Original Article

TIMP4 Modulates ER-α Signalling in MCF7 Breast Cancer Cells

(TIMP4 / RNASeq / oestrogen receptor α / breast cancer)

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Abstract. Tissue inhibitor of metalloprotease 4 (TIMP4) contributes to poor prognosis in breast and other tumours. However, the mechanisms of how TIMP4 influences breast cancer cell behaviour are unknown. Our aim was to explore the signalling pathways modulated by TIMP4 in breast cancer cells. Human recombinant TIMP4 was added to MCF7 breast cancer cells and RNASeq was performed. TIMP4 RNASeq results were validated by RT-PCR. Network analyses of TIMP4-exposed cells showed that ER-α, HIF1A and TGF-β signalling were activated, whereas FOXO3 signalling was downregulated. ER-α protein levels were increased and concordantly, promoters of TIMP4-upregulated genes were significantly enriched in oestrogen-binding sites. We concluded that TIMP4 modulates multiple signalling pathways relevant in cancer in MCF7 cells, including the ER-α cascade.

Introduction

Tissue inhibitors of metalloproteases (TIMPs) are a family of proteins that suppress metalloprotease activity. Four TIMP members, TIMP1–4, are known in humans (Brew et al., 2000). In the past years it has been discovered that TIMPs have functions independent of their characteristic metalloprotease inhibition capability. These proteins are able to act as ligands of transmembrane receptors and to modulate diverse signalling networks (Stetler-Stevenson, 2008). TIMP4 is the most recently identified member of this family. Although a membrane receptor has not been identified, a yeast two-hybrid assay showed that CD63, a member of the tetraspanin family trasmembrane proteins, is able to interact with TIMP4 (Chirco et al., 2006). Nevertheless, co-immunoprecipitation assays in mammalian cervical cancer cells were not able to detect a direct interaction between these proteins, which could be due to specific posttranslational modifications (Lizarraga et al., 2015b). In addition, in clear contrast to other TIMP members, little is known about the signalling networks modulated by TIMP4 (Melendez-Zajgla et al., 2008), although recent work in our group has shown that, in cervical cells, NF-κB could be the main downstream signalling pathway modulated by this protein (Lizarraga et al., 2015b). Overexpression of TIMP4 is correlated with poor prognosis, advanced stage, and disease progression in multiple tumours (Lizarraga et al., 2005; Rorive et al., 2010). In breast cancer, TIMP4 protein overexpression significantly correlates with a decrease in overall and disease-free survival (Liss et al., 2009). These studies suggest that TIMP4 overexpression has oncogenic properties in human cancers. Our aim was to explore the possible TIMP4 signalling pathways in breast cancer cells. For this, we performed whole transcriptome assays by RNASeq in MCF7 breast cancer cells exposed to recombinant human TIMP4 (hrTIMP4).

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Abbreviations: DAVID – Database for Annotation, Visualization and Integrated Discovery, ER-α – oestrogen receptor α, FOXO3 – forkhead box O3, GO – gene ontology, HIF1A – hypoxia inducible factor 1 α subunit, HOMER – Hypergeometric Optimization of Motif EnRichment, TGF-β – transforming growth factor β,TIMP – tissue inhibitor of metalloprotease, TNF-α – tumour necrosis factor α.

Material and Methods

Cell culture

The human breast cancer cell line, MCF7, was obtained from the ATCC (Manassas, VA). Cells were validated at our Institution validation facility using STR as-

says. Cells were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 5% foetal bovine serum (FBS, HyClone™, GE Healthcare Cell Culture, Pittsburgh, PA) in a 5% CO_2 atmosphere at 37 °C.

Recombinant proteins

TIMP4 (974-TSF-010 catalogue) and tumour necrosis factor α (TNF-α) (210-TA-050/CF catalogue) were purchased from R&D (R&D Systems, Minneapolis, MN). TIMP4 was used at a 10 nM concentration and TNF-α at 20 ng/ml.

