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Differential Regulation of Human Thymosin Beta 15 Isoforms by Transforming Growth Factor Beta 1

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Abstract

We recently identified an additional isoform of human thymosin beta 15 (also known as NB-thymosin beta, gene name *TMSB15A*) transcribed from an independent gene, and designated *TMSB15B*. The purpose of this study was to investigate whether these isoforms were differentially expressed and functional. Our data show that the *TMSB15A* and *TMSB15B* isoforms have distinct expression patterns in different tumor cell lines and tissues. *TMSB15A* was expressed at higher levels in HCT116, DU145, LNCaP and LNCaP-LN3 cancer cells. In MCF-7, SKOV-3, HT1080 and PC-3MLN4 cells, *TMSB15A* and *TMSB15B* showed approximately equivalent levels of expression, while *TMSB15B* was the predominant isoform expressed in PC-3, MDA-MB-231, NCI-H322 and Caco-2 cancer cells. In normal human prostate and prostate cancer tissues, *TMSB15A* was the predominant isoform expressed. In contrast, normal colon and colon cancer tissue expressed predominantly *TMSB15B*. The two gene isoforms are also subject to different transcriptional regulation. Treatment of MCF-7 breast cancer cells with transforming growth factor beta 1 repressed *TMSB15A* expression but had no effect on *TMSB15B*. siRNA specific to the *TMSB15B* isoform suppressed cell migration of prostate cancer cells to epidermal growth factor, suggesting a functional role for this second isoform. In summary, our data reveal different expression patterns and regulation of a new thymosin beta 15 gene paralog. This may have important consequences in both tumor and neuronal cell motility.

INTRODUCTION

Thymosin beta 15 is a 5 kDa actin-binding protein that we discovered in highly metastatic rat prostate cancer cells. Antisense thymosin beta 15 reduced tumor cell migration (Bao et al., 1996), and we and other groups showed increased thymosin beta 15 expression in human prostate, breast and lung cancers (Bao et al., 1996; Gold et al., 1997; Chakravatri et al., 2000; Gu et al., 2008). We recently established that the human homolog of thymosin beta 15 is identical to NB thymosin beta (*TMSB15A*, previously known as *TMSNB*) (Banyard et al., 2007), a transcript found in neuroblastoma cells (Yokoyama et al., 1996). This identity was confirmed by another group (Dhaese et al., 2007). We further showed that a second thymosin beta 15 gene exists in the human genome (Banyard et al., 2007). Thymosin beta 15b (gene name *TMSB15B*) has 87% mRNA homology with the previously identified human thymosin beta 15a (gene name *TMSB15A*), and 98% identity across the coding sequence.

In this study we have analyzed the expression differences between *TMSB15A* and *TMSB15B* in various tumor cells and tissues. We also demonstrate that transforming growth factor beta 1 (TGFB1) differentially regulates the expression of the thymosin beta 15 isoforms and

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further show that *TMSB15B* produces a protein functional in motility of prostate cancer cells.

MATERIALS AND METHODS

Sequence Analysis

Sequence analysis was performed using the Ensembl Genome Browser and ClustalW2 programs at the European Bioinformatics Institute/European Molecular Biology Laboratory (EBI-EMBL), and BLAST program at the National Center for Biotechnology Information (NCBI). For human thymosin beta 15a, gene name *TMSB15A*, the Ensembl identifier is ENSG00000158164. For thymosin beta 15b, gene name *TMSB15B*, the Ensembl 52 gene identifier is AL034485.16-201, transcript identifier ENST00000372602. Sequences were aligned using the EMBOSS Pairwise alignment algorithm (EBI-EMBL).

