo sapiens proteoglycan 1, secretory granule (PRG1), mRNA	2.821
BC011878	Homo sapiens, Similar to hypothetical protein FLJ14627, clone MGC:20447 IMAGE:3532898, mRNA, complete cds	2.843
NM_007021	Homo sapiens decidual protein induced by progesterone (DEPP), mRNA	2.875
NM_014622	Homo sapiens loss of heterozygosity, 11, chromosomal region 2, gene A (LOH11CR2A), mRNA	2.936
AK027071	Homo sapiens cDNA: FLJ23418 fis, clone HEP21245, highly similar to HSU35048 Human TSC-22 protein mRNA	2.938

(*continued on next page*)

Table 2 – *continued*

GenBank_ID	Definition	Ratios*
AK027727	Homo sapiens cDNA FLJ14821 fis, clone OVARC1000556, highly similar to RIBOSOMAL PROTEIN S6 KINASE II ALPHA 2 (EC 2.7.1.-)	2.971
AL157424	Homo sapiens mRNA; cDNA DKFZp761E1512 (from clone DKFZp761E1512)	3.025
NM_006818	Homo sapiens ALL1-fused gene from chromosome 1q (AF1Q), mRNA	3.045
L78132	Human prostate carcinoma tumour antigen (pcta-1) mRNA, complete cds	3.083
NM_001085	Homo sapiens serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 (SERPINA3), mRNA	3.253
AK025983	Homo sapiens cDNA: FLJ22330 fis, clone HRC05729, highly similar to AF131821 Homo sapiens clone 24877 mRNA sequence	3.254
NM_002825	Homo sapiens pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1) (PTN), mRNA	4.124
NM_030763	Homo sapiens nucleosomal binding protein 1 (NSBP1), mRNA	4.813
NM_000583	Homo sapiens group-specific component (vitamin D binding protein) (GC), mRNA	4.882
NM_000640	Homo sapiens interleukin 13 receptor, alpha 2 (IL13RA2), mRNA	7.360
AK027847	Homo sapiens cDNA FLJ14941 fis, clone PLACE1010944	10.618
BC008915	Homo sapiens, clone MGC:16086 IMAGE:3618167, mRNA, complete cds	20.706

* Numbers indicate the ratio of Cy5/Cy3, i.e. the changes in expression, and are the average of two independent hybridisations.

metastases observed in the lungs from mice with MDA-MB-435LM/AF1Q tumours was increased three-fold compared with lungs from mice bearing parental MDA-MB-435LM tumours. When these lungs were examined microscopically, large numbers of metastases were observed in the mice bearing MDA-MB-435LM/AF1Q tumours, while the lungs of the mice bearing MDA-MB-435LM/vector and parental MDA-MB-435LM tumours had significantly fewer metastases (Fig. 5(B and C)). In addition, similar effects of AF1Q on MMP-2, MMP-9, Ets-1, and RhoC expression noted in the *in vitro* studies were also observed in the *in vivo* studies (Fig. 5(D and E)).

4. Discussion

In this study, we have identified one set of breast cancer metastasis-associated genes by using two well-established breast cancer cell lines with different metastatic potentials combined with microarray analysis. Some of them have been implicated in the development and progression of breast cancer, but many remain to be characterised. In particular, we demonstrated that one of these identified genes, AF1Q, an oncogene not described previously in breast cancer, might has a functional role in breast cancer metastasis. Over-

