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ORIGINAL ARTICLE

SR proteins ASF/SF2 and SRp55 participate in tissue factor biosynthesis in human monocytic cells

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Summary. *Background:* Human monocytes express two naturally occurring forms of circulating tissue factor (TF) – full-length TF, a membrane-spanning protein, and alternatively spliced TF, a soluble molecule. Presence of the variable exon 5 in TF mRNA determines whether the encoded TF protein is transmembrane, or soluble. Recently, an essential SR protein ASF/SF2 was implicated in TF pre-mRNA processing in human platelets. *Objective:* To examine molecular mechanisms governing regulated processing of TF pre-mRNA in human monocytic cells. *Methods and results: In silico* analysis of the human TF exon 5, present only in full-length TF mRNA, revealed putative binding motifs termed exonic splicing enhancers (ESE) for the SR proteins ASF/SF2 and SRp55, which were found to be abundantly expressed in monocytic cell lines THP-1 and SC, as well as monocyte-enriched peripheral blood mononuclear cells (PBMC). Using a splice competent mini-gene reporter system transiently expressed in monocytic cells, it was determined that weakening of either five closely positioned ASF/SF2 ESE (bases 87–117) or a single conserved SRp55 ESE (base 39) results in severe skipping of exon 5. ASF/SF2 and SRp55 were found to physically associate with the identified ESE. *Conclusions:* SR proteins ASF/SF2 and SRp55 appear to interact with the variable TF exon 5 through ESE at bases 39 and 87–117. Weakening of the above ESE modulates splicing of TF exon 5. This study is the first to identify and experimentally characterize *cis*-acting splicing elements involved in regulated biosynthesis of human TF.

Keywords: monocytic cells, pre-mRNA splicing, SR proteins, tissue factor.

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Introduction

Existence of circulating tissue factor (TF) was postulated over a century ago, yet only confirmed in mid-1990s [1–3]. Circulating TF participates in thrombogenesis, and is elevated in many pro-thrombotic disease states [4]; most circulating TF is functionally inert, and it is only partially understood how TF becomes active, or 'de-encrypted' [5]. The pool of circulating TF comprises the full-length TF (flTF), which is membrane anchored and circulates in cell-derived microparticles [6], and the soluble alternatively spliced TF (asTF), produced via exclusion of exon 5 from the mature mRNA [7].

asTF exhibits properties of a hypomorphic co-factor of the serine protease factor (F)VII/FVIIa: native asTF secreted by cultured endothelial cells exerts very modest pro-coagulant activity compared with native flTF, and it was proposed that asTF may affect the thrombogenic potential of circulating blood either directly, or indirectly through interaction with tissue factor pathway inhibitor (TFPI) [8]. The biologic role of asTF in blood and other tissues is not known. Biosynthesis of the soluble circulating TF via alternative pre-mRNA splicing was shown in mice [9], and systemic microbial challenge of mice results in marked elevation of total circulating TF [10]. Recently, human asTF was shown to promote primary tumor growth via upregulated angiogenesis [11]. In that regard, substantial asTF levels were reported in diverse types of cancer [12,13]: aberrations in alternative pre-mRNA splicing is a prominent feature of many malignancies [14] and may thus lead to abnormal TF/asTF production, which could in turn contribute to various cancer-associated pathologies. Still, the molecular mechanisms that govern biosynthesis of the two

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structurally distinct TF protein variants from the primary RNA transcript remain to be delineated.

Circulating monocytes are known to be the major source of TF in the vasculature, and have been shown to effectively transfer TF protein to platelet surfaces [15]. Platelets themselves may also serve as a source of TF protein: TF pre-mRNA is present in quiescent platelets and can, upon stimulation with fibrinogen in the presence of thrombin, be rapidly spliced into the mature form that produces biologically active flTF; an essential SR protein ASF/SF2 appears to play a role in this process [16]. While platelets seem to produce only flTF [16], circulating monocytes are known to synthesize both TF forms [7]. Monocyte-derived TF is a major contributor to stent thrombosis [17]. Because human monocytes exhibit a highly regulated pattern of TF pre-mRNA processing [7,18], it is plausible that ASF/SF2 and other SR proteins may play a role in regulated TF biosynthesis in monocytes.