RNASeq

Human recombinant TIMP4 or vehicle (phosphate-buffered saline, PBS) was added to MCF7 cells, and after 24 h RNA was extracted using Trizol reagent (Life Technologies, Carlslbad, CA). RNASeq libraries were constructed using Illumina TruSeq RNA sample prep kit (Illumina, San Diego, CA), with RNAs with RNA integrity number (RIN) > 8, according to manufacturer's instructions. Sequencing of libraries was performed in a GAIIx equipment (Illumina). RNASeq data were analysed using the CLC genomics workbench version 7 from CLC Bio. Parameters used were: align with 2 maximum number of mismatches, 0.9 minimum length fraction, 0.8 minimum similarity fraction, and 10 maximum number of hits for a read. Mapped reads range was 16×10^6 to 28×10^6. All transcripts with a relative expression level lower than 0.2 and less than 10 reads were filtered out. Empirical analysis of differential gene expression was calculated using the EdgeR (Robinson et al., 2010) algorithm. Upregulated or downregulated genes with an absolute fold change higher than 1.5 were used for further analyses. Experiments were performed with two biological replicates. Raw data are available upon request.

Bioinformatic analyses

The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to do functional annotation analysis of enriched gene ontology (GO) terms and KEGG pathways (Huang da et al., 2009). Statistical significance was evaluated with a modified Fisher's exact test; GO and KEGG terms with P-values < 0.01 were considered significant (Huang da et al., 2009). Master Regulators Analysis was done using Ingenuity Pathway Analysis (Qiagen, Redwood City, CA) (Kramer et al., 2014). Significant pathways were considered for network corrected bias P values of < 0.05. For motif enrichment we used the Hypergeometric Optimization of Motif EnRichment (HOMER) tool (Heinz et al., 2010).

RT-PCR

RNA was reverse transcribed to cDNA with Superscript III kit (Invitrogen). Products were amplified using Amplitaq Gold (Applied Biosystems, Foster City, CA) and normalized to GAPDH. RT-PCR's were performed in triplicate including biological replicates.

Western blot

Total cell extracts were lysed with RIPA buffer (EMD Millipore, Darmstadt, Germany). Protein was quantified using the Bradford procedure. Equal protein amounts were separated by SDS-PAGE, transferred to PVDF membranes (Amersham, Little Chalfont, UK), blocked, incubated with specific antibodies, washed, and incubated with a secondary goat anti-mouse antibody (Millipore, D00377). Primary antibodies were: ER-α (F-10) sc-8002 (Santa Cruz Biotechnologies, Santa Cruz, CA) at a 1 : 200 dilution and Tubulin 2146 (Cell Signaling, Danvers, MA) at a 1 : 200 dilution.

Results

RNASeq analysis of TIMP4 in MCF7 cells

In order to analyse the transcriptome of MCF7 breast cancer cells after TIMP4 activation, we stimulated the cells with human recombinant TIMP4 (hrTIMP4) at a 10 nM concentration, as reported previously (Lizarraga et al., 2015b) and, after 24 h, we extracted RNA to perform RNASeq analyses. We found 18,476 transcripts expressed in control MCF7 cells and 18,395 transcripts expressed in MCF7 cells exposed to TIMP4. An analysis of differentially expressed genes induced by hrTIMP4 showed a total of 3,172 upregulated and 2,858 downregulated genes with an absolute fold change ≥ 1.5 To obtain a high confidence list, we filtered our results using a $P < 0.05$, which gave us a stricter list of 341 genes (Supplementary Material 1, available at: https://www.dropbox.com/s/63osr6ciigw6e9v/Supplementary_Material_1.xlsx?dl=0). Fig. 1A shows the top 20 up- and downregulated genes, whereas Fig. 1B shows a Volcano plot that combines statistical significance with fold change. The validation rate for randomly selected genes was 100 % (Fig. 1C).

Functional enrichment analysis of genes regulated by TIMP4

Differentially expressed genes induced by hrTIMP4 were categorized using the DAVID gene enrichment analysis (Huang da et al., 2009). Using a high classification clustering stringency and the high confidence list, we found a total of 70 functional annotation clusters, which included, among others, differentiation, mitochondrial activity, RNA metabolism, cell communication and cell death (Supplementary Material 2, available at: https://www.dropbox.com/s/atkdud9vnhgnmyl/Supplementary_Material_2.xlsx?dl=0). These annotations are concordant with the previously proposed stem and apoptotic functions of TIMP4 (Lizarraga et al., 2015a,b) and, as expected, included the NF-κB pathway, which is induced by TIMP4 in cervical cancer cells (Lizarraga et al., 2015b).