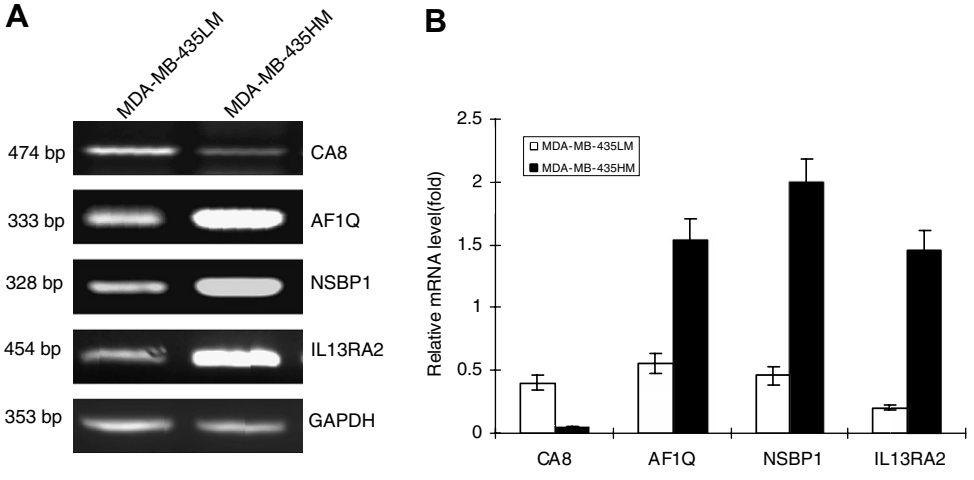

Fig. 1 – (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of selected genes differentially expressed in MDA-MB-435HM and MDA-MB-435LM cell lines according to microarray analyses. RT-PCR was performed with total RNA isolated from both cell lines. PCR products were loaded on a 1.2% agarose gel and stained with ethidium bromide. The expected length of PCR products is indicated on the left. (B) Relative mRNA level of carbonic anhydrase VIII (CA8), ALL1-fused gene from chromosome 1q (AF1Q), nucleosomal binding protein 1 (NSBP1), and interleukin 13 receptor, alpha 2 (IL13RA2) expression in both cell lines was normalised to the signal intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Generally, altered expressions of the representative subset of genes observed in microarray assay were correspondingly verified by RT-PCR.

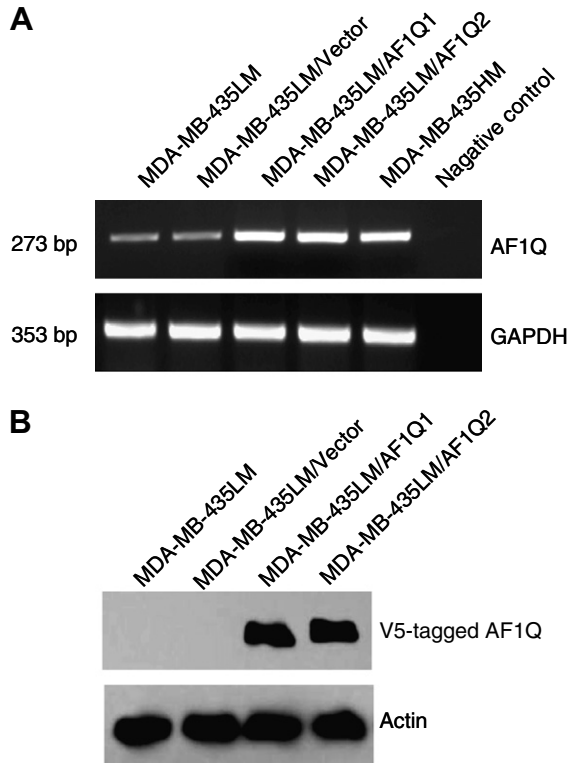

Fig. 2 – Expression of AF1Q in the AF1Q transfectants and control cells. (A) Representative reverse transcription-polymerase chain reaction (RT-PCR) is shown. RT-PCR was performed on total RNA extracts using gene-specific primers for AF1Q. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as an internal control. Negative control was performed using total RNA sample of MDA-MB-435HM cells without reverse transcription. (B) Western blotting analysis of V5-tagged AF1Q expression in the AF1Q transfectants and control cells. Equal amount of total proteins for each sample was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), blotted onto a polyvinylidene difluoride (PVDF) membrane, and probed with the V5 tag monoclonal antibody. The blot was developed using an enhanced chemiluminescence (ECL) detection system.

expression of AF1Q enhances the invasive and metastatic potential of MDA-MB-435LM cells *in vitro* and *in vivo*, accompanied by the up-regulation of MMP-2, MMP-9, transcription factor Ets-1, and RhoC expression. Consistent with this observation, reduced AF1Q expression in MDA-MB-435HM cells by siRNA resulted in a significant decrease in the invasive potential and the expression of MMP-2, MMP-9, Ets-1, and RhoC compared with either parental or non-silencing control cells. These data provide functional evidence that oncogene AF1Q may be a novel mediator of metastasis promotion in human breast cancer through regulation of MMP pathway and RhoC expression.