The structure of flTF, a surface glycoprotein with a single transmembrane domain, resembles that of glycoproteins CD44 and CD45, membrane-spanning receptors that participate in lymphocyte activation [19,20]. ASF/SF2 and SRp55, an SR protein whose expression pattern in human tissues is largely unknown, were both implicated in regulated production of alternatively spliced isoforms of CD44 and CD45 enriched in malignancies [21,22]. We recently noted [9] that the human and murine TF exon 5 features a conserved recognition motif, termed exonic splicing enhancer (ESE), for SRp55. In this report, we demonstrate that exon 5 of the human TF gene contains functional ESE recognized by ASF/SF2 as well as SRp55. Weakening of these motifs results in skipping of exon 5, indicating that these two SR proteins participate in constitutive and, possibly, alternative TF pre-mRNA splicing.

Materials and methods

Cells and Western blotting

THP-1 and SC cells (ATCC) were grown in RPMI-1640 according to the supplier's instructions. Human PBMC were isolated from buffy coat by gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare, Piscataway, NJ, USA) and short term culturing in T75 flasks; monocytes were allowed to adhere to the bottom of the flasks and harvested. Samples were resolved on 10% Precise Protein gels (Pierce Biotechnology, Inc., Rockford, IL, USA), transferred to polyvinylidene difluoride membranes (Roche Diagnostics, Indianapolis, IN, USA), blocked at 4 °C, and signal detected using corresponding primary and HRP-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) followed by treatment with developing reagents and exposure to Bio-Max MR film (Kodak, Rochester, NY, USA).

Reporter constructs and plasmid transfection

The reporter construct pGL-hTF was generated analogously to the system used to study splicing dynamics of the bovine amelogenin gene [23]. The reporter construct pSPL3B-hTF was generated using pSPL3B, a version of the vector pSPL3 [24]. Construct integrity was verified by automated sequencing. Substitutions within ESE motifs were generated using the GeneTailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA, USA). For transient expression of pGL-hTF and pSPL3B-hTF, cells were incubated in the presence of plasmids and TransFectin reagent (Bio-Rad Laboratories, Hercules, CA, USA), transferred to fresh medium for 24 h, and total RNA was collected using the RNeasy mini-kit (Qiagen Inc., Valencia, CA, USA). pGL-hTF correctly recapitulated production of both TF mRNA isoforms as demonstrated by sequencing of subcloned amplicons (not shown). For transient expression of pGL-hTF constructs in monocyte-enriched PBMC, cells were subjected to the nucleofection procedure (Amaxa Biosystems, Cologne, Germany), and total RNA was collected as above.

RT-PCR

For detection of ASF/SF2, SRp55, flTF/asTF, and GAPDH mRNA by conventional reverse transcription polymerase chain reaction (RT-PCR), total RNA (250 ng–2 μg) was reversed transcribed using Transcriptor reverse transcriptase and amplified using FastStart Taq Polymerase (Roche). Quantitative RT-PCR of flTF and asTF mRNA was performed using a previously described TaqMan assay [25]. See Supplementary material for details on specific primer pairs and PCR conditions.

RNA mobility shift assay

Uniformly labeled RNA probes were synthesized via T7 RNA polymerase transcription (T7 MAXIscript kit, Ambion, Inc., Austin, TX, USA) in the presence of [α-^{32}P]UTP and template PCR fragments containing wild-type or mutagenized ESE motifs for ASF or SRp55, with a T7 promoter included in the upstream primer used to generate the templates. Transcription products were purified from 9% denaturing polyacrylamide gels. Unlabeled RNA was synthesized using T7 MEGAscript kit (Ambion) and purified as above. Nuclear extracts of THP-1 cells were prepared as per Andrews and Faller [26], and protein concentrations determined using the Bio-Rad Protein Assay (Bio-Rad). One hundred fmoles of uniformly labeled RNA probes were incubated with various amounts of extract at 25 °C for 15 min in a binding buffer as per Deikus and Bechhofer [27], with omission of tryptophan. After incubation, samples were placed on ice for 15 min and loaded on 10% native polyacrylamide gels as described [27]. To visualize sample migration, 0.001% xylene cyanol was included during the binding reaction. Gels were dried, and bandshift products detected using the Storm 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA).

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Statistical analysis

Results were assessed for statistical significance using the Student *t*-test. *P*-values of less than 0.05 were considered statistically significant.