RNA Extraction, cDNA Synthesis and PCR

RNA was purified using RNeasy Plus Mini kit (Qiagen, Valencia, CA) and transcribed using iScript cDNA synthesis kit (Biorad, Hercules, CA). RT-PCR and quantitative PCR were performed using iQ SYBR Green Supermix (Biorad) using primers TBNBF5 and TBNBR7 to amplify *TMSB15A*, and TBALTF4 and TBALTR6 to amplify *TMSB15B* (Table 1). For RT-PCR the reaction was performed on a MJ Cycler PT200 (Biorad) using a 94°C 3 min activation step, 94°C 15 sec melt step, 60°C 15 sec annealing, 72°C 30 sec extension, with 33 total cycles of amplification, followed by a 72°C 5 min final extension. Housekeeping genes ribosomal protein s9 (*RPS9*) and beta-2-microglobulin (*B2M*) (Table 1) were amplified at 58°C for 29 cycles. Products were separated on 2% agarose gels and visualized using Ethidium Bromide. Real time quantitative PCR was performed on an Opticon 2 instrument (Biorad) using cycling conditions; 95°C 3 min followed by 38 cycles of 95°C 15 sec, 60°C 15 sec, 72°C 30 sec, 78°C 1 sec plate read, followed by 72°C 5 min, melt curve from 65-98°C read every 0.2 °C, 72°C 5 min, 10°C 5 min. Data were collected using the MJ Opticon Monitor 3.1 program (Biorad) and analyzed using the ΔCt method (Pfaffl 2001). The Cancer Survey I qPCR tissue array (OriGene Technologies, Rockville, MA) was used to assess the expression of *TMSB15A* and *TMSB15B* in various tissues. *TMSB15A* and *TMSB15B* were amplified in replicate plates and cycle threshold (Ct) values compared. This was valid assuming that *TMSB15A* and *TMSB15B* amplified with equal efficiencies. To test this, *TMSB15A* and *TMSB15B* were PCR amplified, fragments gel purified using QIAquick gel extraction kit (Qiagen) and quantified using a UV spectrophotometer. A dilution series was qPCR amplified to compare reaction efficiencies. *TMSB15A* and *TMSB15B* levels were compared within each sample and no inter-sample analysis was performed, therefore a housekeeping gene value for normalization was not necessary, and an arbitrary housekeeping gene value was assigned to calculate fold difference using the ΔCt method (Pfaffl, 2001).

Cell Culture, siRNA Transfection and Cell Migration

LNCaP, SKOV-3, Caco-2, PC-3, HT1080, HCT116, MCF-7, HeLa, HT-29 and DU145 were obtained from American Type Culture Collection (Manassas, VA) and grown according to supplier instructions. LNCaPLN3, PC-3MLN4 and NCI-H322 cells were grown in RPMI supplemented with 10% fetal bovine serum (FBS) and 1% glutamine-penicillin-streptomycin (GPS) (Invitrogen). MDA-MD-231 and HaCaT cells were grown in DMEM supplemented with 10% FBS and 1% GPS (Invitrogen, Carlsbad, CA). Control and *TMSB15A* and *TMSB15B* siRNAs were purchased from Thermo Scientific Dharmacon (Lafayette, CO). Cell transfection with siRNAs was performed using Silentfect (Biorad) and cells collected for RNA or migration assays after 48 hr incubation. Cell migration was performed as previously described (Banyard and Symons, 2002) using a 48 well modified

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Boyden chamber (Neuro Probe, Gaithersburg, MD). PC-3 cells were plated on 100 μg/ml collagen I coated 8 μm pore membranes and migration quantified after 4 hr incubation. Recombinant human transforming growth factor beta 1 (TGFB1) (R&D systems, Minneapolis, MN) was resuspended at 10 μg/ml in 4 mM HCl with 0.1% bovine serum albumin carrier. For TGFB1 treatment 2×10^5 MCF-7 cells were plated per 35 mm dish and incubated overnight. Cells were washed twice and media replaced with low serum media (DMEM + 0.5% FBS +1% GPS) for 24 hr. Cells were then treated with 10 ng/ml TGFB1 or resuspension solution for 24 hr and RNA collected as described above.

Statistical Analysis

Migration assay and qPCR data were analyzed using the two-sample Student's *t* test. Two-tailed values of $P < 0.05$ were considered statistically significant for all analyses.

