Article

SRF Rearrangements in Soft Tissue Tumors with Muscle Differentiation

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Abstract: The Serum Response Factor (SRF) is a transcription factor that regulates the expression of a wide set of genes involved in cell proliferation, migration, cytoskeletal organization and myogenesis. Accumulating evidence suggests that SRF may play a role in carcinogenesis and tumor progression in various neoplasms, where it is often involved in different fusion events. Here we investigated SRF rearrangements in soft tissue tumors, along with a gene expression profile analysis to gain insight into the oncogenic mechanism driven by SRF fusion. Whole transcriptome analysis of cell lines transiently overexpressing the SRF::E2F1 chimeric transcript uncovered the specific gene expression profile driven by the aberrant gene fusion, including overexpression of SRF-dependent target genes and of signatures related to myogenic commitment, inflammation and immune activation. This result was confirmed by the analysis of two cases of myoepitheliomas harboring SRF::E2F1 fusion with respect to EWSR1-fusion positive tumors. The recognition of the specific gene signature driven by SRF rearrangement in soft tissue tumors could aid the molecular classification of this rare tumor entity and support therapeutic decisions.

Keywords: myoepithelial neoplasm; SRF; fusion; E2F1; myogenesis

1. Introduction

Gene fusions, which arise from chromosomal rearrangements, have been largely described in literature as pivotal driver mutations in many types of neoplasia, including benign as well as malignant tumors of hematologic, epithelial and mesenchymal origin [1]. In recent years, gene fusion events have been specifically associated with definite tumor histotypes, leading to their possible role as molecular diagnostic markers. Moreover, some chimeric proteins derived from gene fusions could be direct or indirect therapeutic targets, making the detection of gene fusions ever more relevant [2,3].

Translocation events involving the SRF gene have been described in literature for different types of soft tissue tumors. Indeed, SRF is often fused to various 3′ partner genes, including RELA [4–6], ICA1L [7], FOXO [8] and STAT6 [9], leading to the production of aberrant transcription factors and thus to the enrichment of SRF-involving pathways.

In this paper, we analyze the specific gene expression profile induced by SRF gene fusion, focusing on the SRF::E2F1 chimeric transcript that we previously identified in two cases of myoepithelial neoplasms of the soft tissues [10].

The serum response factor (SRF) gene on 6p21.1 encodes a MADS box transcription factor that binds the core sequence of the CArG boxes (CC (A/T) 6 GG) in the promoter...
of the target genes, leading to the regulation of a wide set of genes, including immediate early genes (as C-FOS, JUN and EGR), as well as genes involved in cell growth, migration, angiogenesis, cytoskeletal organization, energy metabolism and myogenesis [11–14]. SRF is also highly expressed in skeletal muscle, where it regulates the expression of skeletal muscle-specific genes, as dystrophin, muscle creatine kinase, myoD, and several genes encoding sarcomeric proteins, like α-skeletal actin, myosin light chain, and tropomyosin [15]. Accumulating evidence suggests that SRF is involved in the carcinogenesis and tumor progression of various neoplasms, especially in the mesenchymal transition of epithelial cells [16,17].

E2F1 is a transcription factor belonging to the E2F family, which is implicated in cell cycle regulation and apoptosis [18,19], and displays dual behavior by promoting or inhibiting tumorigenesis, depending on the cellular context [20,21]. To date, fusion events involving E2F1, like the SRF::E2F1 rearrangement investigated in this study, have not been previously reported in other tumors.

2. Materials and Methods

2.1. Cell Culture and Transfection of SRF::E2F1

HEK293 and HT1080 were obtained from CLS Cell Lines Service (Eppelheim, Germany) and cultured in Dulbecco’s modified Eagle’s medium high glucose (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin-streptomycin (complete medium). Plasmid DNA containing the full-length SRF::E2F1 [10] under control of the CMV promoter was used for transfection experiments. Cells were seeded on 6-well plates (9 × 10^5 cells/well) in DMEM complete medium, and after 24 h, transiently transfected with SRF::E2F1, mock or empty pcDNA3.1 vector using Lipofectamine 2000 (Life Technologies, Delhi, India). Untreated and mock (treated with only Lipofectamine) or pcDNA3.1-transfected cells were used as controls. Total RNA was extracted 48 h after transfection using the Quick-RNA™ Miniprep Kit (Zymo Research, Irvine, CA, USA), and reverse transcribed by PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio, Shiga, Japan) into cDNA.