Results

flTF and asTF mRNA levels in human monocytic cells

TF mRNA was detectable by conventional RT-PCR in THP-1 and SC cells, two human monocytic cell lines; in cells that underwent < 5 passages, levels of flTF and asTF mRNA in THP-1 cells, derived from a patient with acute monocytic leukemia (AML), were found to be substantially higher compared with SC cells, derived from normal peripheral blood monocytes (Fig. 1A, 2A,B). Elevated TF mRNA levels in AML-derived monocytes are consistent with a report documenting high TF mRNA levels in bone marrow samples harvested from patients with acute promyelocytic leukemia [28]. Compared with SC cells, THP-1 cells also exhibited markedly higher flTF protein content (Fig. 1B). SR proteins ASF/SF2 and SRp55 were expressed in abundance in THP-1 and SC cells, as well as monocyte-enriched PBMC (Fig. 1A–E). Treatment of THP-1 cells with Tg003, an inhibitor of Clk kinases known to regulate various SR proteins, including ASF/SF2 [16], resulted in a pronounced decrease of flTF and asTF mRNA levels, indicating that SR proteins may control constitutive TF mRNA generation in monocytic cells (Fig. 2C,D). THP-1 cells, extensively used in TF-related research [15,18], were selected as the main cell line for this study.

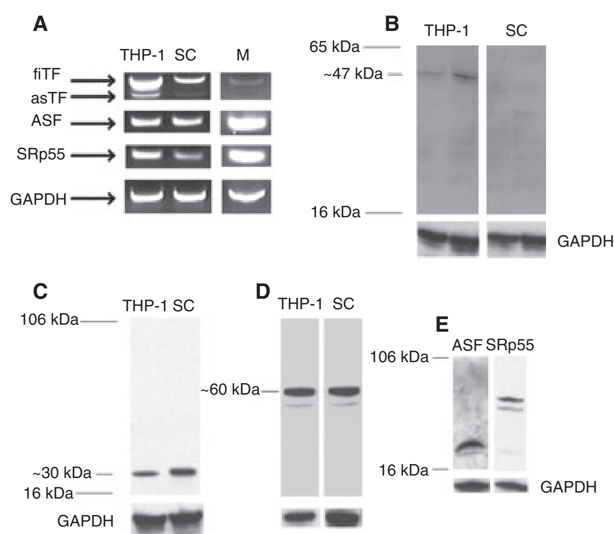

Fig. 1. Expression of tissue factor (TF), ASF/SF2, and SRp55 in THP-1 cells, SC cells, and human monocytes. (A) Representative conventional reverse transcription polymerase chain reaction (RT-PCR); total RNA from THP-1 cells, SC cells, and human monocytes (indicated as M). (B) THP-1 cells express higher quantity of flTF compared with SC cells (for each cell line, two amounts of cell lysate are shown; 1.8×10^6 cells were used to prepare each lysate). (C) Expression of ASF/SF2 protein in THP-1 and SC cells. (D) Expression of SRp55 protein in THP-1 and SC cells. (E) Expression of ASF/SF2 and SRp55 proteins in human monocytes.

Identification of putative binding sites for SR proteins ASF/SF2 and SRp55 in the human TF exon 5

Splicing dynamics of TF exon 5 are of interest because processing of this internal exon by the spliceosomal machinery determines whether the encoded TF protein is membrane anchored (flTF) or soluble (asTF) [7,16]. To conduct an *in silico* analysis of the exon 5 sequence (160 bp), we utilized ESEfinder, a web identifier of putative ESE motifs (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process = home) [29]. Eight ESE motifs specific for ASF/SF2 were identified, as were two ESE motifs specific for SRp55 (Fig. 3A), suggesting that ASF/SF2 as well as SRp55 may play a role in determining the fate of exon 5 during TF pre-mRNA processing. RESCUE-ESE, a distinct web resource designed to identify putative SR binding motifs based on statistical analysis of exon-intron junctures and composition of splice sites (http://genes.mit.edu/burgelab/rescue-ese/) [30], also identified the two putative hexameric binding motifs for SRp55, including the site at position 39, the only putative SRp55 ESE that appears conserved in the murine TF exon 5 [9] (Fig. 3A). We then designed single base pair substitutions at various positions within each putative ESE that rendered it unrecognizable by ESEfinder (Fig. 3A). To assess functional impact of the designed mutations on exon 5 inclusion, two splicing reporter mini-genes were generated: pGL-hTF, featuring complete intronic sequences flanking the variable TF exon 5, and pSPL3B-hTF, featuring the above mutations in the context of a different promoter and a much smaller genomic *TF* segment (Fig. 3B).