RESULTS

Expression of Thymosin Beta 15 Isoforms in Tumor Cells and Tissues

We have identified two independent thymosin beta 15 genes in the human genome, both located on chromosome X. *TMSB15A* is located on the reverse strand at Xq22.1, while the newly identified *TMSB15B* gene paralog is located on the forward strand at Xq22.2 (Banyard et al., 2007). At the cDNA level, *TMSB15A* and *TMSB15B* show 87% identity, with 98% sequence conservation across the coding region. The mRNA alignment of the two human thymosin beta 15 isoforms is shown in Figure 1. The coding region is boxed, with amino acid sequence above and differences in the nucleotide sequences bolded. The two variant nucleotides in the coding sequence are silent, resulting in translation of identical proteins from the *TMSB15A* and *TMSB15B* isoforms. Further genomic analysis revealed an additional copy of the thymosin beta 15 coding sequence on chromosome 6 (Vega Gene RP11-505P4.5, Ensembl Locus ID OTTHUMG00000015035). This latter sequence shows the classical characteristics of a retrotranposed pseudogene; it lacks the intronic sequence and has frameshift mutations, stop codons and codon deletions which would prevent translation of thymosin beta 15 protein. Alignment of this sequence with *TMSB15A* and *TMSB15B* is shown in Supplementary Figure 1. The two variant nucleotides in the thymosin beta 15 coding sequence are conserved between *TMSB15B* and the pseudogene. This indicates that the pseudogene originated as a copy of the *TMSB15B* coding sequence.

In contrast to the coding sequence, numerous nucleotide differences exist in both the 3' and 5' untranslated regions of *TMSB15A* and *TMSB15B*. This allowed us to design isoform-specific PCR amplification primer sets (Table 1) to examine expression patterns. RT-PCR demonstrated that both *TMSB15A* and *TMSB15B* genes were transcribed and the amplicons were sequenced to confirm primer specificity. We found that different thymosin beta 15 isoform expression patterns existed in various human tumor cell lines. Our data show that in HCT116 colorectal carcinoma and DU145, LNCaP-LN3 and LNCaP prostate carcinoma cells, *TMSB15A* was expressed at higher levels than *TMSB15B* (Fig. 2A, left panel). In MCF-7 breast carcinoma, SKOV-3 ovarian carcinoma, HT1080 fibrosarcoma cells and PC-3MLN4 prostate carcinoma, *TMSB15A* and *TMSB15B* showed approximately equivalent levels of expression (Fig. 2A, center panel). In contrast, PC-3 prostate carcinoma, MDA-MB-231 breast carcinoma, NCI-H322 lung carcinoma and Caco-2 colorectal carcinoma cells showed significantly greater *TMSB15B* expression compared to *TMSB15A* (Fig. 2A, right panel). No expression of either thymosin beta 15 isoform was detected in several cell lines, including HaCaT keratinocytes, HeLa cervical carcinoma, HT-29 colorectal adenocarcinoma and NIH:OVCAR5 ovarian carcinoma, under these conditions (data not shown). Our data indicate that *TMSB15B* is expressed in multiple tumor cell types and is the predominant isoform in many cells.

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We next examined expression in various normal and tumor tissues to determine whether *TMSB15A* and *TMSB15B* exhibit any tissue specificity. Replicate 96 well plates of human tissue cDNAs were amplified using quantitative PCR. *TMSB15A* and *TMSB15B* PCR amplicons were of equal length, GC content, and amplified with equal efficiencies (Supplementary Fig. 2) allowing comparison of expression levels. Relative expression of *TMSB15A* and *TMSB15B* were compared using the ΔCt method (Pfaffl, 2001) using a constant housekeeping gene value for normalization, as described in Material and Methods. Data indicated that certain tissues, such as prostate, express predominantly *TMSB15A*, both in normal and tumor tissues (Fig. 2B, left panel). In contrast, normal colon tissue and colon cancer expressed predominantly *TMSB15B* (Fig. 2B, right panel).

TGFB1 Regulation of Thymosin Beta 15 Isoform Expression

Thymosin beta 15 affects cell colony growth, transformation and cell migration (Bao et al., 1996; Abdulrahman et al., 2007; Dhaese et al., 2007) and a key regulator of cell growth in the tumor microenvironment is transforming growth factor beta 1 (TGFB1). We therefore examined thymosin beta 15 expression in response to TGFB1 treatment. MCF-7 breast cancer cells, which express both *TMSB15A* and *TMSB15B* (Fig. 2A), were grown in reduced-serum medium overnight, then treated with TGFB1 for 24 or 48 hours. Thymosin beta 15 isoform RNA levels were determined using quantitative PCR. TGFB1 treatment significantly repressed *TMSB15A* expression in MCF-7 cells, whereas *TMSB15B* level was unaffected (Fig. 3). This was not generalized to all breast cancer cells, however, as TGFB1 did not repress *TMSB15A* expression in T-47D or ZR-75-1 breast cancer cells (data not shown).

