A Splicing-Independent Function of SF2/ASF in MicroRNA Processing

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SUMMARY

Both splicing factors and microRNAs are important regulatory molecules that play key roles in post-transcriptional gene regulation. By miRNA deep sequencing, we identified 40 miRNAs that are differentially expressed upon ectopic overexpression of the splicing factor SF2/ASF. Here we show that SF2/ASF and one of its upregulated microRNAs (miR-7) can form a negative feedback loop: SF2/ASF promotes miR-7 maturation, and mature miR-7 in turn targets the 3’UTR of SF2/ASF to repress its translation. Enhanced microRNA expression is mediated by direct interaction between SF2/ASF and the primary miR-7 transcript to facilitate Drosha cleavage and is independent of SF2/ASF’s function in splicing. Other miRNAs, including miR-221 and miR-222, may also be regulated by SF2/ASF through a similar mechanism. These results underscore a function of SF2/ASF in pri-miRNA processing and highlight the potential coordination between splicing control and miRNA-mediated gene repression in gene regulatory networks.

INTRODUCTION

MicroRNAs (or miRNAs) are small noncoding RNAs of great importance in posttranscriptional gene regulation. With few known exceptions (Vasudevan et al., 2007), miRNAs typically bind to the 3’ untranslated regions (UTRs) of protein-coding genes and repress their expression by translational inhibition and/or promoting mRNA degradation. It is estimated that each miRNA can have hundreds of potential functional targets (Bartel, 2004); thus, miRNAs are broadly involved in diverse cellular processes to establish and/or maintain cell identity (Alvarez-Garcia and Miska, 2005). Alterations in miRNA expression often lead to severe pathological consequences and are frequently observed in human diseases (Esquela-Kerscher and Slack, 2006; Lee and Dutta, 2009; He et al., 2007).

MiRNAs are primarily transcribed by RNA polymerase II as long primary transcripts called pri-miRNAs. Pri-miRNAs are first processed in the nucleus by an RNase III-like enzyme, Drosha, to liberate ~70 nt miRNA precursors (pre-miRNAs). Pre-miRNAs are then exported to the cytoplasm, where they are further cleaved by Dicer to produce mature miRNAs, which are subsequently loaded into an RNA-induced-silencing complex (RISC) to guide downstream gene repression (Bartel, 2004). Emerging evidence suggests that miRNA processing is a highly regulated event. For instance, multiple pri-miRNAs are detected in tumor cells but are not processed to precursor or mature miRNAs (Thomson et al., 2006). It has also been shown that Drosha cleavage is initiated cotranscriptionally (Morlando et al., 2008), and retention of pri-miRNAs at the transcriptional sites enhances their conversion to pre-miRNAs (Pawlacki and Steitz, 2008). Several RNA-binding proteins have been implicated in regulating pri-miRNA maturation (Guil and Caceres, 2007; Trabucchi et al., 2009); however, the detailed mechanisms remain elusive.

Pre-mRNA splicing, including regulated alternative splicing, is another layer of eukaryotic gene regulation. More than 90% of human genes might be alternatively spliced (Wang et al., 2008); in fact, alternative splicing is one major contributor to mammalian proteome diversity (Lander et al., 2001; Maniatis and Tasic, 2002). Intron removal is catalyzed by the spliceosome, a macromolecular complex that consists of 5 different snRNAs and over 300 associated proteins (Wahl et al., 2009). Among them, SR proteins are a family of key splicing factors involved in both constitutive and alternative splicing. In addition to splicing regulation, SR proteins are also involved in diverse events during the life cycle of mRNAs, including transcription elongation, mRNA export, nonsense-mediated mRNA decay, and translational regulation (Long and Caceres, 2009).

