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# Regulation of fibrillin-1 gene expression by Sp1

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ABSTRACT

Mutations in the fibrillin-1 gene (*FBN1*) cause Marfan Syndrome (MFS), a hereditary disorder of connective tissue. The transcription of *FBN1* has been reported to be driven by a short ultraconserved region (SUPR) in the 5′ untranslated exon A of *FBN1*, but the nature of other factors involved in *FBN1* gene regulation has not been clarified. In this study, we characterized the transcription factors involved in *FBN1* gene regulation. The results show that Sp1 protein binds to two putative binding sites in the promoter of *FBN1*. Overexpression of Sp1 resulted in a significant increase in both promoter activity and *FBN1* mRNA level in HEK 293 cells, whereas inhibition or knockdown of Sp1 decreased *FBN1* gene expression. In addition, we found that Poly [ADP-ribose] polymerase 1 (PARP1) binds to the palindromic sequence TCTCGCGAGA in the ultraconserved region of the *FBN1* promoter and that the regulation of *FBN1* expression by PARP1 is dependent on Sp1. These results indicate that both Sp1 and PARP1 contribute to *FBN1* gene expression. These observations add to our understanding of the transcriptional regulation of *FBN1* gene expression.



## 1. Introduction

Fibrillin-1 is a large glycoprotein component of the 10–12 nm extracellular microfibrils (Sakai et al., 1986). Mutations in the gene for fibrillin-1 (*FBN1*) cause Marfan syndrome (MFS), a hereditary disorder of connective tissue with prominent manifestations in the ocular, skeletal, and cardiovascular systems including a risk for ascending aortic dissection (Robinson et al., 2006).

Differences in allele specific *FBN1* gene expression have been proposed as a candidate modifying factor in MFS (Mátyás et al., 2007), but currently little is known about *FBN1* gene expression. *FBN1* possesses three alternatively spliced 5′ upstream exons initially termed exon B, A, and C, each of which can be spliced to the first coding exon (exon 1, previously termed exon M), with a strong bias towards transcription initiation from exon A followed by exon 1. It has been shown that transcripts from several sources (placenta, neonatal fibroblasts, and osteosarcoma cells) have a strong bias for the utilization of exon A; no clones containing more than one upstream exon were found suggesting either initiation from alternative transcription initiation sites or mutually exclusive splicing from an as yet undetermined additional upstream exon (Corson et al., 1993). The three upstream exons are embedded in an approximately 1.8 kb long CpG island (Corson et al., 1993). In previous work, we showed that a short ultraconserved promoter region (SUPR) in the region of the 5′ untranslated exon A of *FBN1* drives transcription of *FBN1* using in vitro promoter assays (Guo et al., 2008). SUPRs are present in about 5% of all human genes and are enriched in genes involved in regulation and development. SUPR-associated transcripts show a significantly higher mean expression than transcripts associated with non-SUPR-containing promoters (Rödelsperger et al., 2009). Computational and biochemical analysis showed the presence of an ultraconserved sequence in the area of the core promoter of exon A containing a putative initiator element (Inr), a downstream promoter element (DPE), and a transcriptionally active 10-nucleotide palindromic element previously found mainly in the upstream regions of genes encoding ribosomal proteins (Colombo and Fried, 1992; Perry, 2005; Roepcke et al., 2006; Wyrwicz et al., 2007; Xie et al., 2005).

Sp1 (specificity protein 1) is a transcription factor containing a GC-rich binding zinc finger protein motif. In TATA-less promoter regions, Sp1 is able to activate transcription through recruiting the TATA-binding protein and associated TFIID, thereby bringing the initiation complex to the correct transcriptional start site (Pugh and Tjian, 1991). In the majority of promoters containing Sp1-binding sites, Sp1 contributes to their basal level of transcription (Cook et al., 1999). We computationally predicted several Sp1 binding sites in a region of intron B and the first 50 nucleotides of exon A, which we will denote as promoter A (Guo et al., 2008).