TMSB15B Functions in Cell Migration

As both thymosin beta 15a and thymosin beta 15b have identical amino acid sequences, protein expression could not be distinguished using thymosin beta 15 antibodies. Detection of endogenous thymosin beta 15 protein by Western blot has also proved technically difficult due to its small size of 5 kDa. Rat thymosin beta 15 is required for tumor cell migration (Bao et al., 1996), and increases branching in developing rat neurons (Choe et al., 2005). The human TMSB15A isoform has previously been established as a functional protein. TMSB15A expression increases mouse fibroblast migration (Dhaese et al., 2007), increases human tumor cell colony formation and anchorage-independent growth, and is required for colony formation (Abdulrahman et al., 2007). To determine whether TMSB15B protein is expressed and functional, we used RNA interference followed by a cell migration assay.

The activity of siRNA oligonucleotides was first determined in PC-3MLN4 cells, which expressed both *TMSB15A* and *TMSB15B*. Cells were transfected with 20 nM siRNAs targeting *TMSB15A* (siRNA *TMSB15A* #9 or siRNA *TMSB15A* #10), *TMSB15B* (siRNA *TMSB15B* #2, siRNA *TMSB15B* #3 or siRNA *TMSB15B* #4) or non-binding siRNA controls (control siRNA#1, control-ONTARGET siRNA or control siRNA#3). Isoform expression was analyzed using RT-PCR. Multiple siRNAs were used against each target to ensure specificity. Figure 4A shows effective and specific knockdown of *TMSB15A* and *TMSB15B* isoforms at the RNA level.

The role of *TMSB15B* in cell motility was then determined by siRNA knockdown in PC-3 cells, which expressed only *TMSB15B* (Fig. 2A). Untreated or control siRNA-transfected PC-3 cells showed directional migration in response to epidermal growth factor (EGF) in the 48-well modified Boyden chamber assay. Three individual *TMSB15B* siRNAs significantly decreased cell migration in response to EGF, $P < 0.001$ (Fig. 4B). *TMSB15A* siRNAs had no effect on PC-3 cell migration, as expected, due to their lack of *TMSB15A* expression. RT-

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PCR confirmed knockdown of *TMSB15B*, and absence of *TMSB15A* in PC-3 cells transfected in parallel (Fig. 4C). Our data provide important evidence that *TMSB15B* produces a functional protein in prostate cancer cell motility.

DISCUSSION

In this report we show that the newly discovered gene paralog of thymosin beta 15, *TMSB15B* (Banyard et al., 2007) shows a different expression pattern in tumor cells and in normal and tumor tissues, compared to *TMSB15A*. We show that TMSB15B is a functional protein in tumor cell motility and is subject to differential gene regulation; TGFB1 suppressed *TMSB15A* expression in MCF-7 breast cancer cells, but did not affect *TMSB15B*. *TMSB15A* and *TMSB15B* genes only have two silent changes in their coding regions and thus produce identical proteins. The occurrence of a second coding copy of the thymosin beta 15 gene in the human genome is unexpected and intriguing although there are numerous mechanisms and functions of gene duplication (Conant and Wolfe, 2008). Two potential advantages to retaining an additional thymosin beta 15 gene are suggested by the current data.

Firstly, gene subfunctionalization at the regulatory level may be responsible for our observations that TGFB1 repressed *TMSB15A* and not *TMSB15B* in MCF-7 cells and the observed tissue specificity of thymosin beta 15 isoform expression. This could be mediated through transcription factor, methylation or microRNA binding site sequence differences in the cis regulatory regions of the *TMSB15A* and *TMSB15B* genes. There is considerable sequence variation between the putative promoter regions of the thymosin beta 15 isoforms. The conserved putative core promoter regions have 76% identity between *TMSB15A* and *TMSB15B* within 250 bp upstream of the transcriptional start sites, but only 59% identity when aligned across the 500 bp upstream sequence. We have previously shown promoter activity within a region 500 bp upstream of the transcriptional start site of the rat thymosin beta 15 promoter (Bao and Zetter, 2000). Similarly, other regions of the *TMSB15A* and *TMSB15B* genes show sequence variability; there is 72% identity between the two introns 1, 76% identity between the two introns 2, and 82% identity between the two 3’ untranslated regions (UTR) of the thymosin beta 15 isoforms (Sequence alignments shown in Supplementary Fig. 3). The UTR sequence variations are of particular interest, as these regions are often targeted for epigenetic regulation by microRNAs. Subfunctionalization has often been reported in developmental genes, and it may be notable that thymosin beta 15 has been shown to affect the branching of developing neurons in the brain (Choe et al., 2005).