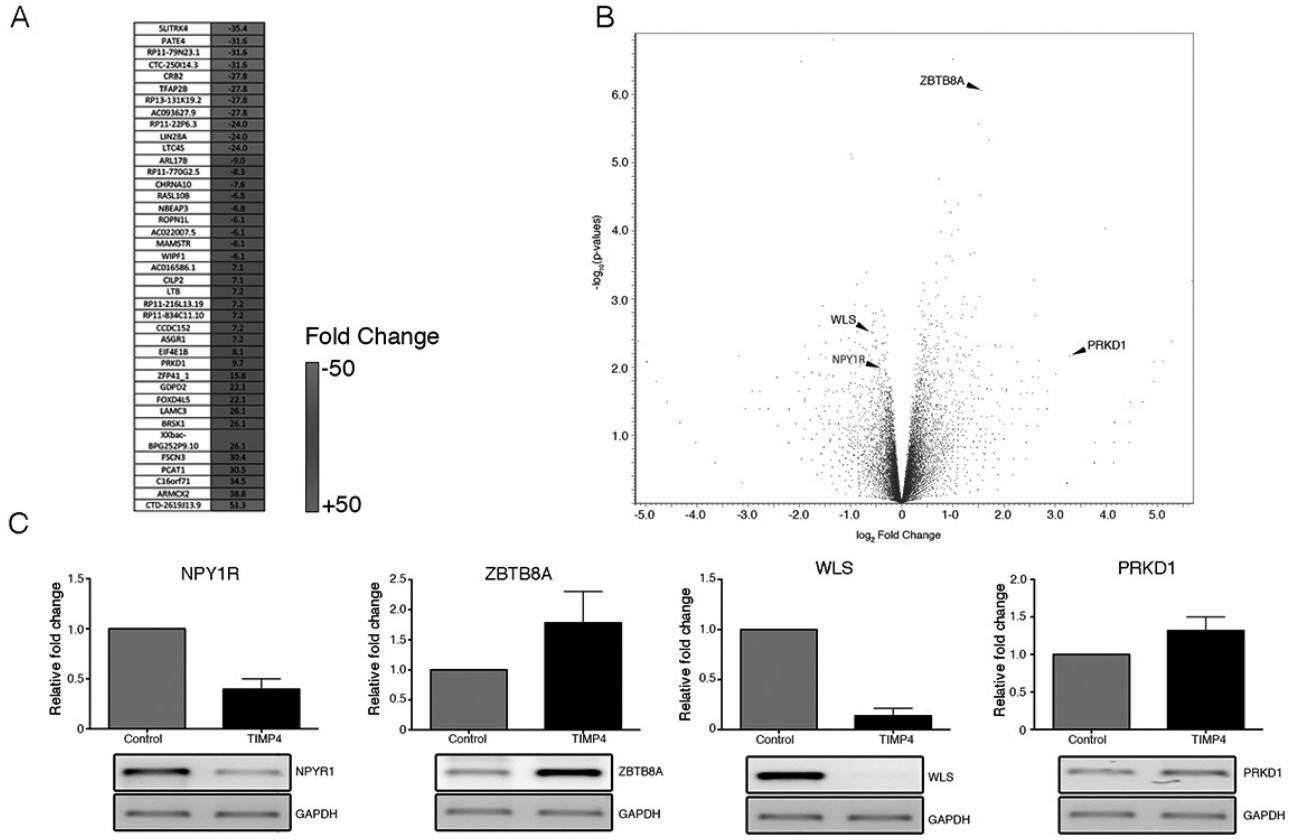

Fig. 1. **A)** Top 20 regulated genes after hrTIMP4 exposure in MCF-7 cells. The relative fold change of hrTIMP4 versus vehicle-treated cells is shown in a heatmap plot. Volcano Plot. The X axis is the log2 fold change between control and TIMP4-exposed cells. The -log10 (P value) is plotted on the Y axis. Each point on the graph represents a gene. An arrow marks the validated genes**. B)** Volcano Plot of validated genes. **C)** RT-PCR validation of four randomly selected genes (*NPY1R*, *ZBTB8A*, *WLS*, *PRKD1*). In the upper panels the densitometric analyses of three replicates are shown.