We show here that SF2/ASF, a prototypical SR protein splicing factor encoded by the SFRS1 gene (Ge and Manley, 1990; Krainer et al., 1990), is intimately involved in pri-miRNA processing. We found that SF2/ASF and miR-7 can form a negative feedback circuit: SF2/ASF promotes the maturation of miR-7, which negatively regulates SFRS1 expression at the translational level. We further show that SF2/ASF directly binds to pri-miR-7 and has a splicing-independent function to enhance Drosha cleavage. Lastly, SF2/ASF-enhanced miRNA production is not limited to miR-7, suggesting that SF2/ASF might be a key regulator that coordinates splicing regulation and miRNA-mediated gene repression.
RESULTS

Identification of SFRS1-miR-7 Feedback Circuit
SF2/ASF is a well-known splicing factor involved in both constitutive and alternative splicing. Given the functional importance of SF2/ASF in tissue- and cell-type-specific splicing, the expression level of its gene, SFRS1, has to be tightly regulated (Grosso et al., 2008; Kari et al., 2007). It is known that SF2/ASF can negatively regulate its own expression, and several mechanisms are involved at the posttranscriptional and translational steps. First of all, SF2/ASF regulates its own splicing to generate unproductive mRNA isoforms (Lareau et al., 2007; Ni et al., 2007; Sun et al., 2008; Karni et al., 2007). It is known that SF2/ASF can decrease the mature miR-7 level (p < 0.05, Figure 1C). Taken together, these data suggest that endogenous SF2/ASF and miR-7 potentially form a negative feedback circuit.

SF2/ASF Is Required for Efficient Production of miR-7
As a logical step to validate the SF2/ASF-miR-7 circuit, we first examined whether miR-7 expression can indeed be enhanced by elevated SF2/ASF levels. Northern blotting was performed with the inducible HeLa cell line (Figure 1B and data not shown). The results agreed well with the miRNA profiling data (~2-fold change). Three additional upregulated miRNAs (miR-29b, miR-221, and miR-222) were also validated (Figure S1F). Together, these data suggest that deep sequencing is a reliable method to detect differentially expressed miRNAs.

In addition, loss-of-function analysis was carried out by interfering RNAs (siRNAs) against either luciferase or SFRS1. Two days after transfection, radiolabeled RT-PCR and western blotting were used to monitor the SFRS1 mRNA level (top two panels; GAPDH as an internal control) and protein level (middle two panels; α-tubulin as an internal control), respectively. Northern blotting analysis of endogenous miR-7 level with U6 as an internal control (bottom two panels). Results from triplicate experiments are plotted in the right panel. The * indicates p < 0.05 (t test, n = 3). Error bar represents SEM.

Figure 1. Differentially Expressed miRNAs upon SF2/ASF Induction
(A) Normalized counts of individual miRNAs at 24, 48, or 72 hr after SF2/ASF induction were compared to that of cells without induction. Differentially expressed miRNAs (>1.5-fold change and q < 0.01) are shown, and z scores are plotted in the heat map. (B) Northern blotting analysis of mature miR-7 in the stable HeLa cell line with inducible SF2/ASF expression. U6 snRNA was used as an internal control. The changes in relative miR-7 expression levels are shown at the bottom of the panel. (C) HeLa cells were transfected with small interfering RNAs (siRNAs) against either luciferase or SFRS1. Two days after transfection, radiolabeled RT-PCR and western blotting were used to monitor the SFRS1 mRNA level (top two panels; GAPDH as an internal control) and protein level (middle two panels; α-tubulin as an internal control), respectively. Northern blotting analysis of endogenous miR-7 level with U6 as an internal control (bottom two panels). Results from triplicate experiments are plotted in the right panel. The * indicates p < 0.05 (t test, n = 3). Error bar represents SEM.
SFRS1 Is a Physiological Target of miR-7

We next examined whether SFRS1 mRNA is a bona fide target of miR-7. Dual luciferase assay was initially used to confirm the target relationship. TargetScan predicts that the 3′ UTR of SFRS1 has a highly conserved seed match (m6+u1A) for miR-7 (Figure 2A). We cloned the putative target site and its surrounding region into a Firefly luciferase reporter (Figure 2B). The resulting construct or the empty vector control was cotransfected into HeLa cells together with synthetic miR-7 precursors or scrambled RNAs. A Renilla luciferase construct was also transfected into HeLa cells together with synthetic miR-7 precursors. Two days after transfection, the levels of mature miR-7 and endogenous SFRS1 mRNA were quantified by northern (U6 as internal control; top two panels) and western blotting (α-tubulin as an internal control; bottom two panels), respectively. The relative levels of S2/ASF protein from three separate transfections are plotted (t test, p < 0.05; middle panel). Quantitative RT-PCR results of endogenous SFRS1 mRNA in the transfected cells are shown in the right panel; GAPDH mRNA was used as an internal control. Error bars represent SEM.