An alternative model favoring the retention of duplicate genes is an advantage of gene dosage. Increasing thymosin beta 15 level may confer advantage to tumor cells by increasing motility, transformation and colony formation (Bao et al., 1996; Abdulrahman et al., 2007; Dhaese et al., 2007). We note that several human cancer cell lines expressed both *TMSB15A* and *TMSB15B* isoforms.

As we have shown, thymosin beta 15 level may also be influenced by the microenvironment, with TGFB1 regulating expression. This may be responsible for the thymosin beta 15 isoform expression differences in various cells and tissues. However, we may find that other signals also contribute to differentially regulate thymosin beta 15 isoform expression. Not all cells examined showed *TMSB15A* repression following TGFB1 treatment. TGFB1 inhibition of cell proliferation is lost or reduced in many tumor cells, including T-47D and ZR-75-1 (Wilson et al., 2005; Stoika et al., 2008) in which *TMSB15A* was not significantly affected, and in other cells that express both thymosin beta 15 isoforms, such as SKOV-3 (Dunfield et al., 2002). MCF-7 cells remain highly sensitive to TGFB1 (Mazars et al., 1995) and are widely used in breast cancer research as a model of

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estrogen receptor positive cancer. Whether TGFB1 responsiveness is the parental gene state or an evolved gain of function is currently unclear.

The importance of understanding thymosin beta 15 isoform RNA expression has become more critical as gene associations in cancer and various other diseases are made using gene microarray analysis. For example, recent reports have shown that *TMSB15A* is amplified in cervical cancer (Narayan et al., 2007), is associated with von Hippel-Lindau disease mutations in renal cell carcinoma (Abdulrahman et al., 2007), and shown to be a tumor protein p53-response gene (Kannan et al., 2001; Bertheau et al., 2007). *TMSB15A* RNA level was also reflective of chemotherapeutic drug response in breast cancer (Bertheau et al., 2007). Considering the fact that thymosin beta 15 protein can be detected in human urine using an enzyme-linked immunosorbent assay (ELISA) (Hutchinson et al., 2005), it may be a clinically useful biomarker of drug response.

