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Construction and evaluation of an adenoviral vector for the liver-specific expression of the serine/arginine-rich splicing factor, SRSF3

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Abstract

Serine/arginine-rich splicing factor-3 (SRSF3), alternatively known as SRp20, is a member of the highly-conserved SR protein family of mRNA splicing factors. SRSF3 generally functions as an enhancer of mRNA splicing by binding to transcripts in a sequence-specific manner to both recruit and stabilize the binding of spliceosomal components to the mRNA. In liver, expression of SRSF3 is relatively low and its activity is increased in response to insulin and feeding a high carbohydrate diet. We sought to over-express SRSF3 in primary rat hepatocytes to identify regulatory targets. A standard adenoviral shuttle vector system containing an epitope-tagged SRSF3 under the transcriptional control of the CMV promoter could not be used to produce infectious adenoviral particles. SRSF3 over-expression in the packaging cell line prevented the production of infectious adenovirus particles by interfering with the viral splicing program. To circumvent this issue, SRSF3 expression from the shuttle vector was blocked by placing its expression under the control of the liver-specific albumin promoter. In this system, the FLAG-SRSF3 transgene is only expressed in the target cells (hepatocytes) but not in the packaging cell line. An additional benefit of the albumin promoter is that expression of the transgene does not require the addition of hormones or antibiotics to drive SRSF3 expression in the hepatocytes. Robust expression of FLAG-SRSF3 protein is detected in both HepG2 cells and primary rat hepatocytes infected with adenovirus prepared from this new shuttle vector. Furthermore, abundances of several known and suspected mRNA targets of SRSF3 action are increased in response to over-expression using this virus. This report details the construction of the albumin promoter-driven adenoviral shuttle vector, termed pmAlbAd5-FLAG.SRSF3, that can be used to generate functional adenovirus to express FLAG-SRSF3 specifically in liver. This vector would be suitable for over-expression of other splicing factors that could inhibit virus production. In addition, this vector would allow only liver-specific expression of other cargo genes when used in a whole-animal paradigm.

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Keywords

SRSF3; adenovirus; liver-specific over-expression; liver; mRNA splicing; hepatocytes

1. Introduction

Efficient transfection of target cells both *in vitro* and *in vivo* is highly desirable to address the function of specific proteins in the most physiological context. In this regard, adenoviral vectors are an extremely valuable tool for the study of gene expression in a wide variety of tissues [1-4]. Tail vein injection of adenoviral constructs can efficiently deliver a gene of interest to the liver, with measureable expression of the transgene observed as early as 24 h after injection—in stark contrast to the months required to generate transgenic animals. In both liver and hepatocytes in primary culture, the efficiency of target cell infection is greater than 95% [5]. In addition to their exceptionally high tropism, adenoviral vectors also have a relatively high packaging capacity in comparison to other virus-based gene delivery systems. Thus, adaptation of adenoviral delivery systems to permit delivery of a wide spectrum of transgenes is highly desirable.

Our laboratory has reported that expression and activity of serine/arginine-rich splicing factor-3 (SRSF3), an important pre-mRNA splicing enhancer protein, is regulated by nutrients in liver [6]. In order to better understand and define the role of SRSF3 on splicing patterns and gene expression in the liver in response to nutrients, we sought to express a N-terminal FLAG-tagged SRSF3 protein in primary rat hepatocytes using adenovirus.

The serine/arginine rich (SR) proteins are a family of *trans*-acting splicing factors that generally act as enhancers of splicing [7]. The canonical SR protein family members include SRSFs 1-12, previously known as SF2/ASF, SC35, SRp20, SRp75, SRp40, SRp55, 9G8, SRp46, SRp30c, SRp38, p54, and SRp35, respectively [8]. These proteins regulate both constitutive and alternative splicing in both a tissue- and developmentally-specific manner [9-18]. Extracellular signaling molecules, such as dietary fat, hormones, and growth factors can affect the splicing process by altering the expression, activity and/or intracellular distribution of SR proteins [19-24]. With more than 95% of human transcripts now thought to undergo alternative splicing [25], deciphering the regulatory mechanisms governing SR protein function is critical to our understanding of the role of RNA splicing in regulating physiological processes.

SRSF3 is the smallest of the 12 SR proteins, at 164 amino acids. It contains one N-terminal RNA recognition motif (RRM) and an arginine-serine (RS) dipeptide repeat domain spanning approximately 60 amino acids at the C-terminus. SR proteins are concentrated in nuclear compartments called speckles, but a few—SRSF3 included—shuttle between the nucleus and the cytoplasm to participate in non-splicing functions including mRNA transport and cap-dependent translation [17]. In mouse models, SRSF3 knockout embryos die prior to blastocyst formation, thus, while structurally similar to other SR proteins, SRSF3 is essential and functionally non-redundant [26]. Furthermore, conditional knockout of SRSF3 in mouse liver interferes with hepatocyte development and negatively affects hepatic lipogenic and cholesterol biosynthetic pathways [27]. SRSF3 is also known to play

critical roles in the production of viral particles from several families, including *picornaviridae*, *retroviridae*, *adenoviridae*, *papillomaviridae*, and *herpesviridae* [28-33].