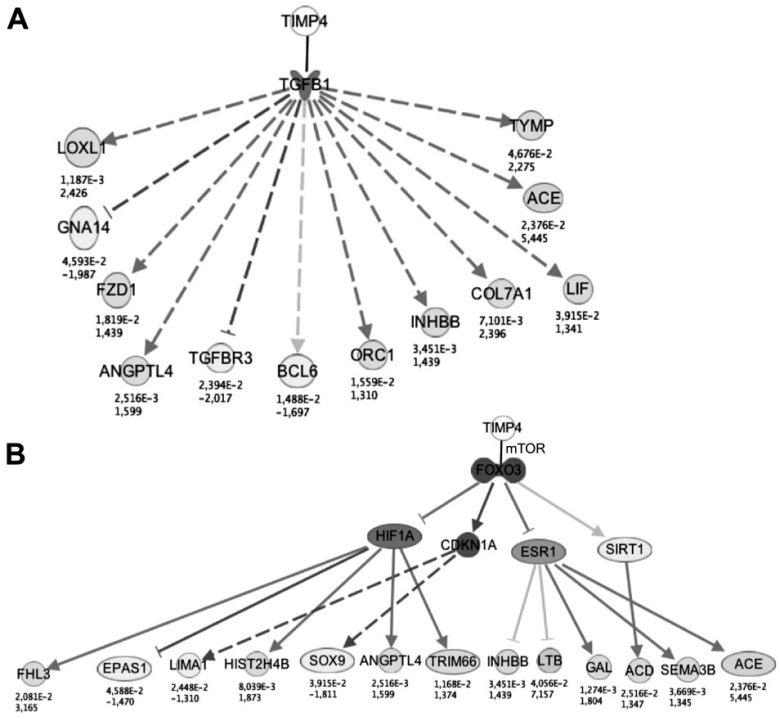

Fig. 2. Master regulator analysis of Timp4-regulated signaling pathways. Ingenuity pathway analysis was used to determine the master regulators. **A)** TGF-β and downstream targets. **B)** FOXO3 and downstream targets. Below each gene the P value and expression changes are depicted.

Master regulator network analysis of differentially expressed genes

To get insight into the possible upstream signalling pathways that were responsible for the gene expression changes found upon hrTIMP4 exposure, we performed a master regulator analysis (Kramer et al., 2014), using the complete list of deregulated genes. Among the regulators, we found two master regulators relevant to breast cancer biology: transforming growth factor β (TGF-β) and forkhead box O3 (FOXO3) (Fig. 2). These regulators were, in turn, predicted to activate hypoxia inducible factor 1 α subunit (HIF1A), oestrogen receptor (ESR (ER-α)) and sirtuin 1 (SIRT1) transcription factors, and to decrease signalling to cyclin-dependent kinase inhibitor 1A (CDKN1A) (Fig. 2). In addition, several other enriched networks were found (Supplementary Material 3, available at: https://www.dropbox.com/s/q08av4hdp71gbcb/Supplementary%20Material%203.pdf?dl=0).

TIMP4 upregulates an ER-α module

Due to the oestrogen receptor signalling relevance in breast cancer and the predicted activation found in the master regulator network analysis, we decided to further validate the effect of hrTIMP4 exposure on this signalling cascade. First, we analysed the promoters of the regulated genes to find whether ER-α-responsive elements were enriched. For this, we used the HOMER tool, which calculates motifs enriched in a subset of gene promoters. Supporting the participation of oestrogen receptor signalling, we found that its consensus recognition sequence was present among the top 50 enriched motifs (Table 1). In accordance with this result, we found that ER-α mRNA levels were increased in hrTIMP4-exposed MCF7 cells (Fig. 3A). To further validate this change, ER-α protein levels were measured at 30 min and 24 h after hrTIMP4 exposure. As expected, after 30 min the ER-α protein increased and returned to basal levels at 24 h (Fig. 3B). Recombinant human TNF-α (hrTNF-α) was used as a response control, since it is able to downregulate the ER-α protein (Lee and Nam, 2008). hrTIMP4 could not rescue the ER-α protein decrease induced by hrTNFα at either 30 min or 24 h, perhaps due to an overlapping regulatory mechanism, as suggested by the previously described effects of TNF on NF-κB activation (Lizarraga et al., 2015b).