The Domain Requirements of SF2/ASF for Promoting miR-7 Expression

We next sought to characterize the molecular mechanism underlying SF2/ASF-enhanced miR-7 expression. Mature miR-7 is encoded by three distinct loci in the human genome. One of them, hsa-miR-7-1, is embedded in the last intron of the hnRNPK gene, which is alternatively spliced via two duplicated 3′ UTRs. The reciprocal targeting between SF2/ASF and miR-7 confirmed that they can potentially form a negative feedback loop. SF2/ASF promotes the production of miR-7, which in turn targets SFRS1 mRNA to repress its translation. The circuit structure implies that miR-7 might in part contribute to the negative feedback regulation observed for SF2/ASF. Conversely, SF2/ASF may play a role in maintaining the steady-state level of mature miR-7, an important regulatory molecule involved in diverse cellular processes (see the Discussion).
and distal variants, without affecting the overall mRNA level (data not shown). Because splicing efficiency could affect the processing of intronic miRNAs (Pawlicki and Steitz, 2008), one attractive model is that SF2/ASF might enhance miR-7 production through alternative splicing regulation.

To test this hypothesis, we constructed a minigene in which the miR-7-containing intron and its flanking exons were inserted downstream of the EGFP open reading frame (Figure 3A). The use of a minigene avoids potential complications due to multiple endogenous miR-7 loci and unforeseen transcriptional or post-transcriptional regulations. When transfected into HeLa cells, the hnRNPK minigene produced both mature miR-7 and the two expected splicing variants, of which the distal 3′ ss was preferred (Figure S3; Figure 3B, lane 1). Cotransfection of SF2/ASF cDNA increased the level of mature miR-7 (1.7-fold) as well as the ratio between proximal and distal splicing variants (Figure 3B, lanes 1 and 2), indicating that the minigene can recapitulate the effects of SF2/ASF at the endogenous hnRNPK locus. Similar results were obtained for an SF2/ASF variant with a nuclear retention signal (SF2-NRS) (Cazalla et al., 2002), except that it was even more active in promoting miR-7 production (2.4-fold; Figures 3B and 3C, lanes 1–3). Moreover, the level of miR-7 precursors was also increased (Figure S3D), suggesting that enhanced miR-7 expression is likely to take place at the nuclear processing step (Drosha cleavage).

To further dissect the domain requirement of SF2/ASF for promoting miR-7 expression, three mutants (SF2ΔRS, SF2ΔRRM1, and SF2ΔRRM2) were examined (Caceres and Krainer, 1993). All mutants failed to promote proximal splice-site usage, indicating that both the RS domain and the RNA-recognition motifs are required for the splicing regulation. In contrast, SF2ΔRS reproducibly repressed miR-7 expression to two-thirds of its normal level (p < 0.05), whereas deletion of RRM1 or RRM2 rendered SF2/ASF inactive in promoting miR-7 production (Figure 3B, lane 1 and lanes 4–6; Figure 3C). The dominant-negative effect of SF2ΔRS suggests a previously uncharacterized function of SF2/ASF in miRNA biogenesis, which is separable from its activities in splicing regulation.

Two additional SR proteins, SFRS2 (SC35) and SFRS7 (9G8), were also tested. Despite the fact that both SR proteins showed much stronger activation of proximal splicing than SF2/ASF, their activities in miR-7 promotion were not correspondingly increased (Figure 3, lanes 7 and 8). Notably, 9G8 had little effect on miR-7 expression, suggesting that different SR proteins may have distinct substrate specificities in regulating miRNA expression. In agreement with the observations above, these results demonstrate that enhanced miR-7 expression is unlikely due to increased proximal splice-site usage, indicating that there are two separate functions of SF2/ASF, in alternative splicing and in miRNA processing.