Weakening of closely positioned ESE for ASF/SF2 impacts inclusion of TF exon 5

Whereas previous studies indicated that ASF/SF2 may participate in TF pre-mRNA splicing in human platelets [16], it is unknown whether this SR protein plays a role in TF exon 5 processing in this and/or other cell types. pGL-hTF (Fig. 3B) was chosen to perform a complete functional analysis of the putative ASF/SF2 ESE in TF exon 5, because it features the entire endogenous intronic environment of this exon. Site-directed mutagenesis of each putative ESE was performed individually and in series where these sites were in close proximity (≤ 20 bp); the resultant constructs were expressed in THP-1 cells, and expression patterns assessed by conventional RT-PCR. Weakening of the ESE at the 5′ and 3′ termini of the exon did not affect its inclusion; in contrast, an appreciable decrease in exon 5 inclusion was observed when four sites at positions 100–117 (termed 'Series 2', S 2), were weakened (Fig. 3C). Next, we generated a pGL-hTF construct in which the high-score ESE at position 87 and the 'S 2' ESE were weakened together, and expressed it in THP-1 and SC cells; similar changes in exon 5 inclusion were observed (Fig. 3D),

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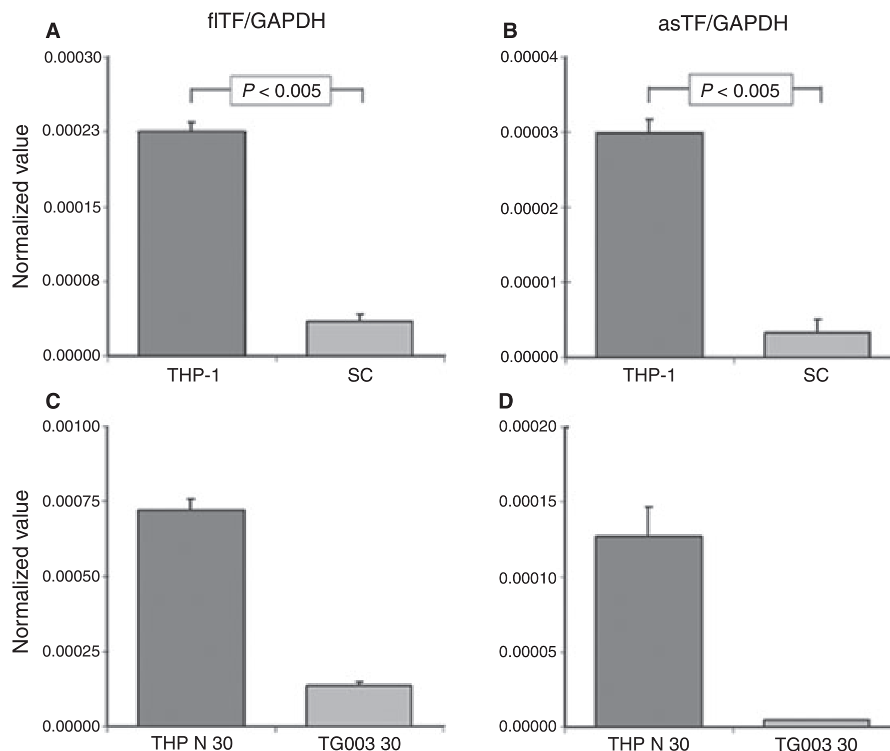

Fig. 2. Quantitative reverse transcription polymerase chain reaction (RT-PCR) of flTF and asTF mRNA. Expression of flTF mRNA (A) and asTF mRNA (B) in THP-1 and SC cells. Error bars are standard deviation ($n = 4$). Treatment of THP-1 cells with Tg003 (100 μM) for 30 min reduces levels of flTF mRNA (C) and asTF mRNA (D). N, non-treated controls (vehicle).

suggesting that the obtained results were not specific to a single cell line. pSPL3B-hTF (Fig. 3A), featuring weakened ASF/SF2 ESE at positions 87 and S 2, also exhibited similar shifts in the pattern of the produced mRNA species when transiently expressed in THP-1 cells (Fig. 3E); this finding demonstrates that the observed effects of ESE weakening are not confined to a particular promoter and/or reporter construct, and do not require distal intronic sequences.