2.2. RNA-Sequencing

Total RNA (250 ng) extracted from HEK293 and HT1080 transfected cells was used to prepare the RNA libraries with the Illumina Stranded mRNA Prep, Ligation Kit (Illumina, San Diego, CA, USA) following manufacturers’ instructions. Libraries were quantified with the Qubit dsDNA BR Assay Kit (Invitrogen, Carlsbad CA, USA) and sized with Agilent 2100 Bioanalyzer System. Pooled libraries were sequenced at 75 bp in paired end on a NextSeq 500/550 High Output V2 flow cell with an Illumina NextSeq 500 instrument (Illumina).

2.3. Bioinformatic Analysis

Paired reads were mapped on the reference human genome hg38 using STAR (https://github.com/alexdobin/STAR accessed on 2 September 2022); duplicates removal, sorting and indexing were performed with Samtools (http://www.htslib.org accessed on 2 September 2022). Gene expression was quantified and normalized as counts per million (CPM) using the python package HTseq-count to obtain the raw gene counts (https://htseq.readthedocs.io/ accessed on 2 September 2022). Subsequently, normalization factors were computed with the R-bioconductor package edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html accessed on 2 September 2022). CPM were employed to perform the principal component analysis (PCA) and the evaluation of differential expression (DE). The R package pcomp (https://cran.r-project.org/package=nsprcomp accessed on 2 September 2022) was adopted to perform the PCA. The DE analysis between controls and samples overexpressing the SRF::E2F1 transcript was conducted with the R-bioconductor package edgeR (https://bioconductor.org/packages/release/bioc/html/edgeR.html accessed on 2 September 2022), setting a p-value < 0.05. The Gene Set Enrichment Analysis
Bioinformatics analyses were likewise performed on SRF::E2F1 fusion-positive tumor samples (L107 and L108) versus EWSR1-positive samples (L161 and L162), previously published [10].

2.4. Real-Time PCR Analysis

Fusion gene expression and mRNA expression level of SRF target genes (EGRI, FOS, CALD1, and ACGT2) were evaluated in transfected HEK293 and HT1080 cells by quantitative RT-PCR (CX96 Touch Real-Time-PCR Detection System, Biorad) with Premix Ex Taq™ DNA Polymerase (Takara Bio). Fold changes were evaluated in comparison to untreated samples using the ΔΔCt method. GAPDH was used as housekeeping gene. GraphPad PRISM Software was used for statistical analysis. The P value was estimated against pcDNA3.1 by a one-way ANOVA (** p < 0.01; *** p < 0.001).

3. Results

3.1. SRF Rearrangement in Myoepithelial Neoplasms

We have previously reported [10] a novel in-frame SRF::E2F1 fusion in two cases of myoepithelial neoplasms (MN) of the soft tissue: a mixed-type tumor harboring FUS::KLF17-rearrangement (case L108) and a spindle cell myoepithelioma with no evidence of other gene fusions (case L107), both lacking pathological evidence of malignancy.

In the two SRF::E2F1 positive patients, the breakpoint was detected in the middle of intron 3 of SRF and the middle of exon 5 of E2F1, leading to a chimeric protein retaining the MAD box domain of SRF and the TAD domain of E2F1 (Figure 1).

![Figure 1](image-url)

**Figure 1.** Schematic representation of SRF::E2F1 fusion transcript and predicted chimeric protein, depicting the exons and protein domains involved. The genomic exon structure of SRF and E2F1 are reported on the left and on the right, respectively. Black arrows indicate the breakpoint. The resulting chimeric protein is shown below [10]. MADS: MAD box DNA binding domain; TAD: Transactivation domain.

3.2. Gene Expression Analysis Driven by SRF Fusion

To gain further insight into the oncogenic mechanism driven by SRF gene fusion and to uncover its specific gene expression profile, we performed gene expression analysis on SRF::E2F1-transfected cell lines. We transiently overexpressed the SRF::E2F1 chimeric transcript into HEK293 and HT1080 recipient cell lines and profiled the whole transcriptome 48 h after transfection. Unsupervised PCA analysis showed a distinct clustering of the samples, with SRF::E2F1 overexpressing cells separating along the second principal component in both HEK293 and HT1080 with respect to control cells, and displaying a specific gene expression profile driven by SRF-fusion de novo expression (Figure 2).
were equally upregulated in samples harboring SRF::E2F1 fusion transcript analyzed by RNA-sequencing. The unsupervised PCA analysis (Figure 4), as reported in our previous work for the HEK293 cell line [10].