The differences in metastatic potential and the genetic relationship of MDA-MB-435HM and MDA-MB-435LM cells allowed the use of cDNA microarray to define potentially important genes for breast cancer metastasis. In the cDNA microarray comparisons presented herein, we identified 60 genes that were differentially expressed between both cell lines. By review of the literature, functions of these differentially expressed genes are involved in cytoskeleton and mobility, signal transduction, transcription regulation, immune surveillance, etc. The results are consistent with current views on breast cancer metastasis, an especially complex process that requires the coordinated expression of many different genes in multiple steps for both tumour cells and the surrounding stromal cells.[25] The outlier genes identified in the model included many that have not previously been described in breast cancer. This is to be expected and is an advantage of a cDNA microarray that included a wide variety of genes.[26]

The development and progression of breast cancer are accompanied by genetic alterations of multiple oncogenes and tumour suppressor genes.[27] A putative oncogene identified by array analysis, AF1Q, was of particular interest, as it is important in leukaemia development and thyroid tumourigenesis, but has not been reported in breast cancer.[13–18] More interestingly, induction of AF1Q expression in primary normal mammary epithelial cells when exposed to chemical carcinogens was demonstrated recently.[19] In our model, we observed that AF1Q was constitutively up-regulated expression in the highly metastatic MDA-MB-435HM cells relative to MDA-MB-435LM cells with low metastatic potential. To investigate whether AF1Q is an instigator of metastasis or merely a correlative product during breast cancer progression, we generated stable MDA-MB-435LM/AF1Q transfectants and tested them for any alterations in the invasive and metastatic phenotype. Our results showed that induced expression of AF1Q significantly enhances the invasive and metastatic potential of MDA-MB-435LM cells *in vitro* and *in vivo*. Consistent with this observation, reduced AF1Q expression resulted in a significant decrease in the invasive potential of MDA-MB-435HM cells *in vitro*. All these may be related to MMP-2, MMP-9, Est-1, and RhoC expression regulated by AF1Q.

MMPs are a family of more than 20 members of zinc-dependent neutral endopeptidases that play a key role in degrading the extracellular matrix and basement membrane in various cancers, and therefore promote metastasis and angiogenesis.[28–30] In this study, we proved that the enhancement of invasion of MDA-MB-435LM cells by AF1Q was accompanied by up-regulation of MMP-2 and MMP-9 mRNA and protein expression. However, no significant differences in MMP-1, MMP-7, TIMP-1, and TIMP-2 mRNA and protein expression were found among AF1Q transfectants, vector-transfected, and parental cells. Recent studies have revealed that Ets-1, which is one of the Ets family members, activates promoters of different subclasses of the MMP genes.[31] Some investigators have reported that the high expression of Ets-1 was correlated with tumour progression and poor prognosis.[32] Our data showed that the expression of Ets-1 was accelerated by AF1Q and exhibited a tendency similar to MMP-2 and MMP-9. These suggest that the up-regulation of MMP-2 and MMP-9 may be stimulated by AF1Q via activation of Ets-1 expression. In addition, previous studies have demonstrated that up-regulated expression of uPA, uPAR and cathepsin D, and down-regulated expression of serine protease inhibitors maspin and cystatin C are associated with increased tu-