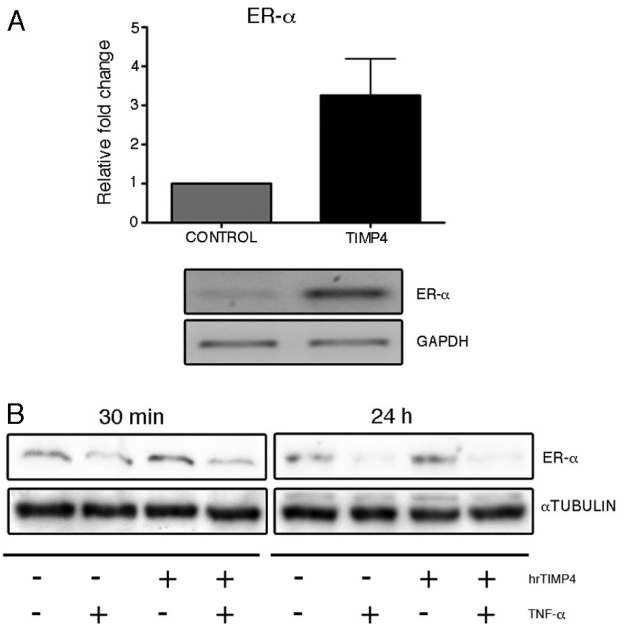

Fig. 3. **A)** Semi-quantitative RT-PCR of ER-α in TIMP4-treated cells. **B)** Western blot assays for ER-α in MCF7 cells exposed to TIMP4 (10 nM), TNF-α (20 ng/ml) or TNF-α+TIMP4 for 30 min or 24 h. Tubulin was used as a loading control.

Discussion

The goal of this report was to explore the signalling pathways used by TIMP4 to regulate breast cancer cells, using the RNASeq whole genome transcriptome. TIMP4 overexpression is known to confer poor clinical outcome in breast cancer patients and other cancers (Liss et al., 2009; Rorive et al., 2010). However, the mechanisms of how it influences cancer biology have not been determined. Our RNASeq data from MCF7 breast cancer cells show that TIMP4 regulates multiple pathways involved in cancer.

Gene ontology enrichment results showed that TIMP4 modulates mRNA levels of genes that participate in differentiation, mitochondrial activity, RNA metabolism, cell communication and cell death. Interestingly, all these processes are strongly interconnected (Green et al., 2014). We then performed a master regulator analysis, aimed to show which upstream signalling pathways are responsible for the gene expression changes (Kramer et al., 2014). This analysis revealed that TIMP4 changes could be mediated by the upregulation of TGF-β, HIF1A, and ESR1 (ER-α) and downregulation of FOXO3 signalling pathways. TGF-β activation of mTOR, the upstream FOXO3 regulator, is known to promote epithelial-mesenchymal transition and cancer invasion (Lamouille et al., 2012), and our results suggest that both of these pathways are upregulated by TIMP4, and thus might be a mechanism of how TIMP4 contributes to the reported poor prognosis.

We also found that promoters of TIMP4-modulated genes are enriched in ER-α-binding sites. This promoter enrichment is similar to other processes/pathways known to be regulated by oestrogens (Liu et al., 2013), and significantly higher than randomly retrieved human gene promoters. The ER-α role in breast cancer progression is well defined, as it stimulates proliferation, inhibits apoptosis, and enhances motility and invasion (Manavathi et al., 2013). Interestingly, genes known to be upregulated by ER-α and to have well-defined roles in breast cancer biology were modulated by TIMP4. Protein kinase D1 (PRKD1) and hes family bHLH transcription factor 6 (HES6) genes, which were upregulated by TIMP4, increase breast cancer cell proliferation and sig-