Spliceosomal protein SRp55 recognizes a bona fide ESE in TF exon 5

To date, no information exists regarding involvement of SRp55 in TF biosynthesis at any step, in any cell type. Weakening of the conserved SRp55 ESE at position 39, using the above techniques, also resulted in considerable exon 5 skipping, whereas weakening of the other putative SRp55 motif at position 111 produced no appreciable effect (Fig. 4A). To verify that the putative SRp55 recognition motif at position 39 is a *bona fide* ESE, we attempted to restore its functionality in the pGL-hTF system by re-introducing an SRp55 binding motif, with a score far stronger than that of the wild-type ESE, at the same location where the endogenous ESE was weakened via a single-base substitution (Fig. 3A). The observed robust restoration of exon 5 inclusion (Fig. 4A) confirmed functionality of the SRp55 ESE at position 39 of human TF exon 5. To verify that the identified functional ESE for ASF/SF2 and SRp55 exert their activity in human monocytes, representative pGL-hTF constructs were transiently expressed in monocyte-enriched human PBMC; the obtained results did not differ from those observed in THP-1 or SC cells (Fig. 4A).

Weakening of functional ASF/SF2 and SRp55 ESE affects RNA-protein interactions

To verify that ASF/SF2 and SRp55 proteins physically associate with the identified ESE, RNA gel shifts were performed using nuclear extracts of THP-1 cells and *in vitro* transcribed RNA probes comprising the two regions of interest, that is ASF/SF2 sites 87–117 and SRp55 site 39. For each of the two RNA probes comprising the wild-type sequence, a counterpart featuring ESE-weakening mutations (Fig. 3A) was also generated. As shown in Fig. 5A, specific complexes were formed between ASF protein, SRp55 protein, and the corresponding wild-type probes; as expected, interaction strength of the probes featuring ESE-weakening mutations was much less pronounced compared with wild-type probes (Fig. 5B).

Discussion

This is the first study documenting expression of the SR proteins ASF/SF2 and SRp55 in human monocytes, as well as the first demonstrating the role of SRp55 in TF biosynthesis. Furthermore, it is also the first study utilizing a splicing minigene system to characterize the mechanisms governing TF

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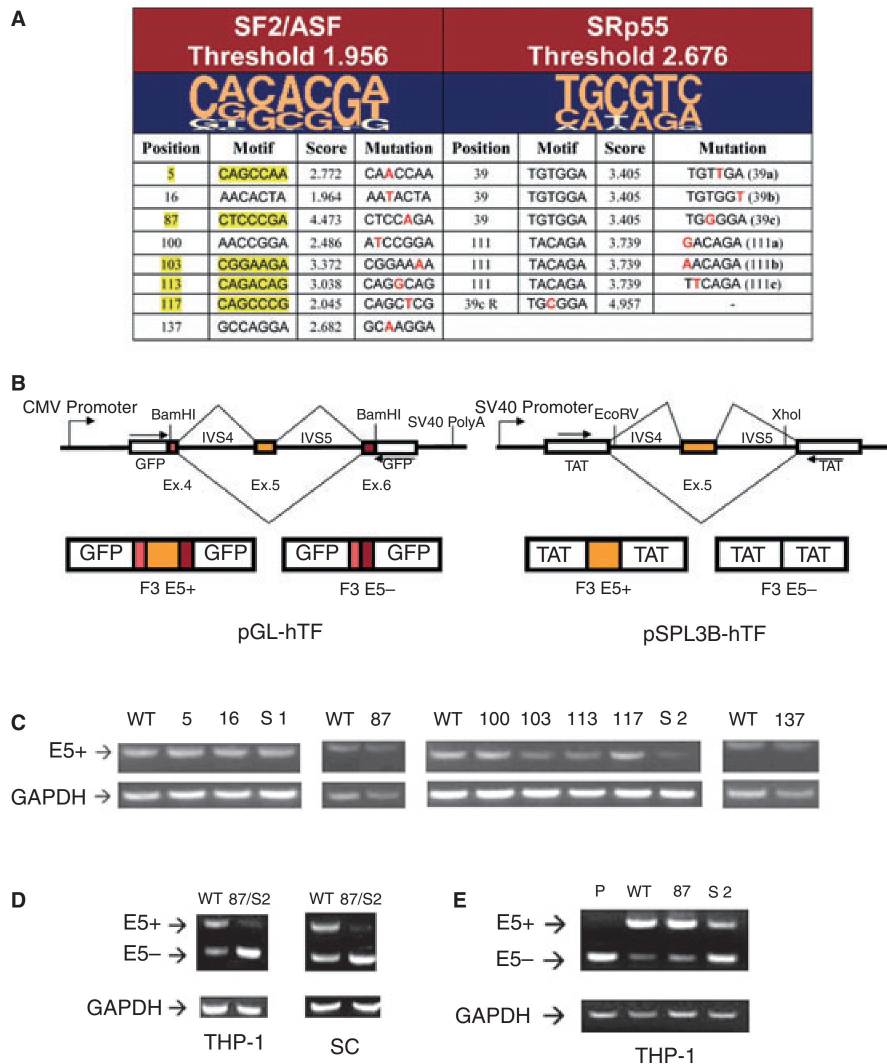