The specific cellular functions of SRSF3 are not as extensively characterized as some other SR proteins, with only a handful of regulatory targets having been identified *in vitro*—its own transcript included [34-37]. In liver, changes in pathway activity due to lack of SRSF3 expression have not identified direct targets of this protein's splicing regulatory activity. Our laboratory recently demonstrated that splicing of the glucose-6-phosphate dehydrogenase (G6PD) mRNA is enhanced by SRSF3 in liver [6]. Because SRSF3 directly binds to G6PD mRNA in a sequence-specific manner, and SRSF3 phosphorylation and binding is increased during feeding, we sought to develop a system in which to over-express SRSF3 in primary hepatocytes and identify additional direct targets of its action.

Hepatocytes in primary culture recapitulate liver-specific traits better than cell lines or hepatomas; however, disadvantages to their use include their poor transfection efficiency and relatively short lifespan in culture (< 5 days) [38]. Adenoviral infection provides a mechanism to express proteins in > 95% of hepatocytes in as little as 24 h after infection, and can also be used in intact animals for liver-specific gene expression. This report details the construction of an adenoviral expression vector that 1) can be used to produce functional SRSF3 adenovirus in HEK-293T cells, and 2) can be used to express SRSF3 in primary rat hepatocytes. Importantly, expression of SRSF3 in this vector is driven by a tissue-specific promoter and standard adenovirus production protocols can be used to generate functional adenovirus stocks.

2. Materials & Methods

2.1 Construction of FLAG-SRSF3 shuttle vectors

Unless otherwise specified, all enzymes were purchased from New England Biolabs (Ipswich, MA).

The SRSF3-FLAG adenoviral shuttle vector was prepared using the RapAd® CMV Adenoviral Expression System (Cell Biolabs, San Diego, CA). We used an N-terminal FLAG-tagged SRSF3 cDNA (a kind gift from Dr. Rozanne Sandri-Goldin, University of California-Irvine, [30]) in a pCS2 vector backbone. To begin to excise the FLAG-SRSF3 cDNA from the pCS2.FLAG-SRSF3 plasmid, the DNA was linearized by partial digestion with *XhoI*, due to a second *XhoI* site in the coding region of SRSF3 (Fig. 1a). The pacAd5 CMV K-N pA vector was digested with *XbaI*. The ends of both *XhoI*-digested pCS2.FLAG-SRSF3 DNA and *XbaI*-digested pacAd5 CMV K-N pA DNA were blunted using DNA Polymerase I, Large (Klenow) Fragment. Both DNA fragments were then digested with *BamHI* to remove the FLAG-SRSF3 insert in the case of the pCS2.FLAG-SRSF3 vector and to provide the complementary sticky end in the pacAd5 CMV K-N pA vector and the products separated on a 1% agarose gel. The 903bp FLAG-SRSF3 insert and 6.2kb pacAd5 CMV K-N pA vector bands were excised from the gel and the DNA purified. The excised vector DNA was treated with Antarctic Phosphatase. Overnight ligations were done at ambient room temperature using an 8:1 ratio of insert to vector DNA. The identity of the

constructed vector was verified by DNA sequencing and analytical restriction digest and is henceforth referred to as pacAd5-CMV-FLAG.SRSF3 (Fig. 1a).

A second vector was constructed that contains FLAG-SRSF3 downstream of the liver-specific albumin promoter. The pLIVE vector (Mirrus Bio, Madison, WI) contains a mouse albumin promoter downstream of the mouse α -fetoprotein enhancer element followed by a multiple-cloning site (MCS). FLAG-SRSF3 was excised from the pCS2 vector backbone as described above. The resulting FLAG-SRSF3 insert was ligated into pLIVE vector that was also cut with *Bam*HI and *Xho*I. The resulting vector is referred to as pLIVE.FLAG-SRSF3 (Fig. 1b).

Final construction of the adenoviral vector required the removal of the CMV promoter from the pacAd5 CMV K-N pA shuttle vector by restriction digest with *Sbf*I and *Not*I (Fig. 1c). The digested products were separated on a 1% agarose gel and the 5.6kb fragment containing the pacAd5 vector DNA without the CMV promoter was excised and purified (Fig. 1c). DNA containing the mouse albumin promoter, α -fetoprotein enhancer and FLAG-SRSF3 was produced by amplification of the bracketed region of pLIVE.FLAG-SRSF3 (Fig. 1c) using PCR. The Gibson Assembly® kit was used to ligate the 2.3kb PCR product and the pacAd5 shuttle vector cut with *Sbf*I and *Not*I (Fig. 1c). The new shuttle vector is henceforth referred to as pmAlbAd5-FLAG.SRSF3. To prepare the empty vector control (henceforth referred to as “control vector”), the PCR primers used to amplify the bracketed region indicated in Fig. 1c were also used to amplify the pLIVE parent plasmid lacking the SRSF3-FLAG cDNA. The 1.4kb PCR product was ligated into the pacAd5 shuttle vector as above. The identities of the pmAlbAd5-FLAG.SRSF3 and control vectors were verified by DNA sequencing and analytical restriction digest.