The *FBN1* SUPR contains a 10-nucleotide palindromic element, TGTCGCGAGA (Guo et al., 2008), which has previously been identified

*Abbreviations:* MFS, Marfan syndrome; *FBN1*, fibrillin-1; SUPR, short ultraconserved promoter region; Inr, Initiator; DPE, downstream promoter element; Sp1, specificity protein 1; PARP1, Poly [ADP-ribose] polymerase 1.

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as a common, conserved motif in a genome-wide human–mouse assessment of six to eight nucleotide segments, and it was therefore suggested that this element may be involved in the regulation of nearly 5% of human genes, mostly those transcribed from TATA-less promoters (Wyrwicz et al., 2007). Using EMSA and mass spectrometry analysis, Mikula et al. (2010) identified a number of proteins that interact with this palindromic motif. Remarkably, PARP1, a ubiquitous and abundant nuclear protein, was found to be among the most abundant proteins binding to TCTCGCGAGA (Mikula et al., 2010). PARP1 plays critical functions in many biological processes, including DNA repair, maintenance of genomic integrity, and regulation of telomerase activity, and is able to regulate gene transcription through chromatin remodeling or by direct interaction with target regulatory elements present in the promoters (Huletsky et al., 1989; Zaniolo et al., 2007). Interestingly, this activity of PARP1 in transcription enhancement is independent of its better known role in poly(ADP)-ribosylation of target proteins such as p53 and DNA-dependent protein kinase (DNA-PK) after DNA damage. Indeed, ADP-ribosylation, specifically, PARP1 auto-ADP-ribosylation, inhibits its coactivator function in transcription (Meisterernst et al., 1997). Since PARP1 has affinity for both double stranded DNA ends and DNA hairpins, PARP1 may be attracted to the hairpin structure formed by TCTCGCGAGA (Mikula et al., 2010). PARP1 is known to be able to bind to both 5′- and 3′-recessed ends on double-stranded DNA, as well as to palindromic-like structures often present in DNA (Pion et al., 2005).

In this work, we show that Sp1 binds to the two putative Sp1 binding sites in promoter A and that these two Sp1 binding sites, proximal to the SUPR region, are critical for *FBN1* expression. Moreover, we provide evidence that PARP1 binds to the palindromic sequence TCTCGCGAGA and co-regulates *FBN1* gene expression with Sp1.

## 2. Material and methods

### 2.1. Cell culture

HEK 293 cells were grown in DMEM medium supplemented with 5% fetal bovine serum and 100 IU/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37 °C in an atmosphere of 5% $CO_2$. For RNA extraction or luciferase experiments, HEK 293 cells were treated with 100 nM mithramycin A, 1 μM PJ43 or combination of both for 48 h. Cell viability was determined by Trypan Blue. No loss of cell viability was observed for up to 48 h following treatment with mithramycin A or PJ43.

### 2.2. Plasmids and luciferase assay

The pCMV-Sp1 expression plasmid was a kind gift from Prof. Suske (IMT, Marburg). The P1, P1b and P1c constructs have been described in our previous study (Guo et al., 2008). Of note, the P1 construct includes intron B and the first 50 nucleotides of exon A. Mutated construct P1m (see Fig. 2) was generated using the GeneTailor Site-Directed Mutagenesis kit (Invitrogen) according to manufacturer's protocol. Primers for mutagenesis were: 5′-TGGATGGGGCCGGGG**AA**TGGGGT GGTGATG-3′ and 5′-CCCCGGCCCCATCCATCCTCCCTTTCCTGC-3′.

Bold letters indicate mutated nucleotides. Transfection and luciferase assay were conducted as previously described (Guo et al., 2008). For siRNA and plasmid cotransfection, HEK 293 cells were first transfected with siRNA as described below, and after 24 h transfection was continued with plasmids for a further 48 h.