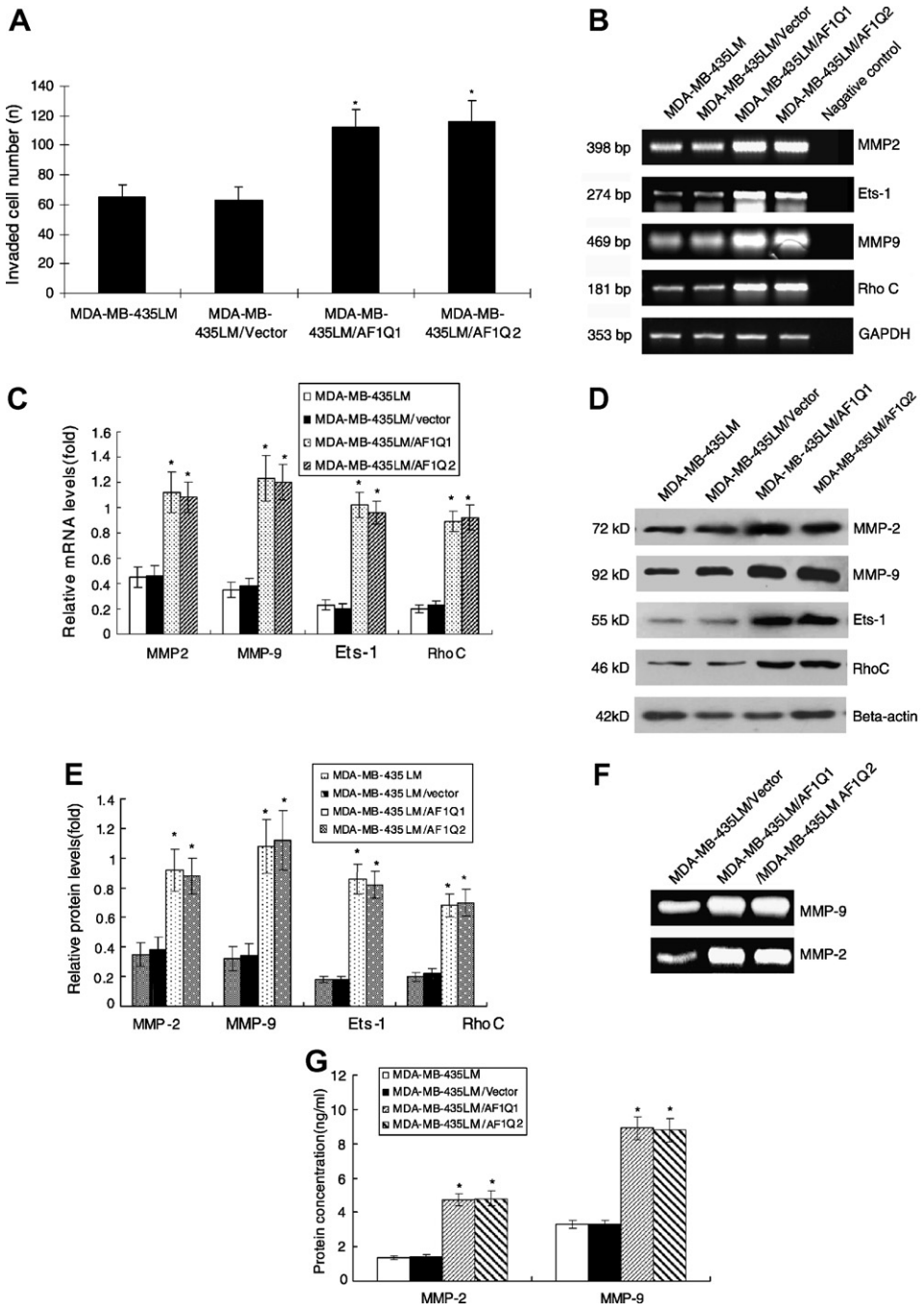

Fig. 3 – Stimulation of the invasive potential of MDA-MB-435LM cells by AF1Q in *in vitro* studies. (A) Results of Matrigel invasion assay. Cells in serum-free medium were seeded into the top of a Transwell chamber onto a Matrigel-coated filter. Serum-containing medium was placed into the low chamber as a chemo-attractant. The number of invading cells was counted 24 h later. The MDA-MB-435LM/AF1Q cells were, on average, two-fold more invasive than either parental or vector-transfected cells. *P < 0.05 versus control (Student's *t*-test). (B) Representative reverse transcription-polymerase chain reaction (RT-PCR) analysis of MMP-2, MMP-9, Ets-1, and RhoC mRNA expression in the AF1Q transfectants and control cells. Total RNA sample of MDA-MB-435LM/AF1Q1 cells without reverse transcription was taken as negative control. (C) Relative mRNA expression of MMP-2, MMP-9, Ets-1, and RhoC in different cell lines was normalised to the signal intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. *P < 0.05 versus control (Student's *t*-test). (D) Representative Western blotting analysis of MMP-2, MMP-9, Ets-1 and RhoC expression in AF1Q transfectants and control cells. (E) Relative protein expression of MMP-2, MMP-9, Ets-1 and RhoC in different cell lines was normalised to the signal intensity of β-actin as an internal control. *P < 0.05 versus control (Student's *t*-test). (F) The activity levels of MMP-2 and MMP-9 were detected by gelatin zymography in the AF1Q transfectants and vector control-transfected cells. (G) Enzyme-linked immunoassay (ELISA) analysis of MMP-2 and MMP-9 protein levels produced per millilitres of medium in 48 h by AF1Q transfectants, vector-transfected, and parental MDA-MB-435LM cells. *P < 0.05 versus control (Student's *t*-test).