2.2 Production of recombinant FLAG-SRSF3 adenovirus in HEK-293T cells

Production of recombinant adenovirus was an adaptation of the RapAd® CMV Adenoviral Expression System manufacturer's instructions. Approximately 2×10^6 HEK-293T cells were plated in a 60 mm tissue culture dish in 3 mL OptiMEM® (Life Sciences, Carlsbad, CA) supplemented with 4% fetal bovine serum and without antibiotics. Cells were allowed to attach to the plate overnight in a 37°C + 5% CO₂ incubator. The shuttle vectors (pacAd5-CMV-FLAG.SRSF3, empty pacAd5-CMV-pA, pacAd5-CMV.GFP (a shuttle vector containing GFP provided as part of the RapAd® system), pmAlbAd5-FLAG.SRSF3, and empty pmAlbAd5) and the pacAd5 9.2-100 Ad backbone vector (Cell Biolabs) were linearized with *Pac*I. The DNA was purified by phenol extraction followed by ethanol precipitation and was resuspended in sterile water.

HEK-293T cells were transfected with 5 μ g of linearized DNA. The appropriate shuttle vector (4 μ g) and pacAd5 9.2-100 (1 μ g) were combined in a 400 μ L-volume of serum-free OptiMEM®. FuGENE® 6 Transfection Reagent (9 μ L, Promega, Madison, WI) was added to the DNA and mixed. Complex formation was allowed to proceed for 40 min at room temperature. The HEK-293T cell medium was aspirated and replaced with 2.5 mL fresh OptiMEM® supplemented with 4% fetal bovine serum, without antibiotics. The DNA-FuGENE® 6 complexes were then added drop-wise to each plate of cells and incubated for 24 h at 37°C + 5% CO₂.

The following day, the media/transfection reagent was aspirated from the HEK-293T cell plates and replaced with 3 mL complete medium (OptiMEM® supplemented with 4% fetal bovine serum and antibiotic/antimycotic). One mL of additional fresh media was added on day 7 and on day 10 post-transfection. Crude viral lysates were harvested on day 12 post-transfection. Following amplification, virus was purified (ViraBind™ Adenovirus Purification Kit, Cell Biolabs) and titered (QuickTiter™ Adenovirus Titer ELISA Kit, Cell Biolabs). Adenovirus produced using the pmAlbAd5-FLAG.SRSF3 shuttle vector DNA is referred to as “albumin FLAG-SRSF3 adenovirus,” and adenovirus produced using the pmAlbAd5 empty vector DNA is referred to as “control adenovirus.”

2.3 Adenoviral infection of HepG2 cells

One day prior to infection, 5×10^6 HepG2, human hepatoma cells (ATCC, Manassas, VA) were seeded in 100 mm tissue culture dishes in 10 mL serum-free Minimum Essential Media (MEM, Gibco®). The next day, the medium was aspirated, replaced with fresh MEM containing adenovirus at the titers indicated in the figure legends and incubated at 37°C + 5% CO₂. After 2 h, the infection medium was removed and replaced with 10 mL complete medium (MEM supplemented with 10% fetal bovine serum and antibiotic/antimycotic). Whole-cell extracts and RNA were collected 48 h later. This procedure is defined as one experiment; replicate experiments represent the same procedure but performed on different days.

2.4 Adenoviral infection of primary rat hepatocytes

All animal experiments were conducted in conformity with the Public Health Service policy on Human Care and Use of Laboratory Animals. Additionally, the Institutional Animal Care and Use Committee of the Division of Laboratory Animal Resources at West Virginia University approved all experimental procedures.

Male Sprague-Dawley rats (180-200 g, Harlan Laboratories, Indianapolis, IN) were fed a standard chow diet (Harlan Teklad). Rats were starved for 18 h prior to hepatocyte isolation. Hepatocyte isolation and incubation in primary culture was as previously described [39].

Two different infection protocols were tested. In the first, following a 2 h attachment period at 37°C + 5% CO₂, the hepatocytes were washed twice with 1× HiWoBa medium to remove the newborn calf serum used for cell attachment, and then 1.5 mL serum-free 1× HiWoBa was added. Purified adenovirus was added dropwise to each plate at the multiplicities of infection (MOIs) indicated in the figure legends and incubated at 37°C + 5% CO₂. After a 2 h infection period, the virus-containing media was removed and replaced with a Matrigel overlay (0.3 mg/mL) in 1× HiWoBa and incubated overnight. The next morning, the cells were washed twice with 1× HiWoBa medium, and then incubated in 1× HiWoBa medium containing 80 nM insulin for 48 h. Medium was replenished at 24 h, and total RNA and whole-cell extracts were collected 48 h after the addition of insulin.