Figure 2. Principal component analysis (PCA) performed on HEK293 and HT1080 cells transfected with SRF::E2F1, we performed a supervised analysis (Supplementary Table S1), identifying 331 differentially expressed genes \( p \text{-value} < 0.05 \) in SRF::E2F1-transfected samples, as well as the immediate early genes \( EGR1, EGR2, EGR \) and \( EGR4 \), with an overexpression of at least seven-fold over control cells. To confirm that this gene expression signature was present and relevant also in SRF fusion-positive tumors, we analyzed the gene expression profile of two myoepitheliomas carrying SRF::E2F1 chimeric fusion with respect to two EWSR1-rearranged MN.

Supervised analysis on MN tumor samples identified 2,249 differentially expressed genes \( p \text{-value} < 0.05 \) in SRF-fused positive tumors with respect to EWSR1-fused positive tumors (Supplementary Table S2). Among the selected genes, SRF, E2F1, EGR2, EGR3 and EGR4 were equally upregulated in samples harboring SRF rearrangement, with a fold induction of at least four times over EWSR1-fused positive tumors.

Interestingly, the overriding activation of the SRF signaling pathway was confirmed by enrichment analysis of genes harboring specific transcription factor binding sites (GSEA TFT database). Figure 3 shows that in both transfected cell lines and tumor samples, the prevalent transcription-factor-activated signature is the one driven by SRF, since all the different SRF-binding motif lists are significantly enriched in SRF-fused positive samples versus controls.

Additionally, \( FOS \) and \( FOSB \), two other SRF immediate early genes, were among the most enriched genes in both SRF::E2F1-transfected cell lines and SRF fusion-positive MN. In line with the gene expression results, real time PCR performed on HEK293 and HT1080 samples showed a remarkable increase of \( EGR1 \) and \( FOS \) mRNA levels in SRF::E2F1 overexpressing cells with respect to untreated, mock-transfected and empty vector controls (Figure 4), as reported in our previous work for the HEK293 cell line [10].
Biomolecules 2022, 12, x FOR PEER REVIEW 5 of 7

Figure 3. GSEA analyses of transcription factor binding sites' enrichment in SRF-fusion positive samples. Tables and hierarchical clustering showing that SRF binding sites were among the top scoring enriched gene sets in (a) SRF::E2F1 transfected HEK293 and HT1080 cells and (b) SRF-fused positive tumors. NES: Normalized Enrichment Score; NOM p-val: Nominal p-value; FDR q-val: False Discovery Rate q-value.

Figure 4. SRF::E2F1 expression in transfected cell lines. mRNA relative expression of SRF::E2F1, EGR1, FOS, ACTG2 and CALD1, target genes of SRF, in HEK293 (a) and HT1080 (b) cell lines, 48 h after transfection. Fold changes were evaluated in comparison to untreated samples. GAPDH was used as housekeeping gene. P value was estimated against pcDNA3.1 by a one-way ANOVA (** p < 0.01; *** p < 0.001).
Extending pathway analysis to the hallmarks gene set (GSEA hallmarks database), we found that the most significantly enriched biological processes in the cell lines transfected with SRF:E2F1 were related to myogenesis, inflammation and IFN-response (Figure 5a). Real-time PCR analysis confirmed, in both HEK293 and HT1080 SRF:E2F1 samples, a significant increase in myogenic marker expression (ACTG2 and CALD1) when compared to all the control samples (Figure 4). Notably, the same hallmark pathways were upregulated in SRF-fusion positive myoepitheliomas (L107 and L108) with respect to EWSR1-fused MN (Figure 5b). Additional significantly enriched gene sets referring to immune activation and inflammation are shown in Supplementary Figure S1.

4. Discussion

The involvement of SRF in different rearrangement events has been identified in various soft tissue tumor types, as in perivascular myoid tumors and rhabdomyosarcomas. Little is known regarding gene fusions in myofibromas, and up to now the few cases analyzed led to the identification of recurrent SRF rearrangements with various 3’ partner genes in a subset of cellular variants of myofibroma [4,5]. SRF fusion events have been observed in myopericytomas, a group of tumors composed of relatively monomorphic, oval-spindle-shaped myoid-like cells, originating from perivascular myoid cells, and showing overlapping morphological features with myofibromas [22]. In particular, Antonescu and colleagues [4] detected SRF rearrangements in eight cases of myofibroma/myopericytoma (among which six cases had identical SRF::RELA fusions) displaying a significant down-regulation of SRF mRNA (Figure 6a). These tumors exhibited a clear smooth-muscle-like...
immunophenotype showing diffuse reactivity and co-expression of SMA and desmin and abundant expression of actin and caldesmon.