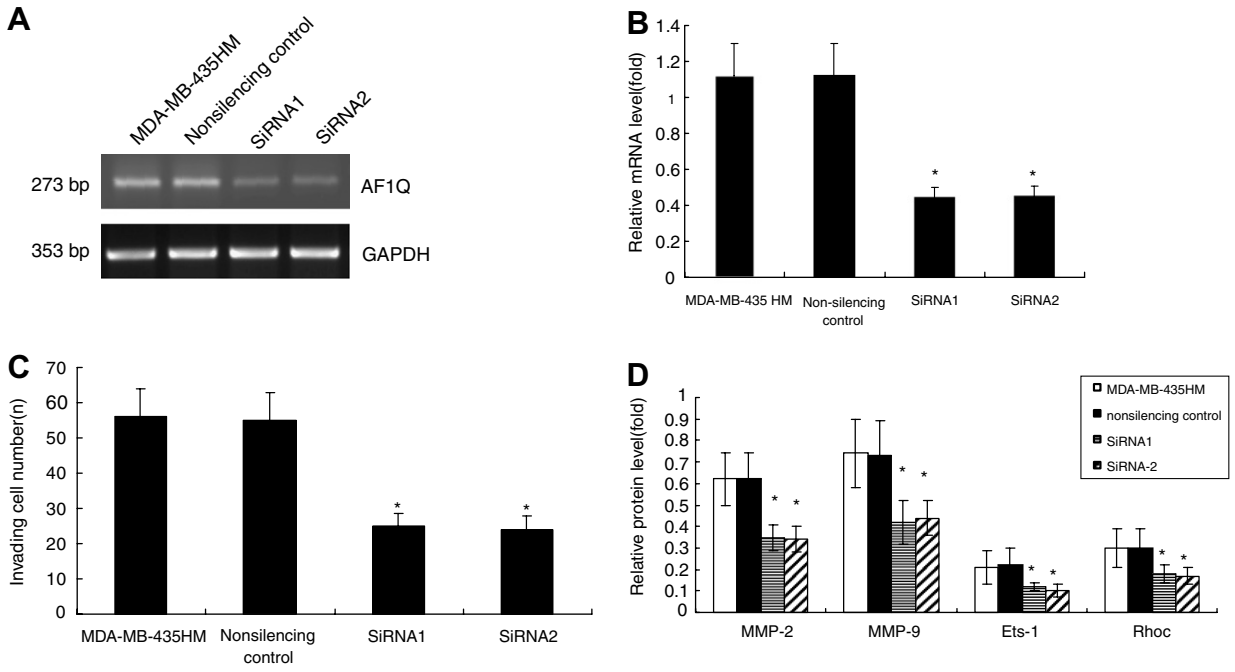

Fig. 4 – Reduced AF1Q expression in MDA-MB-435HM cells results in a significant decrease in the invasion capacity *in vitro* and in the protein expression of MMP-2, MMP-9, Ets-1, and RhoC. (A) Reverse transcription-polymerase chain reaction (RT-PCR) analysis of AF1Q expression in the siRNA-targeted MDA-MB-435HM cells, non-silencing control, and parental cells. (B) Relative mRNA expression of AF1Q in different cell lines was normalised to the signal intensity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. *$P < 0.05$ versus control (Student's *t*-test). (C) *In vitro* invasion assays of the invasive potential of MDA-MB-435HM, non-silencing control, and siRNA-targeted MDA-MB-435HM cells using Matrigel invasion chamber *in vitro*. *$P < 0.05$ versus control (Student's *t*-test). (D) Relative protein expression of MMP-2, MMP-9, Ets-1, and RhoC in different cell lines was normalised to the signal intensity of β-actin as an internal control.