In summary, our data reveal a functional role for the newly identified *TMSB15B* gene paralog, and show that the expression of this gene is under different regulatory controls compared to *TMSB15A*. This may have important consequences in light of the tumor cell and tissue expression differences, as regulatory signals will vary in different tumor microenvironments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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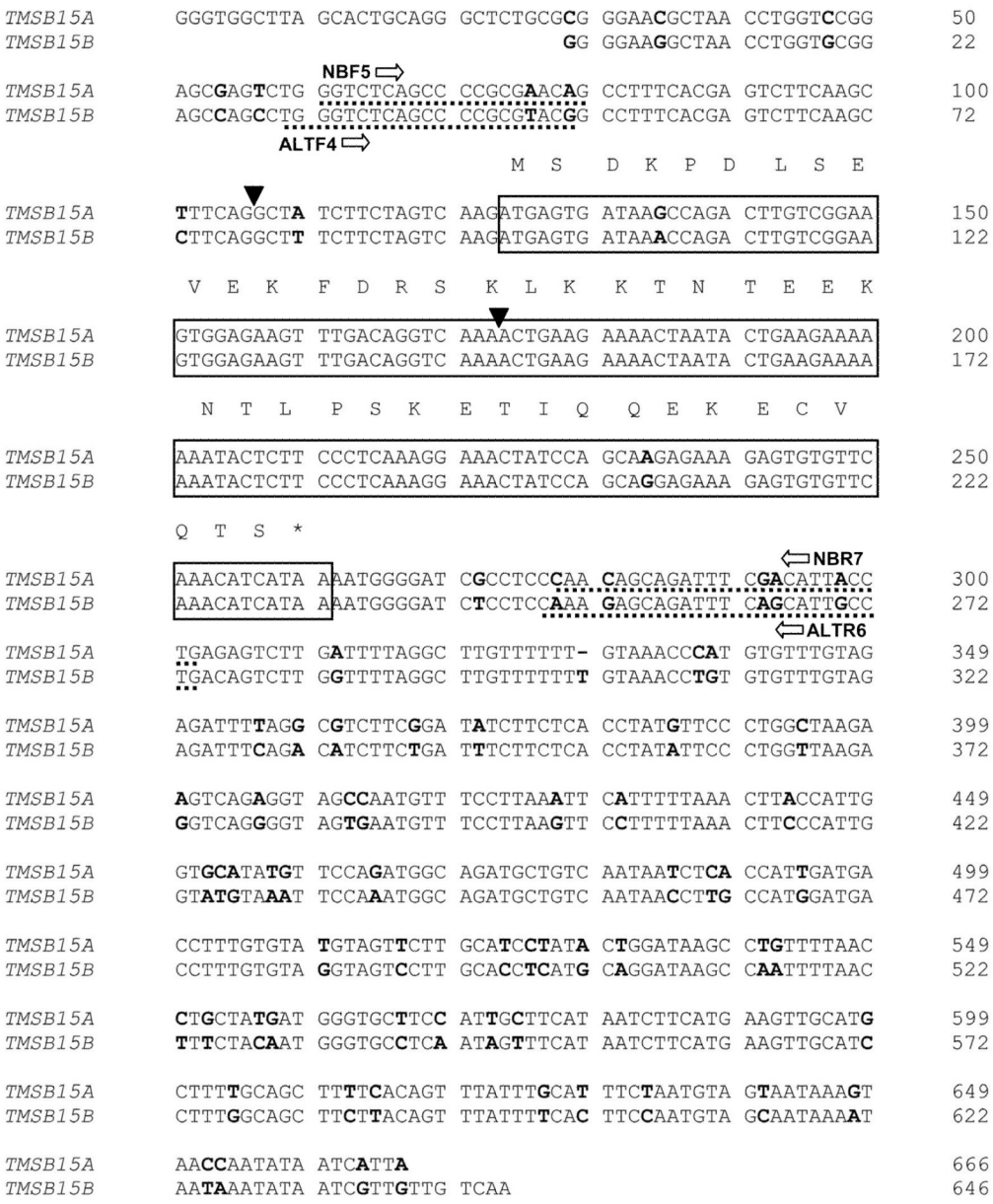

Figure 1.

Alignment of *TMSB15A* and *TMSB15B* isoform mRNAs. The coding sequence is boxed, with the amino acid sequence shown above. Nucleotide sequence differences are indicated in bold and exon splice sites are marked with arrowheads. Primer locations are underlined.

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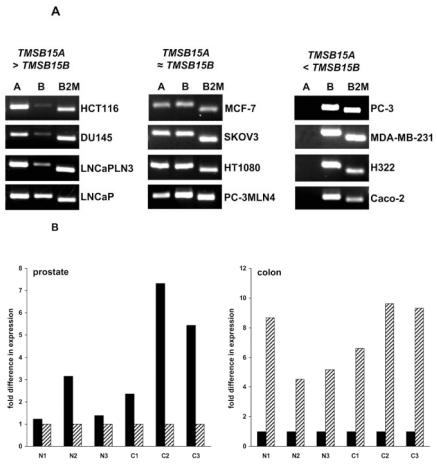

Figure 2.

TMSB15A and *TMSB15B* are expressed at different levels in different human tumor cells and normal tissues. (A) Specific primers were used to amplify *TMSB15A* or *TMSB15B* in various tumor cells. HCT116, DU145, LNCaP-LN3 and LNCaP cells expressed more *TMSB15A* (labeled A) than *TMSB15B* (labeled B) (left panel). MCF-7, SKOV-3, HT1080 and PC-3MLN4 cells expressed approximately equivalent levels of each (center panel), while PC-3, MDA-MB-231, NCI-H322 and Caco-2 cells expressed more *TMSB15B* than *TMSB15A* (right panel). *B2M* amplified as control. (B) Left panel; quantitative PCR analysis of *TMSB15A* (solid bars) and *TMSB15B* (striped bars) in prostate showed *TMSB15A* was the main isoform expressed in either normal (N) or cancer (C) tissue. Data shown as fold expression relative to *TMSB15B* C(t) value for each prostate tissue sample. Right panel; quantitative PCR analysis in colon showed *TMSB15B* was the major isoform expressed in either normal or tumor tissue. Data shown as fold expression relative to *TMSB15A* C(t) value for each colon tissue sample.