Table 1. Top enriched promoter motifs in promoters from TIMP4-regulated genes

Motif Name	Consensus	P value	Log P value	q value (Benjamini)	# of Target Sequences with Motif (of 3244)	% of Target Sequences with Motif	# of Background Sequences with Motif (of 39364)
Tcf12(bHLH)/GM12878-Tcf12-ChIP-Seq(GSE32465)/Homer	VCAGCTGYTG	1.00E-05	-1.24E+01	0.0011	1606	49.51%	17879.3
Atoh1(bHLH)/Cerebellum-Atoh1-ChIP-Seq(GSE22111)/Homer	VNRVCAGCTGGY	1.00E-04	-1.14E+01	0.0014	1591	49.04%	17775.7
Ap4(bHLH)/AML-Tfap4-ChIP-Seq(GSE45738)/Homer	NAHCAGCTGD	1.00E-04	-1.02E+01	0.0034	1751	53.98%	19819.6
MyoD(bHLH)/Myotube-MyoD-ChIP-Seq(GSE21614)/Homer	RRCAGCTGYTSY	1.00E-04	-9.64E+00	0.0043	1343	41.40%	14947.2
E2A(bHLH)/proBcell-E2A-ChIP-Seq(GSE21978)/Homer	DNRCAGCTGY	1.00E-03	-8.22E+00	0.0143	2079	64.09%	24013.4
Ascl1(bHLH)/NeuralTubes-Ascl1-ChIP-Seq(GSE55840)/Homer	NNVVCAGCTGBN	1.00E-03	-7.11E+00	0.0295	2136	65.84%	24825.2
EKLF(Zf)/Erythrocyte-Klf1-ChIP-Seq(GSE20478)/Homer	NWGGGTGTGGCY	1.00E-03	-7.21E+00	0.0295	456	14.06%	4765.4
Myf5(bHLH)/GM-Myf5-ChIP-Seq(GSE24852)/Homer	BAACAGCTGT	1.00E-03	-7.31E+00	0.0295	1115	34.37%	12443.5
REST-NRSF(Zf)/Jurkat-NRSF-ChIP-Seq/Homer	GGMGCTGTCCATGG-TGCTGA	1.00E-03	-7.01E+00	0.0295	34	1.05%	219.3
Mef2a(MADS)/HL1-Mef2a.biotin-ChIP-Seq(GSE21529)/Homer	CYAAAAATAG	1.00E-02	-6.35E+00	0.0418	529	16.31%	5663.4
Mouse_Recombination_Hotspot(Zf)/Testis-DMC1-ChIP-Seq(GSE24438)/Homer	ACTYKNATTCGTGN-TACTTC	1.00E-02	-6.45E+00	0.0418	92	2.84%	794
MyoG(bHLH)/C2C12-MyoG-ChIP-Seq(GSE36024)/Homer	AACAGCTG	1.00E-02	-6.37E+00	0.0418	1638	50.49%	18817.3
Tcf3(HMG)/mES-Tcf3-ChIP-Seq(GSE11724)/Homer	ASWTCAAAGG	1.00E-02	-6.40E+00	0.0418	324	9.99%	3323
HOXA2(Homeobox)/mES-Hoxa2-ChIP-Seq(Donaldson et al.)/Homer	GYCATCMATCAT	1.00E-02	-5.65E+00	0.0663	123	3.79%	1146.8
Tcf4(HMG)/Hct116-Tcf4-ChIP-Seq(SRA012054)/Homer	ASATCAAAGGVA	1.00E-02	-5.61E+00	0.0663	541	16.68%	5861.9
TEAD(TEA)/Fibroblast-PU.1-ChIP-Seq(Unpublished)/Homer	YCWGGAATGY	1.00E-02	-5.62E+00	0.0663	831	25.62%	9250.9
Pit1+1bp(Homeobox)/GCrat-Pit1-ChIP-Seq(GSE58009)/Homer	ATGCATAATTCA	1.00E-02	-5.30E+00	0.0738	377	11.62%	3998.4
Ptf1a(bHLH)/Panc1-Ptf1a-ChIP-Seq(GSE47459)/Homer	ACAGCTGTTN	1.00E-02	-5.35E+00	0.0738	2676	82.49%	31736.8
PPARE(NR),DR1/3T3L1-Pparg-ChIP-Seq(GSE13511)/Homer	TGACCTTTGCCCCA	1.00E-02	-5.24E+00	0.0739	1506	46.42%	17354.2
RXR(NR),DR1/3T3L1-RXR-ChIP-Seq(GSE13511)/Homer	TAGGGCAAAGGTCA	1.00E-02	-5.02E+00	0.0831	1741	53.67%	20229.8
TATA-Box(TBP)/Promoter/Homer	CCTTTTAWAGSC	1.00E-02	-5.07E+00	0.0831	1503	46.33%	17339.9
Rfx1(HTH)/NPC-H3K4me1-ChIP-Seq(GSE16256)/Homer	KGTTGCCATGGCAA	1.00E-01	-4.55E+00	0.1263	405	12.48%	4378.8
Pax7(Paired,Homeobox),long/Myoblast-Pax7-ChIP-Seq(GSE25064)/Homer	TAATCHGATTAC	1.00E-01	-4.42E+00	0.1388	34	1.05%	264.6
Rfx5(HTH)/GM12878-Rfx5-ChIP-Seq(GSE31477)/Homer	SCCTAGCAACAG	1.00E-01	-4.15E+00	0.1727	518	15.97%	5727.5
X-box(HTH)/NPC-H3K4me1-ChIP-Seq(GSE16256)/Homer	GGTTGCCATGGCAA	1.00E-01	-3.91E+00	0.2114	223	6.87%	2343.4
Bcl6(Zf)/Liver-Bcl6-ChIP-Seq(GSE31578)/Homer	NNNCTTTCCAGGAAA	1.00E-01	-3.58E+00	0.2776	1734	53.45%	20347.9
Erra(NR)/HepG2-Erra-ChIP-Seq(GSE31477)/Homer	CAAAGGTCAG	1.00E-01	-3.58E+00	0.2776	2354	72.56%	27936.7
GSC(Homeobox)/FrogEmbryos-GSC-ChIP-Seq(DRA000576)/Homer	RGGATTAR	1.00E-01	-3.58E+00	0.2776	1191	36.71%	13787