A Splicing-Independent Function of SF2/ASF in Pri-miR-7 Processing

To uncouple SF2/ASF’s functions in alternative splicing and miRNA processing, we mutated the proximal or distal splice site of the hnRNPK minigene (PM or DM). When transfected into HeLa cells, the two mutants gave rise to opposite splicing patterns (Figure 4A, lanes 1, 3, and 5). Notably, we could detect both proximal and distal variants, due to background expression of the endogenous hnRNPK gene, which contributes to less than 5% of the total hnRNPK transcripts and can be ignored in miR-7 expression analysis (Figure 4A and data not shown). As expected, cotransfection of SF2/ASF no longer affected alternative splicing choice. In contrast, both precursor and mature miR-7 levels were significantly increased by SF2/ASF for the PM and DM constructs (Figure 4B and Figure S4A). The levels of miR-7 enhancement were slightly different between the DM and PM constructs (p = 0.076, Figure 4B), indicating possible context dependence (see the Discussion). We also made a construct for which both the proximal and distal splice sites were mutated; however, it led to several cryptic splicing variants (Figure S4B). As an alternative, we cloned the miR-7-1 precursor and its flanking regions into a heterologous context to eliminate all possible splicing (Figure 4C). Once again, the levels of both precursor and mature miR-7 were increased by SF2/ASF over-expression, and the enhancement level was comparable to that in the endogenous context (Figures 4C and 4D; Figure S4C). Together, these data clearly demonstrate that enhanced miR-7 expression is mediated by a splicing-independent function of SF2/ASF.
Regulated miR-7 Biogenesis by SF2/ASF

SF2/ASF Directly Binds to Pri-miR-7 In Vivo
Two RNA-binding protein splicing factors (hnRNPA1 and KSRP) were recently shown to directly interact with primary miRNA transcripts and serve as auxiliary factors for more efficient Drosha cleavage (Michlewski et al., 2008; Trabucchi et al., 2009). A genome-wide survey found that SF2/ASF may bind in close proximity to the stem loop of three miRNAs in HEK293T cells, although the functional significance remains unclear (Sanford et al., 2009). We therefore examined whether SF2/ASF can directly bind to the primary miR-7 transcript to promote its cropping. To this end, a UV crosslinking and immunoprecipitation (CLIP) assay was employed, which can capture in vivo interactions between an RNA-binding protein and its cognate RNAs (Ule et al., 2003).

Since the activities of SF2/ASF in splicing and miRNA processing converge at the endogenous hnRNPK locus, the intronless miR-7-expressing minigene (Figure 4C) was used for CLIP analysis to avoid potential binding of SF2/ASF to neighboring exons. We transfected HeLa cells with a moderate level of the pCG-miR-7 construct. UV crosslinking was carried out 48 hr after transfection, followed by partial RNase digestion and immunoprecipitation with a monoclonal antibody against SF2/ASF (Hanamura et al., 1998). The antibody is highly specific for SF2/ASF with an excellent IP efficiency (Figures S5A and S5B). RNA fragments associated with SF2/ASF were then analyzed by radiolabeled RT-PCR against the EGFP and miR-7 regions. The primer pair specific for miR-7 is located in the flanking regions of its stem loop, such that the primary transcripts rather than the miR-7 precursors are probed. The EGFP region, on the other hand, serves as an internal control to estimate the enrichment of SF2/ASF binding.

As expected, correct PCR products were detected only in the SF2/ASF CLIP, but not the mock immunoprecipitation (Figure S5C, lanes 3–6, and data not shown). We observed a 2-fold enrichment of the pri-miR-7-1 fragment by CLIP compared to the EGFP amplicon (Figure S5D). Although we cannot rule out that the EGFP region might also be bound by SF2/ASF, our data demonstrate that SF2/ASF preferentially binds to pri-miR-7-1 RNA. Using ESEfinder (Cartegni et al., 2003), we identified a putative SF2/ASF-binding site within the stem-loop region of miR-7-1 (Figure 5A). The motif was then mutated and the CLIP experiment was repeated to examine its effect on SF2/ASF binding. In contrast to the wild-type minigene, the mutant construct showed similar ratios between the EGFP and miR-7 amplicons before and after CLIP, suggesting that the binding of SF2/ASF to pri-miR-7 transcripts is mediated by the predicted binding site (Figure 5B, lanes 4 and 8; Figure 5C).

The CLIP results were further corroborated by an RNA affinity purification assay. The stem-loop regions of the corresponding miR-7-1 transcripts were in vitro transcribed and covalently coupled to agarose beads, followed by incubation with HeLa cell extract. Whereas SF2/ASF protein could be pulled down by both RNAs, the pull-down efficiency was markedly reduced when the mutant RNA was used (Figure 5D). Extending the CLIP data, these results confirmed that the interaction between SF2/ASF and miR-7 is mediated at least in part by the SF2/ASF-binding site within the miR-7 stem loop.