SF2/ASF Threshold 1.956				SRp55 Threshold 2.676			
Position	Motif	Score	Mutation	Position	Motif	Score	Mutation
5	CAGCCAA	2.772	CAACCAA	39	TGTGGA	3.405	TGTTGA (39a)
16	AACACTA	1.964	AATACTA	39	TGTGGA	3.405	TGTGGT (39b)
87	CTCCCGA	4.473	CTCCAGA	39	TGTGGA	3.405	TGGGGA (39c)
100	AACCGGA	2.486	ATCCGGA	111	TACAGA	3.739	GACAGA (111a)
103	CGGAAGA	3.372	CGGAAAA	111	TACAGA	3.739	AACAGA (111b)
113	CAGACAG	3.038	CAGGCAG	111	TACAGA	3.739	TTCAGA (111c)
117	CAGCCCG	2.045	CAGCTCG	39c R	TGCGGA	4.957	-
137	GCCAGGA	2.682	GCAAGGA				

Fig. 3. Exon 5 of human tissue factor (TF) contains functional exonic splicing enhancer (ESE) motifs. (A) Results of *in silico* analysis performed using the web resource ESEfinder. Putative hexameric ESE for SRp55 were also identifiable using web resource RESCUE-ESE (see text for web links). Detailed description of the SELEX-based approach used to develop the ESE prediction algorithm can be found in ref. 29; pictograms of the ESE consensus motifs are reproduced with permissions. The thresholds are values above which a score assigned to a given sequence is deemed significant; the shown default threshold values are 'the median of the highest scores for each sequence in a set of 30 randomly chosen 20 nt sequences from the starting pool used for functional SELEX experiments' [29]. Base position for each ESE denotes nucleotide 1 of the corresponding sequence; the scores assigned by ESEfinder are as indicated. Highlighted in yellow: putative ASF/SF2 motifs recognized by two distinct search algorithms (ESEfinder versions 2.0 and 3.0). Single-base pair substitutions in each putative ESE, shown in red, eliminate ESE recognition by ESEfinder. In the SRp55 column, letters 'a, b, c' indicate three distinct single base pair substitutions that result in elimination of ESE recognition by ESEfinder. Replacement of the mutagenized base [G] in the SRp55 motif '39c' by a non-native base [C] restores ESE consensus and yields assignment of a score higher than that of the endogenous motif. (B) Diagrams of splicing reporter mini-genes developed to assess functionality of putative ESE motifs. Arrows indicate locations of primers used in conventional reverse transcription polymerase chain reaction (RT-PCR) assays. (C) Representative ($n \geq 4$) RT-PCR assay of exon 5 containing amplicons generated by wild-type pGL-hTF, and the pGL-hTF mutants with ASF/SF2 ESE weakened by site-directed mutagenesis. S 1, series 1 (mutagenized sites 5 and 16), S 2, series 2 (mutagenized sites 100, 103, 113, and 117). (D) Representative ($n \geq 4$) RT-PCR assay, wild-type pGL-hTF and the pGL-hTF mutant with five weakened ASF/SF2 ESE at positions 87-117. (E) Amplicons generated by wild-type pSPL3B-hTF, and pSPL3B-hTF mutants in which ASF/SF2 ESE functional in the pGL-hTF system were weakened. P, empty plasmid (pSPL3B).

mRNA expression in human monocytes, a cell type known to produce high levels of TF after stimulation with various agonists [4], and an important indicator of increased thrombotic risk [31]. The reporter mini-gene pGL-hTF, developed for this study, capitalizes on the recently discovered regulatory nature of the variable TF exon 5 [7], facilitating investigation of TF pre-mRNA processing events, the complete understanding of which is likely required for appropriately designed strategies to elucidate the biologic role(s) and biochemical properties of the two naturally occurring circulating TF isoforms *in vivo*.