In the second protocol, a 4 h cell attachment period was used. After the 4 h attachment, cells were washed twice, purified adenovirus was added dropwise to each plate as described above, and incubated for an additional 6 h. The second protocol matched the first from this point forward.

3. Results & Discussion

To discover new targets of SRSF3 in liver, we adopted the approach of adenoviral over-expression to produce tagged SRSF3. Using standard techniques, FLAG-SRSF3 was sub-cloned into an adenoviral shuttle vector (pacAd5) and the linearized vector was transfected into HEK-293T cells. Unexpectedly, HEK-293T cells transfected with pacAd5-CMV-FLAG.SRSF3 (Fig. 1a) failed to produce virus, while cells transfected with control vector (pacAd5 CMV K-N pA or pacAd5-CMV.GFP) produced virus with titers of 7.0×10^9 and 5.5×10^{10} ifu/mL, respectively. Increasing the ratio of shuttle vector DNA to backbone from 3:1 to 2:1 also did not yield measurable viral titers. To determine if the problem was transfection efficiency or viral production, Western analysis was performed on cell lysates using two different ratios of shuttle vector to backbone. In both cases, FLAG-SRSF3 was detected (Fig. 2a, lanes 2 and 3) and its abundance was greater than 1.5-fold over endogenous SRSF3 as measured with the endogenous SRSF3 antibody. This indicated that transfection was occurring and FLAG-SRSF3 was robustly expressed in HEK-293T cells. Thus, the lack of virus production was not due to problems with transfection efficiency.

The expression of adenovirus genes is tightly regulated by changes in pre-mRNA splicing patterns [40]. Distinct mRNA species accumulate at specific times during adenovirus infection depending on viral splice-site selection. Splice-site switching is a temporally regulated process that occurs when the viral splicing program switches from producing transcripts that encode the early viral proteins—those involved in suppression of the host immune response and replication of the viral genome—to transcripts that encode the late viral proteins—proteins involved in packaging of the newly-produced virus [40, 41]. To accomplish this, the viral splicing machinery favors proximal splice sites during early infection, and distal splice sites at later time points. As a result, the viral transcription units produce alternatively spliced RNA transcripts that are translated into functionally distinct proteins [42]. Previous studies have shown that splice-site selection is significantly influenced by the balance of active SR proteins present in host cells [41]. Over-expression of the SR protein, SRSF1, in HEK-293T cells impairs virus production by inhibiting the proximal-to-distal splice site switch in the early viral E1A and E1B transcription units, resulting in decreased accumulation of mRNA necessary for late viral protein (L1) expression [41]. These studies, coupled with the over-expression of CMV-driven SRSF3 in HEK-293T cells in our hands (Fig. 2a, lanes 2 and 3), are consistent with the hypothesis that SRSF3 may be inhibiting production of functional adenovirus.

We considered several alternative approaches to circumvent these issues. The first was to use a lentivirus expressing a myc-tagged SRSF3. We were able to produce this lentivirus in HEK-293T cells, but primary rat hepatocytes are exceptionally resistant to lentiviral infection ([38, 43] and data not shown). Another potential alternative we considered was to use an adeno-associated vector (AAV), but AAV systems also require splice-site switching [44] and thus we would likely encounter the same issues.

Potentially, functional adenovirus would be produced if we blocked SRSF3 over-expression in the HEK-293T packaging cells. To accomplish this, we constructed an adenovirus shuttle vector in which the CMV promoter is replaced with a liver-specific promoter (Fig. 1c). In

this vector (pmAlbAd5-FLAG.SRSF3), expression of FLAG-SRSF3 is driven by the mouse albumin promoter downstream of the mouse α -fetoprotein enhancer. HEK-293T cells transfected with this vector do not express FLAG-SRSF3 (Fig. 2b, lane 1). Adenovirus was produced at titers ranging from 1.1×10^{10} – 2.9×10^{10} ifu/mL for both pmAlbAd5-FLAG.SRSF3 (albumin FLAG-SRSF3 adenovirus) and pmAlbAd5 only (control adenovirus).

To ensure that our newly-produced adenovirus would express FLAG-SRSF3 in liver cells, we first infected a human hepatoma cell line, HepG2. FLAG-SRSF3 was robustly expressed in the HepG2 cells and its expression was increased 3-fold over endogenous SRSF3 (Fig. 3, lane 1, top panel). Cells infected with control adenovirus or uninfected cells expressed only the endogenous SRSF3 (Fig. 3, lanes 2 and 3, top and middle panels).