SF2/ASF Is Involved in the Drosha Cleavage Step of miR-7 Maturation
We next examined the effect of SF2/ASF binding on enhanced miR-7 production. When the wild-type or mutant pCG-miR-7 minigene was transfected into HeLa cells, the basal levels of miR-7 expression were comparable. This is possibly due to the mutations (G-C to A-T base-pairing) which might relax the local secondary structure and partially compensate for the loss of the SF2/ASF-binding site. However, the two constructs differed substantially under SF2/ASF overexpression conditions: SF2/ASF-enhanced miR-7 expression was significantly reduced with the mutant construct (p = 0.017; Figure 6A), suggesting that the binding site is required for SF2/ASF-enhanced miR-7 production.

Figure 4. A Splicing-Independent Function of SF2/ASF in Promoting miR-7 Expression
(A and B) (A) Wild-type hnRNPK minigene, the proximal 3’ splice-site mutant (PM) or the distal 3’ splice-site mutant (DM), was transfected into HeLa cells together with a control vector or a cDNA expressing SF2/ASF. Alternative splicing of hnRNPK and miR-7 expression were monitored by radiolabeled RT-PCR and northern blotting, respectively. The levels of mature miR-7 were normalized to total hnRNPK levels. Results from triplicate experiments were plotted in (B) (t test, p < 0.05; n = 3).

(C) Intronless minigene expressing miR-7 (pCG-miR-7) was transfected into HeLa cells together with a control vector or SF2/ASF cDNA. The levels of EGFP and miR-7 were monitored by radiolabeled RT-PCR and northern blotting, respectively.

(D) The miR-7 levels were normalized to EGFP, and the results from triplicate experiments were plotted (t test, p < 0.05; n = 3). Error bars represent SEM.
Notably, the levels of both precursor and mature miR-7 were upregulated by SF2/ASF in vivo (Figure 6A), indicating that the Drosha cleavage step is likely to be involved. This is further supported by the observation that SF2-NRS is more potent than wild-type SF2/ASF for promoting miR-7 expression (Figure 3B). As a more direct approach, an in vitro pri-miRNA processing assay was performed, which monitors the conversion of pri-miRNAs to pre-miRNAs (Guil and Caceres, 2007). Whole-cell extracts were prepared from HeLa cells with or without SF2/ASF knockdown (Figure 6B). Consistent with the in vivo data, the processing of wild-type pri-miR-7 was significantly reduced when SF2/ASF-knockdown cell extract was used (Figure 6C, lanes 2 and 3). Adding back purified SF2/ASF protein restored the processing efficiency in a dose-dependent manner (Figure 6C, lanes 4–6). These results strongly argue that SF2/ASF indeed functions at the Drosha cleavage step. Furthermore, depletion and add-back of SF2/ASF had little effect on the miR-7 substrate with a mutated SF2/ASF-binding site (Figure 6D). These data agreed with the in vivo data showing that the level of pre-miR-7 was not significantly changed by cotransfection of the mutant pri-miR-7 construct with an SF2/ASF expression vector. Overall, our data clearly demonstrate that SF2/ASF promotes the Drosha cleavage step of pri-miR-7 processing, although additional effects on postcapping steps cannot be ruled out.

**DISCUSSION**

We show here that SF2/ASF and miR-7 can form a negative feedback circuit. SF2/ASF directly binds to the primary miR-7 transcript to promote its maturation; mature miR-7 in turn represses the translation of SFRS1 mRNA by targeting its 3’UTR (Figure 7B).
Regulated miR-7 Biogenesis by SF2/ASF

Notably, negative feedback does not always lead to a stable steady state, as overcorrection and/or time delay could result in oscillation (Elowitz and Leibler, 2000). Because gene repression by miRNA is relatively modest, with little time delay, miRNA-mediated negative feedback loops are advantageous for noise dampening and have been shown as a recurrent circuit motif in mammalian gene regulatory networks (Tsang et al., 2007).