Cells of the immune system commonly employ pre-mRNA splicing plasticity to perform various physiologic functions [32], yet little is known about the molecular mechanisms employed by these cells to govern these events. In agreement with recent

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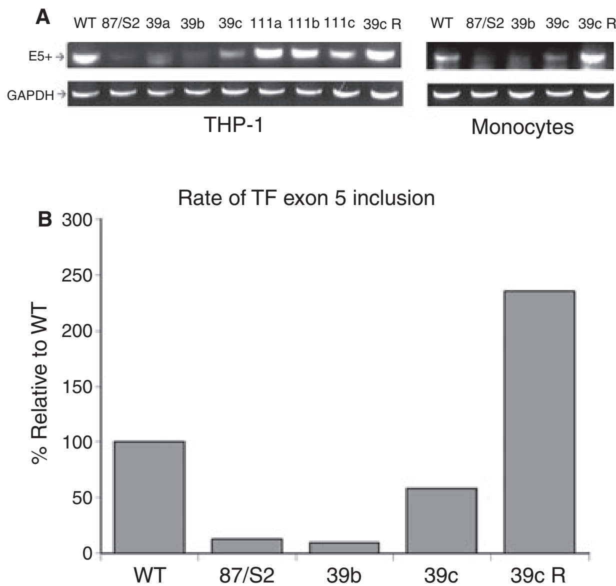

Fig. 4. Exon 5 of human tissue factor (TF) contains a functional exonic splicing enhancer (ESE) motif for SRp55. (A) Representative ($n \geq 5$) conventional reverse transcription polymerase chain reaction (RT-PCR) assay of exon 5 containing amplicons generated by wild-type pGL-hTF, and the pGL-hTF mutants with SRp55 ESE weakened by site-directed mutagenesis as shown in Fig. 3A. (B) Quantitative confirmation of changes in splicing of exon 5 as a result of weakening of functional ESE, expressed as % of E5+ amplicon generated by WT pGL-hTF in THP-1 cells; the bars represent average values of two independent experiments.