We then infected primary rat hepatocytes with albumin FLAG-SRSF3 or control adenoviruses at MOIs of 5, 10, and 15. In hepatocytes infected with albumin FLAG-SRSF3 adenovirus, we observed a dose-dependent increase in FLAG-SRSF3 protein (Figs. 4a and b). The increase in expression was only 15% and 30% more with MOIs of 10 and 15, respectively, as compared to an MOI of 5 (Fig. 4b, left graph). Expression of FLAG-SRSF3 was not detected in the uninfected cells or in cells infected with the control adenovirus (Fig. 4a, right panel, and Fig. 4b, left graph). Expression of endogenous SRSF3 decreased approximately 20% in hepatocytes infected with albumin FLAG-SRSF3 adenovirus at an MOI of 5, but expression decreased with increasing MOI to an amount similar to cells that did not receive virus or received the control virus (Fig. 4a and b, right panel). Total SRSF3 mRNA abundance reflects both the endogenous mRNA and FLAG-SRSF3 mRNA.

SRSF3 expression is known to be tightly controlled by an auto-regulatory mechanism [37, 45]. Increased SRSF3 protein expression promotes the inclusion of exon 4 in the SRSF3 mRNA transcript. SRSF3 exon 4 is known as a “poison exon” because inclusion of this exon creates an in-frame premature termination codon in the transcript, ultimately decreasing the amount of functional SRSF3 protein in the cell [37]. Since the pmAlbAd5-FLAG.SRSF3 shuttle vector contains SRSF3 cDNA lacking exon 4, it is not affected by this negative feedback mechanism. Expression of the FLAG-SRSF3 protein from the albumin FLAG-SRSF3 adenovirus is likely triggering the natural negative feedback loop, thus promoting exon 4 inclusion in the endogenous SRSF3 mRNA and causing the decrease in protein abundance observed in Figure 4.

SRSF3 regulates the splicing and expression of several genes involved in intermediary metabolism [6, 14, 27]. We next measured the activity of the FLAG-SRSF3 at enhancing expression of these targets. SRSF3 binds to exon 12 of the G6PD transcript, enhancing splicing of this exon and causing an increase in the abundance of G6PD mRNA [6]. Over-expression of SRSF3 by the albumin FLAG-SRSF3 adenovirus in primary rat hepatocytes increased G6PD mRNA abundance 2.2-fold over both uninfected and control adenovirus infected cells (Fig. 5a).

In mice with a liver-specific SRSF3 knockout, several pathways of intermediary metabolism were found to be disrupted in liver, including both glucose production and glucose

deposition, and decreases in lipogenesis and cholesterol biosynthesis [14, 27]. In this regard, SREBP-1 is a well-described transcription factor that enhances hepatic lipid metabolism [46], and we measured total SREBP-1 mRNA to determine if SRSF3 over-expression affected the expression of SREBP-1 mRNA. Interestingly, we found that SREBP-1 mRNA was increased approximately 2.5-fold in albumin FLAG-SRSF3 adenovirus-infected hepatocytes relative to uninfected and control adenovirus-infected hepatocytes (Fig. 5b). The PCR primers used to measure SREBP-1 mRNA do not distinguish between SREBP-1a and SREBP-1c isoforms, but the SREBP-1a isoform is much less abundant in hepatocytes [46]. In addition, we measured the abundance of Malic Enzyme (ME1) mRNA, an enzyme that represents an important link between glucose and lipid metabolic pathways in liver. We found that the amount of ME1 mRNA was increased 1.6-fold in hepatocytes infected with albumin FLAG-SRSF3 adenovirus as compared to uninfected and control infected cells (Fig. 5c). The effect of SRSF3 over-expression via the albumin FLAG-SRSF3 adenovirus was specific to distinct transcripts, as the amounts of Nuclear Factor κ -Light Chain Enhancer of Activated B Cells (NF- κ B) and β -glucuronidase (GusB) mRNA did not change (Figs. 5d-e). Taken together, our results are consistent with the decreased lipogenesis in the livers of SRSF3 knockout mice and are supportive of the role of SRSF3 in regulating hepatic lipid metabolism.

4. Conclusions

The primary goal of our laboratory has been to understand the mechanisms by which nutritional cues regulate gene expression in liver at the level of mRNA processing—specifically mRNA splicing. We discovered that the splicing activity of SRSF3 is regulated by nutrients and hormones in liver [6], thus we sought to identify additional targets of SRSF3-regulated splicing. Adenovirus is an effective tool to express transgenes in hard-to-transfect cells, such as primary rat hepatocytes. Expression of the splicing factor, SRSF3, challenged the use of this system because the splicing factor abrogated the production of functional viral particles by the packaging cells (HEK-293T). Use of the liver-specific promoter from the albumin gene circumvented this problem because this promoter is inactive in HEK-293T cells (Fig. 2b), but is active in both primary hepatocytes and hepatoma cells (Figs. 3 and 4). The albumin promoter has a further advantage in the target cells in that it does not require the addition of exogenous compounds to drive transgene expression in the liver. Inducible promoter systems often require addition of hormones and/or antibiotics to the culture media that could have undesirable pleiotropic effects in primary hepatocytes, confounding the interpretation of changes in target gene expression. We have confirmed that the FLAG-SRSF3 protein expressed by this adenovirus is active in the hepatocytes (Figs. 4 and 5). In this regard, over-expression of SRSF3 increased the abundance of transcripts for 3 key proteins in hepatic lipid metabolism: G6PD, SREBP1, and ME1 mRNA (Figs. 5a-c), while expression of two unrelated transcripts, NF- κ B and GusB, remained unchanged (Figs. 5d-e). The adenoviral vector described herein is, to our knowledge, the first to enable over-expression of SRSF3 in primary hepatocytes, and thus represents a novel tool to study the activity of SRSF3 and its critical role in the regulation of hepatic metabolic pathways.