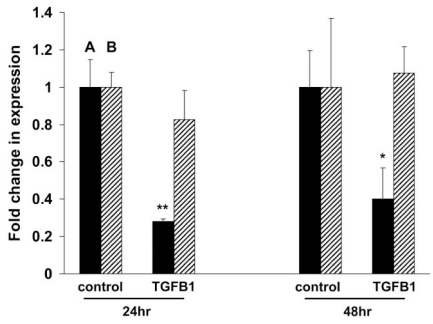

Figure 3.

TMSB15A and *TMSB15B* RNA expression are differentially regulated by TGFB1. MCF-7 breast cancer cells were treated with 10 ng/ml TGFB1 or control conditions for 24 or 48 hrs. qPCR was used to assess RNA expression of *TMSB15A* and *TMSB15B. TMSB15A* levels (black bars, marked 'A') were significantly inhibited by TGFB1, while no effect was observed on *TMSB15B* (striped bars, marked 'B'), * $P < 0.05$, ** $P < 0.01$. Fold change relative to *RPS9* housekeeping gene expression and error bars indicate standard deviation of replicate samples.

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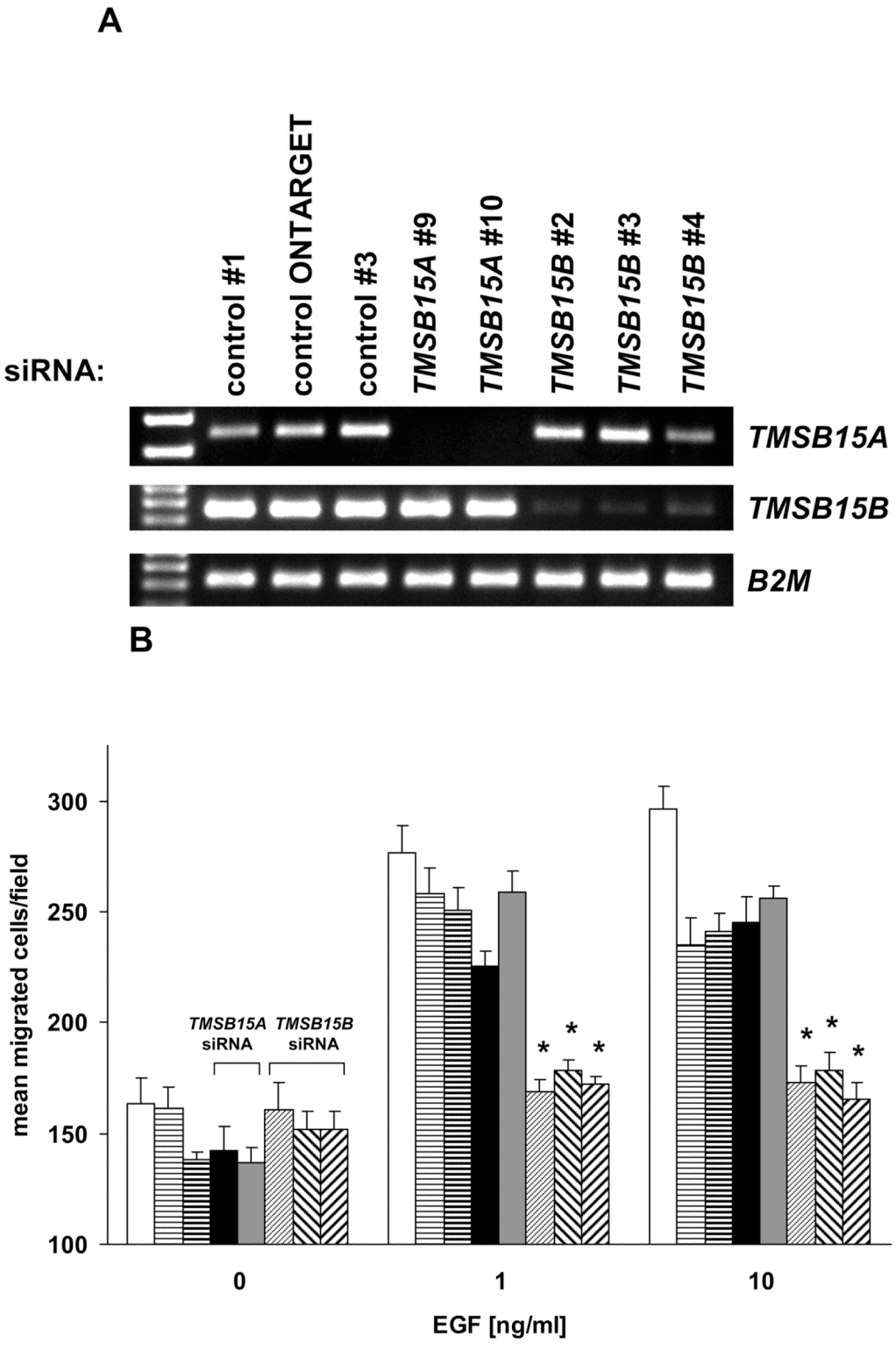