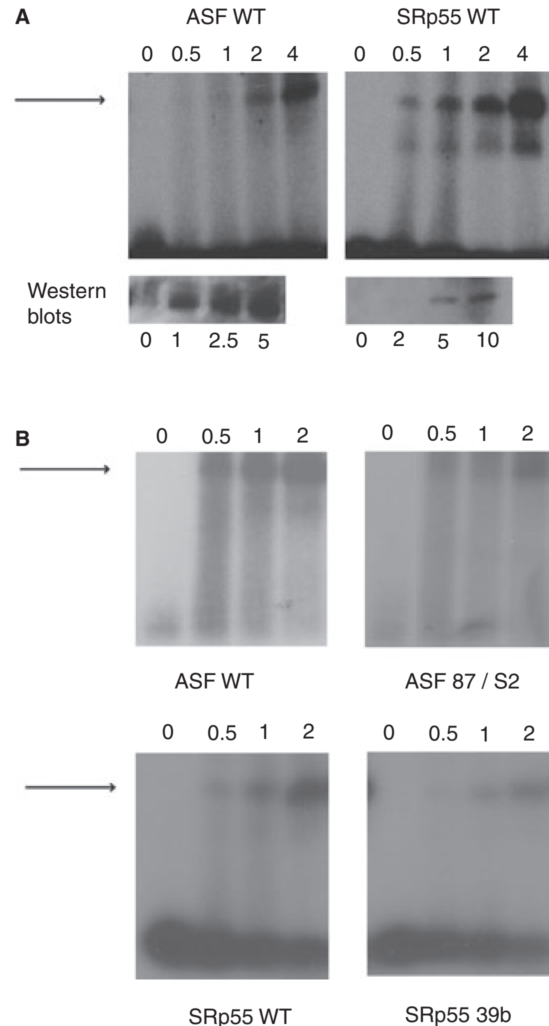

Fig. 5. ASF/SF2 and SRp55 associate with RNA probes featuring identified functional exonic splicing enhancers (ESE). (A) [α-^{32}P]UTP labeled RNA probes comprising wild-type ASF/SF2 ESE 87-117 and SRp55 ESE 39 were incubated with various amounts of THP-1 nuclear extracts (amounts shown are in μg). RNA–protein complexes are indicated by an arrow. The lower panels are Western blots of the unlabeled RNA–protein complexes probed with ASF/SF2- and SRp55-specific antibodies (see Fig. 1). (B) Labeled RNA probes comprising ASF/SF2 ESE 87-117 and SRp55 ESE 39 mutagenized as per Fig. 3A exhibit weakened RNA-protein interaction compared with their wild-type counterparts.

observations suggesting ASF/SF2 involvement in human TF pre-mRNA splicing into the flTF mRNA [16], we here demonstrate that ASF/SF2 binds to ESE located at positions 87–117 of the human TF exon 5; weakening of these ESE modulates splicing of TF exon 5 in the context of a reporter gene. We note that, in addition to exon 5, all constitutive internal exons of the human TF gene (i.e. 2, 3, and 4) contain high-score motifs identifiable by ESEfinder, raising the possibility that ASF/SF2 may participate in spliceosomal processing of multiple exonic sequences of the TF pre-mRNA. SRp55, a less studied SR protein, was found to interact with the ESE at position 39 of TF exon 5, and appears to be required for effective definition of this exon in human monocytic cells. Increased frequency of binding motifs for ASF/SF2 and SRp55 is found in exons with weak 5′ and 3′ splice sites [33]. One function ascribed to functional ESE is recruitment of spliceosomal components to weak splice sites [34]. Weak splice sites have been previously shown to associate with alternative splicing events [35]. Of note, the variable exon 5 of the human TF gene is preceded by a short and thus potentially weak polypyrimidine tract [36], making it tempting to speculate that ASF/SF2 and SRp55 may also govern the extent of exon 5 alternative splicing in tissue types that feature TF pre-mRNA splicing plasticity and, consequently, produce both protein encoding TF mRNA isoforms.

The SR-dependent nature of TF mRNA isoform production opens a possibility to influence blood thrombogenicity through modification of SR protein activity. The phosphorylation state of SR proteins, their activity, and sub-cellular localization are regulated by various SR protein kinases such as Clk [37]. SR protein kinases have distinct substrate specificities and, thereby, control the activity of specific subsets of SR proteins [38]. Activation of different SR protein kinases after external stimulation has been shown to result in altered phosphorylation state of various SR proteins such as ASF/SF2, SC35, SRp40, SRp55, and SRp75 [39]. Differential expression of TF isoforms is likely be dependent on the SR protein phosphorylation pattern, which can change in response to specific environmental stimuli; of note, sub-cellular localization of phosphorylated SR proteins is known to influence their activity

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and substrate specificity, thereby controlling pre-mRNA splicing [40]. Precise signaling pathways that affect ASF/SF2 and/or SRp55 activity in human monocytic cells, while outside of the scope of this study, clearly appear to be of interest as possible therapeutic targets because they may prove to be a tool to modulate the pro-coagulant potential of monocytes in circulation. In that light, further molecular dissection of the mechanisms governing TF pre-mRNA processing is very likely to expand our understanding of hemostasis and its aberrations, and aid in the development of improved risk stratification criteria for thrombotic events. The role of ASF/SF2 and SRp55 in TF pre-mRNA processing in human monocytic cells has now been demonstrated; however, involvement of other spliceosomal proteins is very likely and certainly warrants experimental investigation. Such studies are currently underway.

Acknowledgements

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

Supplementary material

The following supplementary material is available for this article:

Fig. S1. (A) Characterization of pAb-flTF by Western blotting. Human brain TF (lane 3), recombinant $TF_{1–218}$ (lane 5), and recombinant asHTF (lane 7) were probed with pAb-flTF (1.0 μg mL^{-1}). Lane 1, molecular weight markers; lanes 2, 4, and 6, empty. (B) Immunohistochemical evaluation of flTF expression in human heart. IgG: non-immune rabbit IgG (Jackson ImmunoResearch). Brown color indicates positive immunostaining. Original magnification: 10×.

This material is available as part of the online article from http://www.blackwell-synergy.com/doi/abs/10.1111/j.1538-7836.2008.02946.x (This link will take you to the article abstract).

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