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Abbreviations

SRSF3	serine/arginine-rich splicing factor-3
SR protein	serine/arginine-rich protein
RRM	RNA recognition motif
RS domain	arginine-serine repeat domain
G6PD	glucose-6-phosphate dehydrogenase
MOI	multiplicity of infection
AAV	adeno-associated virus
SREBP-1	sterol regulatory element-binding protein 1
ME1	malic enzyme 1
NF-κB	nuclear factor κ-light chain enhancer of activated B cells
GusB	β- glucuronidase

References

1. Rosenfeld MA, et al. Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium in vivo. *Science*. 1991; 252(5004):431–4. [PubMed: 2017680]
2. Alemany R, Suzuki K, Curiel DT. Blood clearance rates of adenovirus type 5 in mice. *J Gen Virol*. 2000; 81(Pt 11):2605–9. [PubMed: 11038370]
3. Sen A, et al. Terminally differentiated neonatal rat myocardial cells proliferate and maintain specific differentiated functions following expression of SV40 large T antigen. *J Biol Chem*. 1988; 263(35): 19132–6. [PubMed: 3198615]
4. Gomez-Foix AM, et al. Adenovirus-mediated transfer of the muscle glycogen phosphorylase gene into hepatocytes confers altered regulation of glycogen metabolism. *J Biol Chem*. 1992; 267(35): 25129–34. [PubMed: 1334082]
5. Schiedner G, et al. Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat Genet*. 1998; 18(2):180–3. [PubMed: 9462752]
6. Walsh CM, et al. Serine arginine splicing factor 3 is involved in enhanced splicing of glucose-6-phosphate dehydrogenase RNA in response to nutrients and hormones in liver. *J Biol Chem*. 2013; 288(4):2816–28. [PubMed: 23233666]
7. Long JC, Caceres JF. The SR protein family of splicing factors: master regulators of gene expression. *Biochem J*. 2009; 417(1):15–27. [PubMed: 19061484]
8. Manley JL, Krainer AR. A rational nomenclature for serine/arginine-rich protein splicing factors (SR proteins). *Genes Dev*. 24(11):1073–4. [PubMed: 20516191]
9. Das R, et al. SR proteins function in coupling RNAP II transcription to pre-mRNA splicing. *Mol Cell*. 2007; 26(6):867–81. [PubMed: 17588520]