Figure 6. Schematic representation of SRF fusion transcripts and predicted chimeric proteins, depicting the exons and protein domains involved. The genomic exon structure of SRF and fusion partner genes is reported on the left and on the right, respectively: SRF::RELA (a) [4–6], SRF::CITED1 (b) [5,9], SRF::NFKBIE (c) and SRF::NCOA2 (d) [5,23,24]. Black arrows indicate breakpoints. Resulting chimeric proteins for each fusion transcript are reported at the bottom of each figure. MADS: MAD box DNA binding domain; DBD: DNA binding domain; DD: dimerization domain; TAD: transactivation domain; CITED: CBP/p300-interacting transactivator with ED-rich tail domain; ANK: ankyrin repeats and N: nuclear receptor coactivator.
Other perivascular myoid tumors harboring SRF:REL fusion (Figure 6a) and displaying pericytic differentiation with expression of the smooth-muscle actin and caldesmon were newly described by Karanian and colleagues [5]. In addition, the histopathological and molecular features of three uterine tumors carrying SRF::REL fusion (Figure 6a) were recently identified and compared to SRF::REL-positive perivascular myoid tumors arising in other anatomical districts [6]. Also in this case, the immunohistochemical analysis showed desmin, caldesmon and SMA expression, supporting the myogenic commitment.

Other genetic alterations involving SRF gene fused to CITED1, CITED2, NFκBIE or NCOA2 (Figure 6b–d) were observed in perivascular myoid tumors [5] and myofibromas [9]. Moreover, SRF::NCOA2 fusion-positive pediatric RMS (rhabdomyosarcoma) showing diffuse staining for desmin and multifocal nuclear positivity for myogenin are reported [23,24].

A novel SRF::ICA1 fusion [7] was also found in a subset of cellular myofibromas of the deep soft tissues arising in adult patients. These SRF-fused tumors displayed an incomplete smooth muscle cell differentiation with a diffuse expression of SMA, calponin, and smooth-muscle-heavy myosin isoform, but no expression of desmin or caldesmon. Lastly, SRF::STAT6 fusion (Figure 7a) was reported in a case of deep soft-tissue tumor of the arm in a 15-year-old boy, expressing a full smooth-muscle phenotype [25] and overlapping features with the SRF::REL myofibromas [4]. Mitotic activity was low, and the tumor showed diffuse expression of α-SMA, desmin, caldesmon, and calponin, without expression of myogenin, MyoD1, CD34, EMA or PS100.

Recently, well-differentiated RMS with SRF::NCOA1 (Figure 7b) or SRF::FOXO1 (Figure 7c) fusions were reported in infantile localized paraspinal muscle tumors showing well-differentiated rhabdomyoblastic proliferations with nuclear atypia, infiltrative borders, and diffuse expression of desmin, myogenin, and MyoD1 [8]. Unsupervised gene expression analysis of these cases of SRF-fused RMSs and a cohort comprising different types of RMS showed that they clustered together and away from other skeletal muscle tumor types. Supervised gene expression analysis comparing SRF-fused RMSs with other types of RMS identified a strong upregulation of genes involved in muscle differentiation and function, and a downregulation of cell cycle/proliferation pathways. A cell model of this specific fusion was recently established from an infantile spindle cell RMS tumor harboring SRF::NCOA2 gene fusion, confirming the rhabdomyoblastic features shown by MYOD1 and myogenin expression [14].

Overall, SRF-fusion transcripts described in the literature mostly retain the MAD domain of SRF necessary for the target genes binding, and the C-terminal transcription activating domains of partner genes. This is the case with SRF::REL [4,5], SRF::ICA1 [7,9], SRF::NCOA2 [5] and SRF::STAT6 [25] neoplasms, which present similar SRF breakpoints. Therefore, in these SRF-fused tumors, aberrant transcription factors are produced, and consequently, pathways involving SRF are enriched [5]. Exploration of the follow-up data regarding SRF-fused tumors demonstrates that neoplasms harboring SRF rearrangements have a generally benign behavior, regardless of SRF partner genes.