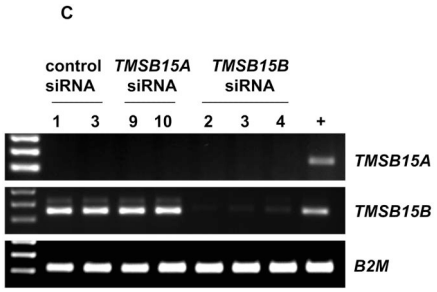

Figure 4.

RNA interference demonstrates function of *TMSB15B* in cell migration to EGF. (A) PC-3MLN4 cells were treated with 3 individual control siRNAs, 2 *TMSB15A* siRNAs (#9 and #10) or 3 *TMSB15B* siRNAs (#2, #3 and #4). RT-PCR analysis using primers to *TMSB15A* (top panel), *TMSB15B* (center panel), or *B2M* housekeeping gene (lower panel) demonstrated isoform specificity of siRNA duplexes. (B) siRNA inhibition of *TMSB15B* significantly reduced PC-3 cell migration to EGF in the modified Boyden chamber assay. Data shown as mean migrated cells/low power field with error bars indicating standard error of the mean. Empty bar; untransfected cells, Horizontal striped bars; control siRNAs (thin stripe control siRNA#1, thick stripe control siRNA#3), Solid bars; *TMSB15A* siRNAs (solid black; *TMSB15A* siRNA#9, solid grey; *TMSB15A* siRNA#9), Striped bars; *TMSB15B* siRNAs (thin upward stripe; *TMSB15B* siRNA#2, thick downward stripe; *TMSB15B* siRNA#3, thick upward stripe; *TMSB15B* siRNA#4). * $P < 0.01$. (C) Parallel transfections were performed in PC-3 cells for RNA collection. RT-PCR showed decreased *TMSB15B* expression in all *TMSB15B* siRNA-treated cells. PC-3MLN4 was used as a positive control for PCR reactions (+). *B2M*; beta-2-microglobulin.

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Table 1

Primer and siRNA Sequences

PCRTarget	**Name**	**Sequence 5'-3'**
TMSB15A	TBNBF5	GGTCTCAGCCCCGCGAACAG
	TBNBR7	CAGGTAATGTCGAAATCTGCTGTTG
TMSB15B	TBALTF4	GGGTCTCAGCCCCGCGTACG
	TBALTR6	CAGGCAATGCTGAAATCTGCTCTTG
B2M	B2Mfwd	CAATCCAAATGCGGCATCTTCAAAC
	B2Mrev	GAATGGAGAGAGAATTGAAAAAGTGGAGCA
RPS9	RIBS9fwd	GATGAGAAGGACCCACGGCGTCTGTTCG
	RIBS9rev	GAGACAATCCAGCAGCCCAGGAGGGACA
siRNA Target	**Name**	**Sense Sequence**
TMSB15A	*TMSB15A* #9	UUUUAGGCGUCUUCGGAUAUU
	TMSB15A #10	CAACAGCAGAUUUCGACAUUU
TMSB15B	*TMSB15B* #2	GUGCCUCAAUAGUUUCAUAUU
	TMSB15B #3	GGUCAGGGGUAGUGAAUGUUU
	TMSB15B #4	GUAAAUUCCAAAUGGCAGAUU