10. Fu XD. Specific commitment of different pre-mRNAs to splicing by single SR proteins. *Nature*. 1993; 365(6441):82–5. [PubMed: 8361546]
11. Sanford JR, et al. A novel role for shuttling SR proteins in mRNA translation. *Genes Dev*. 2004; 18(7):755–68. [PubMed: 15082528]
12. Sanford JR, et al. Splicing factor SFRS1 recognizes a functionally diverse landscape of RNA transcripts. *Genome Res*. 2009; 19(3):381–94. [PubMed: 19116412]
13. Sen S, Jumaa H, Webster NJ. Splicing factor SRSF3 is crucial for hepatocyte differentiation and metabolic function. *Nat Commun*. 2013; 4:1336. [PubMed: 23299886]
14. Sen S, Talukdar I, Webster NJ. SRp20 and CUG-BP1 modulate insulin receptor exon 11 alternative splicing. *Mol Cell Biol*. 2009; 29(3):871–80. [PubMed: 19047369]
15. Tacke R, Manley JL. The human splicing factors ASF/SF2 and SC35 possess distinct, functionally significant RNA binding specificities. *EMBO J*. 1995; 14(14):3540–51. [PubMed: 7543047]
16. Tacke R, Manley JL. Determinants of SR protein specificity. *Curr Opin Cell Biol*. 1999; 11(3):358–62. [PubMed: 10395560]
17. Twyffels L, Gueydan C, Krays V. Shuttling SR proteins: more than splicing factors. *FEBS Journal*. 278(18):3246–3255. [PubMed: 21794093]
18. Wang J, Manley JL. Overexpression of the SR proteins ASF/SF2 and SC35 influences alternative splicing in vivo in diverse ways. *RNA*. 1995; 1(3):335–46. [PubMed: 7489505]
19. Busch A, Hertel KJ. Evolution of SR protein and hnRNP splicing regulatory factors. *Wiley Interdisciplinary Reviews: RNA*. 2011
20. Caceres JF, et al. Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. *J Cell Biol*. 1997; 138(2):225–38. [PubMed: 9230067]
21. Graveley, BR.; Hertel, KJ. SR Proteins, in eLS. John Wiley & Sons, Ltd.; 2001.
22. Misteli T, et al. Serine phosphorylation of SR proteins is required for their recruitment to sites of transcription in vivo. *J Cell Biol*. 1998; 143(2):297–307. [PubMed: 9786943]
23. Blaustein M, et al. Concerted regulation of nuclear and cytoplasmic activities of SR proteins by AKT. *Nat Struct Mol Biol*. 2005; 12(12):1037–44. [PubMed: 16299516]
24. Stamm S. Regulation of alternative splicing by reversible protein phosphorylation. *J Biol Chem*. 2008; 283(3):1223–7. [PubMed: 18024427]
25. Tejedor JR, Valcarcel J. Gene regulation: Breaking the second genetic code. *Nature*. 2010; 465(7294):45–46. [PubMed: 20445621]
26. Jumaa H, Wei G, Nielsen PJ. Blastocyst formation is blocked in mouse embryos lacking the splicing factor SRp20. *Curr Biol*. 1999; 9(16):899–902. [PubMed: 10469594]
27. Sen S, Jumaa H, Webster NJG. Splicing factor SRSF3 is crucial for hepatocyte differentiation and metabolic function. *Nat Commun*. 4:1336. [PubMed: 23299886]
28. Fitzgerald KD, Semler BL. Re-localization of cellular protein SRp20 during poliovirus infection: bridging a viral IRES to the host cell translation apparatus. *PLoS Pathog*. 7(7):e1002127. [PubMed: 21779168]
29. Verma D, et al. Epstein-Barr Virus SM protein utilizes cellular splicing factor SRp20 to mediate alternative splicing. *J Virol*. 84(22):11781–9. [PubMed: 20810723]
30. Sciabica KS, Dai QJ, Sandri-Goldin RM. ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation. *EMBO J*. 2003; 22(7):1608–19. [PubMed: 12660167]
31. Escudero-Paunetto L, et al. SR proteins SRp20 and 9G8 contribute to efficient export of herpes simplex virus 1 mRNAs. *Virology*. 2010; 401(2):155–64. [PubMed: 20227104]
32. Wong RW, et al. Digoxin suppresses HIV-1 replication by altering viral RNA processing. *PLoS Pathog*. 2013; 9(3):e1003241. [PubMed: 23555254]
33. Jia R, et al. Control of the papillomavirus early-to-late switch by differentially expressed SRp20. *J Virol*. 2009; 83(1):167–80. [PubMed: 18945760]
34. Liang H, Tuan RS, Norton PA. Overexpression of SR proteins and splice variants modulates chondrogenesis. *Exp Cell Res*. 2007; 313(8):1509–17. [PubMed: 16140295]

35. Galiana-Arnoux D, et al. The CD44 alternative v9 exon contains a splicing enhancer responsive to the SR proteins 9G8, ASF/SF2, and SRp20. *J Biol Chem*. 2003; 278(35):32943–53. [PubMed: 12826680]
36. Lim LP, Sharp PA. Alternative splicing of the fibronectin EIIIB exon depends on specific TGCATG repeats. *Mol Cell Biol*. 1998; 18(7):3900–6. [PubMed: 9632774]
37. Jumaa H, Nielsen PJ. The splicing factor SRp20 modifies splicing of its own mRNA and ASF/SF2 antagonizes this regulation. *EMBO J*. 1997; 16(16):5077–85. [PubMed: 9305649]
38. Gao S, et al. Ex vivo gene delivery to hepatocytes: techniques, challenges, and underlying mechanisms. *Ann Biomed Eng*. 2012; 40(9):1851–61. [PubMed: 22484829]
39. Tao H, et al. Inhibition of the splicing of glucose-6-phosphate dehydrogenase precursor mRNA by polyunsaturated fatty acids. *J Biol Chem*. 2002; 277(34):31270–8. [PubMed: 12072438]
40. Imperiale MJ, Akusjarvi G, Leppard KN. Post-transcriptional control of adenovirus gene expression. *Curr Top Microbiol Immunol*. 1995; 199(Pt 2):139–71. [PubMed: 7555066]
41. Molin M, Akusjarvi G. Overexpression of essential splicing factor ASF/SF2 blocks the temporal shift in adenovirus pre-mRNA splicing and reduces virus progeny formation. *J Virol*. 2000; 74(19):9002–9. [PubMed: 10982344]
42. Molin M, et al. Unscheduled expression of capsid protein IIIa results in defects in adenovirus major late mRNA and protein expression. *Virus Res*. 2002; 83(1-2):197–206. [PubMed: 11864752]
43. Seppen J, et al. Lentiviral vectors for efficient transduction of isolated primary quiescent hepatocytes. *J Hepatol*. 2002; 36(4):459–65. [PubMed: 11943415]
44. Mouw MB, Pintel DJ. Adeno-associated virus RNAs appear in a temporal order and their splicing is stimulated during coinfection with adenovirus. *J Virol*. 2000; 74(21):9878–88. [PubMed: 11024114]
45. Jumaa H, Nielsen PJ. Regulation of SRp20 exon 4 splicing. *Biochim Biophys Acta*. 2000; 1494(1-2):137–43. [PubMed: 11072076]
46. Horton JD, Goldstein JL, Brown MS. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *J Clin Invest*. 2002; 109(9):1125–31. [PubMed: 11994399]