mour-cell invasion and metastasis in several malignancies including breast cancer.[33–35] In this study, we did not find any significant differences in the expression of these proteolytic enzymes and inhibitors between AF1Q transfectants and control cells. Taken together, it is possible that the up-regulation of MMP-2, MMP-9, and transcriptional factor Ets-1 is partially responsible for the promoting effects of AF1Q on breast cancer metastasis.

RhoC belongs to the Ras superfamily of small GTPases.[36] Recent studies have demonstrated that up-regulation of RhoC plays a key role in metastasis, not only in inflammatory breast cancer,[37] but also in a plethora of malignant neoplasms including bladder,[38] ovary,[39] pancreas[40] and skin (melanoma)[11] through regulation of cell motility, invasion, and tumour-dependent angiogenesis. Therefore, there is an increasing interest in investigating the effect of AF1Q gene on RhoC expression. Interestingly, we found that AF1Q transfectants significantly up-regulate RhoC mRNA and protein expression compared with either parental or vector control-transfected cells. Therefore, it seems reasonable to conclude that the promoting effect of AF1Q on the invasive and metastatic potential of MDA-MB-435LM cells is mediated partially through up-regulation of RhoC expression. The growth and expansion of primary tumours and the development of secondary metastases require a large supply of blood. Thus, increased numbers of blood vessels, and hence increased angiogeneic activity, are essential for tumour growth and metastasis.[41] VEGF and bFGF are the two most potent factors that can stimulate angiogenesis and thus facilitate the malignant dissemination of cancer cells.[42] The increased expression of VEGF and bFGF genes has been detected in various human cancers including breast cancer. However, no significant difference in VEGF and bFGF expression was observed among AF1Q transfectants, vector control cells, and its parental counterparts. Our data suggest that AF1Q-induced metastasis may not be mediated by the regulation of the two important angiogenic factors.

RNAi is the sequence-specific silencing induced by double-stranded RNA (dsRNA).[24] In this study, we demonstrated that reduced AF1Q expression in MDA-MB-435HM cells by siRNA results in a significant decrease in the invasive potential of MDA-MB-435HM cells *in vitro* as well as the protein expression of MMP-2, MMP-9, Ets-1, and RhoC. The results support the data obtained from gene transfection experiments *in vitro*. Taken together, this study provides evidence that oncogene AF1Q has a functional role in primary breast tumour growth and spontaneous pulmonary metastasis in our model, at least in part, through regulation of MMP pathway and RhoC expression. Clearly, further studies are needed to prove whether these genes identified in this analysis have relevance to breast cancer and other solid tumours with high rates of distant metastasis.