### 2.3. Nuclear extracts and EMSA analysis

Nuclear extracts from HEK 293 cells were prepared using NE-PER kit (Pierce) according to manufacturer's protocol. EMSA analysis was performed as described before (Guo et al., 2008). In competition experiments, nuclear extracts were incubated at room temperature for 10 min with a 100 fold molar excess of unlabeled double stranded oligonucleotides before the addition of the labeled oligonucleotides. For supershift analysis, 1 μg of Sp1 specific antibody (Santa Cruz, sc-59X) or 1 μg PARP1 antibody (Active Motif, Catalog No: 39559) was preincubated with nuclear extract proteins at room temperature for 20 min before the addition of the labeled DNA probes. The same biotinylated oligonucleotide containing the palindromic element as in our previous study was used (Guo et al., 2008). Oligonucleotides used in the EMSA are listed in Fig. 1.

### 2.4. Knockdown of endogenous PARP1 and Sp1 protein and treatment of HEK 293 cells

HEK 293 cells maintained between 50% and 70% confluence were used for transfection. siRNA sequences of Sp1 and PARP1 were obtained from the published literature (Sp1 siRNA: 5-GCAACAUGGGAAUUAUGAA-3 (Bin et al., 2011); PARP siRNA:5′-AAGATAGAGCGTGAAGGCGAA-3′ (Kameoka et al., 2004). Scrambled siRNA-A (Santa Cruz) was used as a negative control. Then, siRNA with a final concentration of 50 nM was transfected using Lipofectamin 2000 (Invitrogen) following manufacturer's protocol. To evaluate the knockdown efficiency, a western blot of the whole cell lysate was performed using antibodies against Sp1 (Santa Cruz, sc-59) and PARP1 (Active Motif, Catalog No: 39559). β-Actin was used as a loading control.

### 2.5. RNA extraction and real time PCR analysis

Total RNA was extracted from HEK 293 cells after siRNA transfection using RNAPure (PeqLab), and 500 ng to 1 μg was used for synthesis of cDNA using a Superscript First Strand synthesis kit (Invitrogen). Real-time quantitative PCR was carried out using the ABI 7900HT real-time PCR System in a final volume of 18 μl containing 5 μl cDNA (in a 1:50 dilution), 9 μl of Power SYBR Green PCR Master Mix (Applied Biosystems) and 1 μl primers (0.2 μmol each) using standard PCR conditions. mRNA levels of *FBN1*, *SP1*, *PARP1* or *GAPDH* were detected using appropriate primers. Samples were run in triplicate, and amplification of 18S rRNA served as the internal control. Primer sequences are given in Table 1.

### 2.6. Chromatin immunoprecipitation (ChIP)

ChIP was performed by using the EZ-ChIP™ kit from Upstate Biotechnology according to manufacturer's protocol. The antibody used in the ChIP assays was an anti-PARP1 antibody (Active Motif, Catalog No: 39559), an anti-Sp1 antibody (Santa Cruz, sc-59x) or an anti-RNA polymerase II antibody from the EZ-ChIP™ kit. Rabbit IgG control antibody was used as a negative control. ChIP purified DNA was amplified by standard PCR with primers spanning a region of 206 bp encompassing the palindromic motif. The ChIP primers were as follows: forward 5′-TGCAAAGGGGAGTGGAAAGGG-3′ and reverse 5′-TTGTGGCACCCACAGTCTC-3′. PCR was performed with Taq polymerase from Invitrogen with 1 M of GC melt (Clontech). The PCR program was: 95 °C, 5 min, 30 cycles of (95 °C 30 s, 55 °C 30 s, 72 °C 30 s), 72 °C 10 min, and 12 °C unlimited.

### 2.7. Statistical analysis

Experiments were performed three times each in triplicate. Data presented are means ± standard error (SE) of at least three independent experiments. Pairwise comparison was conducted with a two-sided Student's *t*-test. $p < 0.05$ was considered to be statistically significant.