HIGHLIGHTS

- Splicing factor SRSF3 blocks production of adenovirus in HEK-293T cells.
- SRSF3 expression in HEK-293T cells is blocked by using the albumin promoter.
- SRSF3-albumin adenovirus can be produced.
- Hepatocytes infected with the SRSF3-albumin adenovirus over-express SRSF3.
- SRSF3 over-expression increases the abundance of target RNAs.

FIGURE 1. Cloning schemes used to construct adenoviral shuttle vectors

A) Construction of *pacAd5-CMV-FLAG.SRSF3*, B) Construction of *pLIVE-FLAG.SRSF3*, and C) Construction of *pmAlbAd5-FLAG.SRSF3*. Note: drawings are not to scale.

FIGURE 2. CMV-driven FLAG-SRSF3 is highly expressed in HEK293T packaging cells while albumin-driven FLAG-SRSF3 is not

A) Western blot of HEK-293T transfected with either a 3:1 (lane 2) or 2:1 (lane 3) ratio of pacAd5-CMV-FLAG.SRSF3 shuttle vector DNA to pacAd5 9.2-100 backbone DNA, or transfection reagent alone (lane 4). Whole-cell extracts were collected 48 h after transfection. Lysate (10 μ g protein) was loaded onto 12% SDS-PAGE gels for Western analysis and probed sequentially with anti-FLAG M2 (1:2000), followed by anti-SRSF3 (1:5000) primary antibodies. **B)** HEK-293T cells were transfected with pmAlbAd5-FLAG.SRSF3 vector DNA (lane 2), pmAlbAd5 control vector DNA (lane 3), or transfection reagent alone (lane 4). Whole-cell extracts were collected 48 h after transfection. Lysate (10 μ g protein) was loaded onto a 12% SDS-PAGE gel for Western analysis and probed with the same antibodies as in A).

FIGURE 3. FLAG-SRSF3 is expressed in HepG2 cells infected with albumin FLAG-SRSF3 adenovirus

Representative Western blots of $n = 2$ separate experiments. HepG2 cells were infected with albumin FLAG-SRSF3 adenovirus (lane 1), control (empty vector) adenovirus (lane 2), or no virus (lane 3) for 2 h at a MOI of 10. Whole-cell extracts were collected 48 h after infection. Lysate (10 μ g protein) was loaded onto 12% SDS-PAGE gels for Western analysis. Membranes were probed with the following antibodies: *top panel*: anti-SRSF3 (1:5000), *middle panel*: anti-FLAG (1:2000), *lower panel*: anti- β -tubulin (1:4000).

FIGURE 4. FLAG-SRSF3 is detected in primary rat hepatocytes infected with albumin FLAG-SRSF3 adenovirus

Primary hepatocytes were infected with albumin FLAG-SRSF3 adenovirus, control (empty vector) adenovirus, or no virus for 4 h at MOIs of 5, 10, or 15. Whole-cell extracts and total RNA were collected 48 h after infection. A) Representative Western blots of $n = 3$ separate experiments. Lysate (10 μ g protein) was loaded onto 12% SDS-PAGE gels for Western analysis and probed sequentially with anti-SRSF3 (1:5000) followed by anti-FLAG M2 (1:2000), and anti- β -tubulin (1:4000) primary antibodies. B) Densitometric quantitation of $n = 3$ experiments represented in 4A. The * asterisk indicates $p < 0.05$. Error bars indicate $\pm$ SEM.

FIGURE 5. Overexpression of FLAG-SRSF3 regulates mRNA abundance of mRNA targets
 Primary rat hepatocytes were infected with albumin FLAG-SRSF3 adenovirus, control (empty vector) adenovirus, or no virus for 4 h at a MOI of 10 and total RNA was collected 48 h later. A-E) The mRNA abundance of each transcript was measured using real-time PCR. Fold changes were calculated using the C_t method with cyclophilin as the endogenous control and expressed relative to the No Virus control. The * asterisk indicates $p < 0.05$ and error bars represent SEM of $n = 3$ separate experiments.