Here, we further investigated the novel SRF::E2F1 fusion in myoepitheliomas, which, together with the mixed tumors/chondroid syringomas, represent a class of myoepithelial tumors of soft tissue with benign behavior [26]. In these samples, the expression of SRF::E2F1 transcript led to the production of a functionally active chimeric protein [10]. Bioinformatic analyses performed herein revealed a distinct expression profile of MN harboring SRF rearrangement with respect to EWSR1 fusion-positive tumors, with the overexpression of SRF target genes as the immediate early genes EGR2, EGR3, EGR4, FOS and FOSB. This specific SRF signature is shared by tumor subtypes carrying other SRF-fused partner genes, such as the perivascular tumors harboring SRF::REL fusion [5]. Indeed, GSEA performed by Karanian and colleagues on SRF::REL tumors [5] revealed that the top enriched gene sets were the SRF binding sites, similar to our SRF::E2F1 MN cases.
Proteins for each fusion transcript are reported at the bottom of each figure. MADS: MAD box DNA-binding domain; (protein interacting domain) and TA: transcription activator domain.

Figure 7. Schematic representation of SRF-fusion transcripts and predicted chimeric proteins, depicting the exons and protein domains involved. The genomic exon structure of the SRF and fusion partner genes are reported on the left and on the right, respectively: SRF::STAT6 (a) [9], SRF::NCOA1 (b) [8] and SRF::FOXO1 (c) [8]. Black arrows indicate breakpoints. Resulting chimeric proteins for each fusion transcript are reported at the bottom of each figure. MADS: MAD box DNA-binding domain; SH2: Src homology domain 2; TAD: transactivation domain; N: nuclear receptor coactivator; D: domain of unknown function; FKHR: forkhead DNA-binding domain; KBD: KIX-binding domain; (protein interacting domain) and TA: transcription activator domain.

Additionally, myoepithelial tumors harboring SRF::E2F1 fusion displayed a strong overexpression of genes involved in myogenic differentiation, as myosin heavy/light chain, troponin and actin gene families; some of these genes were also identified as differentially expressed in SRF-fused perivascular tumors [5].

Interestingly, we observed an upregulation of genes involved in inflammation and immune response in SRF fusion-positive myoepitheliomas with respect to EWSR1-fused MN. These results seem to be in line with the few reports showing SRF’s role in immune activation [27–31], despite that SRF involvement in the regulation of inflammation and immunity has not been systematically explored yet. As proof, the comparison between the differentially expressed genes in SRF::RELA tumors [5] and our SRF::E2F1 myoepitheliomas showed 356 overlapping genes, including those involved in myogenesis (MYH11, TAGLN and MYLK), inflammation (TNSF10, IFI27, CXCL11, BST2 and IFI44L) and the immediate early genes EGR2 and EGR4. The specificity of gene expression signature induced by the fusion transcript in myoepitheliomas was confirmed by bioinformatics analyses on HEK293 and HT1080 transiently expressing SRF::E2F1 fusion.

Of note, the SRF::E2F1-positive MN carrying the FUS rearrangement (L108) could display a specific genetic signature induced by the FUS::KLF17 fusion. However, the reduced number of cases in the SRF::E2F1 group and the lack of published data concerning
the FUS::KLF17-activated pathways prevents the identification of a specific gene expression profile determined by FUS rearrangement in this sample.

This work proves the central role of the SRF chimeric fusion protein in inducing the myogenic differentiation program in SRF-fused tumors, as well as the inflammatory and immune signature, with the upregulation of the Interferon-induced genes (Figure 8).

**Figure 8.** Schematic representation of the proposed pathways involving SRF::E2F1. In SRF fusion-positive myoepitheliomas, the SRF::E2F1 fusion encodes a chimeric transcript, and consequently, an aberrant transcription factor is produced. The chimeric protein binds the CArG sequence on the DNA, inducing the expression of immediate early genes and those involved in myogenesis and inflammation.

In conclusion, it is tempting to speculate that the relatively benign behavior of this molecular subtype of myoid tumors could at least be partly dependent on the induction of an inflammatory response and immune recognition. The identification of the specific gene signature driven by SRF fusion could aid the molecular diagnostic process and guide the therapeutic decisions with respect to the clinical behavior of this rare tumor entity.

**Supplementary Materials:** The following supporting information can be downloaded at: [https://www.mdpi.com/article/10.3390/biom12111678/s1](https://www.mdpi.com/article/10.3390/biom12111678/s1), Table S1: Differentially expressed genes between SRF::E2F1-transfected cells and controls (logFC = log2 fold change; logCPM = log2 counts/million); Table S2: Differentially expressed genes between SRF-Fused and EWSR1-Fused tumors (logFC = log2 fold change; logCPM = log2 counts/million); Figure S1: GSEA analyses of SRF::E2F1 myoepitheliomas versus EWSKI-Fused positive tumors.

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**Conflicts of Interest:** The authors declare no conflict of interest.
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