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Oct4:Sox17(POU,Homeobox,HMG)/F9-Sox17-ChIP-Seq(GSE44553)/Homer	CCATTGTATGCAAAT	1.00E-01	-3.46E+00	0.2776	147	4.53%	1514.1
TCFL2(HMG)/K562-TCF7L2-ChIP-Seq(GSE29196)/Homer	ACWTCAAAGG	1.00E-01	-3.51E+00	0.2776	100	3.08%	989.6
Tlx?(NR)/NPC-H3K4me1-ChIP-Seq(GSE16256)/Homer	CTGGCAGSCTGCCA	1.00E-01	-3.53E+00	0.2776	679	20.93%	7689.5
Unknown-ESC-element(?)/mES-Nanog-ChIP-Seq(GSE11724)/Homer	CACAGCAGGGGG	1.00E-01	-3.60E+00	0.2776	1057	32.58%	12178.1
Phox2a(Homeobox)/Neuron-Phox2a-ChIP-Seq(GSE31456)/Homer	YTAATYNRATTA	1.00E-01	-3.35E+00	0.2822	414	12.76%	4595.1
Otx2(Homeobox)/EpiLC-Otx2-ChIP-Seq(GSE56098)/Homer	NYTAATCCYB	1.00E-01	-3.15E+00	0.3333	912	28.11%	10511.8
HNF4a(NR),DR1/HepG2-HNF4a-ChIP-Seq(GSE25021)/Homer	CARRGKBCAAAGT-YCA	1.00E-01	-3.07E+00	0.3486	705	21.73%	8057.8
NeuroD1(bHLH)/Islet-NeuroD1-ChIP-Seq(GSE30298)/Homer	GCCATCTGTT	1.00E-01	-2.88E+00	0.4123	1173	36.16%	13682.3
TEAD2(TEA)/Py2T-Tead2-ChIP-Seq(GSE55709)/Homer	CCWGGAATGY	1.00E-01	-2.87E+00	0.4123	696	21.45%	7979.3
AR-halfsite(NR)/LNCaP-AR-ChIP-Seq(GSE27824)/Homer	CCAGGAACAG	1.00E-01	-2.69E+00	0.4129	2895	89.24%	34780.4
ERE(NR),IR3/MCF7-ERa-ChIP-Seq(Unpublished)/Homer	VAGGTCACNSTGACC	1.00E-01	-2.71E+00	0.4129	401	12.36%	4512.4
FOXA1(Forkhead)/LNCAP-FOXA1-ChIP-Seq(GSE27824)/Homer	WAAGTAAACA	1.00E-01	-2.55E+00	0.4129	1137	35.05%	13306.6
HNF6(Homeobox)/Liver-Hnf6-ChIP-Seq(ERP000394)/Homer	NTATYGATCH	1.00E-01	-2.75E+00	0.4129	591	18.22%	6750.8
Mef2c(MADS)/GM12878-Mef2c-ChIP-Seq(GSE32465)/Homer	DCYAAAAATAGM	1.00E-01	-2.63E+00	0.4129	499	15.38%	5677.1
Nkx2.1(Homeobox)/LungAC-Nkx2.1-ChIP-Seq(GSE43252)/Homer	RSCACTYRAG	1.00E-01	-2.77E+00	0.4129	2555	78.76%	30539.9
Olig2(bHLH)/Neuron-Olig2-ChIP-Seq(GSE30882)/Homer	RCCATMTGTT	1.00E-01	-2.67E+00	0.4129	2015	62.11%	23924.8
p53(p53)/Saos-p53-ChIP-Seq/Homer	RRCATGYCYRGRCAT-GYYYN	1.00E-01	-2.77E+00	0.4129	107	3.30%	1106
RFX(HTH)/K562-RFX3-ChIP-Seq(SRA012198)/Homer	CGGTTGCCATGGC-AAC	1.00E-01	-2.75E+00	0.4129	218	6.72%	2376
Rfx2(HTH)/LoVo-RFX2-ChIP-Seq(GSE49402)/Homer	GTTGCCATGGCAACM	1.00E-01	-2.82E+00	0.4129	228	7.03%	2484.8
SCL(bHLH)/HPC7-Scl-ChIP-Seq(GSE13511)/Homer	AVCAGCTG	1.00E-01	-2.65E+00	0.4129	3069	94.61%	36984.7
Unknown(Homeobox)/Limb-p300-ChIP-Seq/Homer	SSCMATWAAA	1.00E-01	-2.68E+00	0.4129	740	22.81%	8530.8
Esrrb(NR)/mES-Esrrb-ChIP-Seq(GSE11431)/Homer	KTGACCTTGA	1.00E-01	-2.44E+00	0.4527	856	26.39%	9955.4