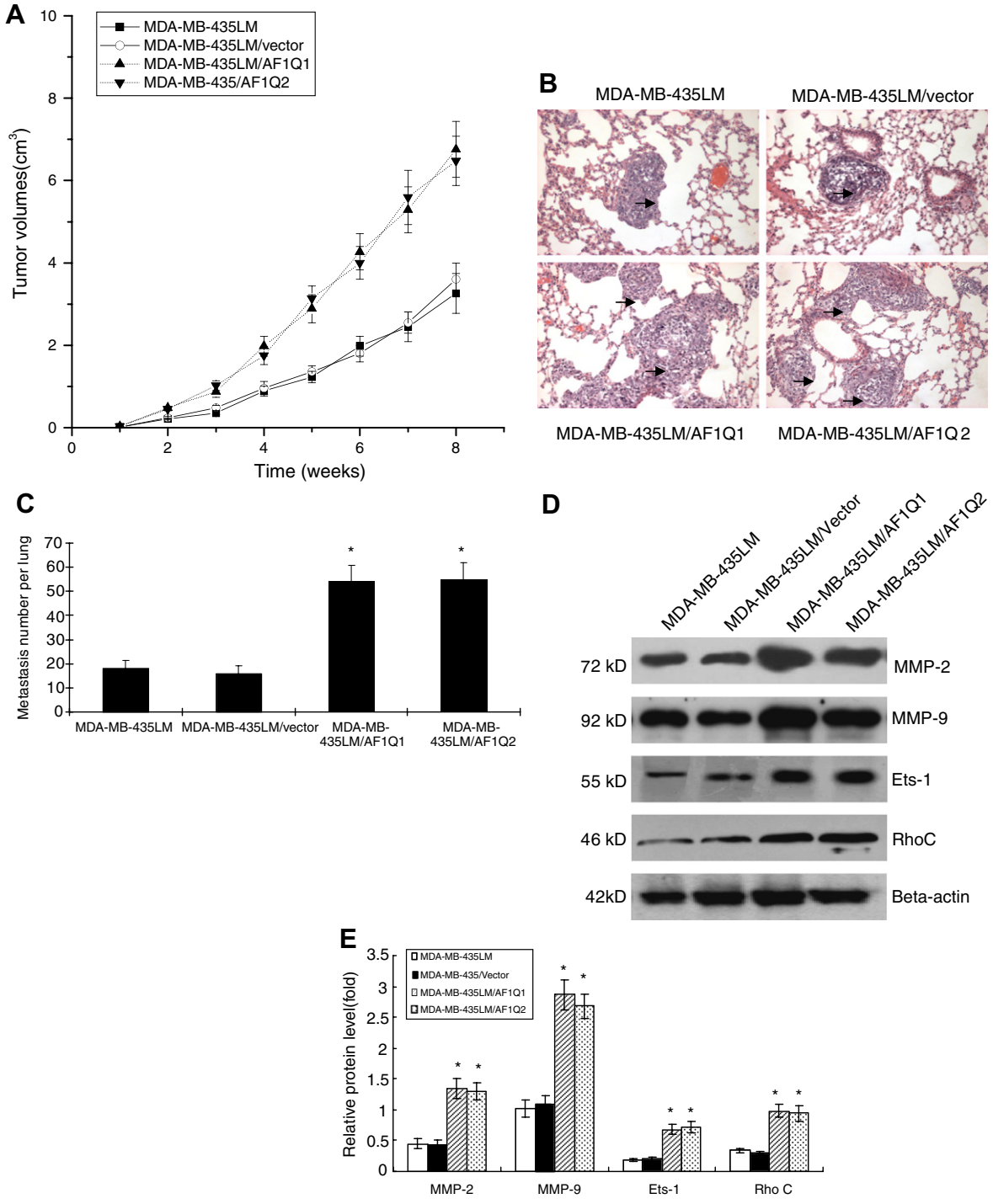

Fig. 5 – Stimulation of the growth and pulmonary metastasis of MDA-MB-435LM cells by AF1Q in *in vivo* studies. (A) Xenograft growth curves of MDA-MB-435LM transfectants and control. (B) Photomicrographs demonstrating the marked differences in metastatic nodules in lung sections obtained from mice bearing MDA-MB-435LM, MDA-MB-435LM/vector, MDA-MB-435LM/AF1Q1, and MDA-MB-435LM/AF1Q2 tumours (H& E × 200). Metastatic nodules are marked by arrows. (C) Representative metastasis numbers per lung in athymic mice bearing MDA-MB-435LM, MDA-MB-435LM/vector, MDA-MB-435LM/AF1Q1, and MDA-MB-435LM/AF1Q2 tumours by 8 weeks of post-inoculation. *$P < 0.05$ versus either parental or vector control cells (Mann-Whitney *U* test). (D) The protein levels of MMP-2, MMP-9, Ets-1, and RhoC were detected by Western blotting analysis in the xenografts of AF1Q transfectants and control. (E) Relative protein expression of MMP-2, MMP-9, Ets-1, and RhoC in different xenograft tumours was normalised to the signal intensity of β-actin as an internal control. *$P < 0.05$ versus control (Student's *t*-test).

Conflict of interest statement

None declared.

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