nificantly increase metastasis in breast cancer patients (Karam et al., 2014). Ras-related C3 botulinum toxin substrate 3 (Rac3), which was also upregulated by TIMP4, promotes proliferation and migration of breast cancer cells (Walker et al., 2011). Finally, MHC class I polypeptide-related sequence B (MICB), which is downregulated by oestrogens (Sroijak and Ponglikitmongkol, 2013), was downregulated by TIMP4, and is a key component that promotes cancer cell death by natural killer cells (Tsukerman et al., 2012). It is also worth mentioning that the TIMP4 promoter contains active oestrogen receptor-binding sites (Pilka et al., 2004), which suggest a positive feedback loop between oestrogen receptors and TIMP4. Other TIMP family members are responsive to oestrogen signalling. For example, TIMP1 serum levels predict response to oestrogen receptor modulator, tamoxifen, in breast cancer patients (Lipton et al., 2008). In turn, oestrogen upregulates TIMP2 levels (Wang and Ma, 2012).

This is the first study that explores TIMP4 signalling in breast cancer cells. Our RNASeq results shows that TIMP4 upregulates ER-α mRNA and protein levels and ER-α-dependent gene expression changes, and suggest upregulation of HIF1A signalling and downregulation of FOXO3 signalling. This study paves the way to identify how TIMP4 influences breast cancer biological behaviour.

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