Negative feedback is also a common mechanism to maintain the steady-state levels of SR proteins (Jumaa and Nielsen, 1997; Sureau et al., 2001). In the case of SF2/ASF, autofeedback has been proposed to occur at multiple levels, including unproductive alternative splicing (Lareau et al., 2007; Ni et al., 2007) and inhibition of translation initiation (Sun et al., 2010). Thus, the miR-7-mediated negative feedback loop is expected to synergize with other feedback mechanisms to precisely control the protein level of SF2/ASF in cells. The relative contribution of each mechanism may vary under different conditions, such as in different cell types or physiological states. This may explain the different phenotypes we observed when knocking down the protein level of SF2/ASF in cells. The relative contribution of each mechanism may vary under different conditions, such as in different cell types or physiological states. This may explain the different phenotypes we observed when knocking down the protein level of SF2/ASF in cells.

Given the reciprocal nature of the SFRS1/miR-7 circuit, an alternative possibility is that SF2/ASF may be critical for the homeostasis of miR-7, an important regulatory molecule orchestrating diverse cellular functions (Li et al., 2009). In this scenario, SF2/ASF serves as a “rheostat” to sense the cellular level of mature miR-7. Fluctuations in miR-7 level are expected to drive the expression of endogenous SF2/ASF in an opposite direction. Because SF2/ASF is required for efficient pri-miR-7 maturation, the negative feedback loop in effect buffers the noise in miR-7 expression to better maintain its steady state.

Besides the SFRS1/miR-7 feedback loop, a circuit with the same architecture has been reported between the transcriptional factor E2F and the miR-17-92 cluster (Woods et al., 2007). Mathematical modeling showed that miR-17-92 is essential for the E2F/Myc cancer network to balance between cell proliferation and apoptosis (Aguda et al., 2008). Therefore, it will be of interest to further investigate the SFRS1/miR-7 feedback loop from a network perspective in order to fully understand its functional significance.

One key finding of this study is a splicing-independent function of SF2/ASF in pri-miRNA processing. We provided multiple lines of evidence that SF2/ASF promotes miR-7 maturation at the Drosha cleavage step. HnRNP A1, another well-known alternative splicing factor, serves as an auxiliary factor in pri-miR-18a processing (Gull and Caceres, 2007). In fact, binding of hnRNP A1 to pri-miR-18a introduces a conformational change in its terminal loop to allow more efficient Drosha cleavage (Michlewski et al., 2008). It is plausible that SF2/ASF may function in a similar manner in promoting pri-miR-7 maturation. In addition, we observed a dominant-negative effect of SF2/ASF on miR-7 production. Since the RS domain of SR proteins often mediates protein-protein interactions, this result suggests that an additional factor(s) might also be involved to enhance pri-miRNA maturation.
Both alternative splicing and miRNA processing coincide in the last intron of the hnRNPK gene, which provides a unique opportunity to examine the potential cooperation and/or competition between the spliceosome and microprocessor (Drosha/DGCR8 complex). Our results showed that SF2/ASF promotes proximal 3' splice-site usage of the miR-7-containing intron. However, the ability of SF2/ASF to promote miR-7 expression is slightly reduced when the proximal 3'ss is used (Figures 4A and 4B), suggesting a context dependence. It has been shown that processing of intronic miRNAs takes place before intron removal (Kim and Kim, 2007). Therefore, our data imply that spliceosome assembly at the nearby splice sites may affect the processing of intronic miRNAs. One attractive model is that the spliceosome might compete with the miRNA processing machinery for common auxiliary factors (e.g., SF2/ASF). Alternatively, intronic miRNAs might adopt different local conformations depending on alternative splice-site usage. We therefore propose that the functions of SF2/ASF in mRNA and miRNA processing might not be mutually exclusive; instead they might modulate each other in a context-dependent manner.

Both splicing factors and miRNAs regulate the expression of a large number of protein-coding genes. Therefore, they may share common downstream targets and/or signaling pathways. Such a “wiring” structure has been reported between transcriptional factors and their regulated miRNAs and is a recurring motif in transcriptional gene networks (Lee et al., 2007; Shalgi et al., 2007). One well-known example is the miR-34 family of miRNAs, which are direct transcriptional targets of p53 and act in concert with other p53 downstream effectors to inhibit inappropriate cell proliferation (Chang et al., 2007; He et al., 2007). Notably, SF2/ASF can also act as an oncprotein by activating the mTOR pathway (Karni et al., 2007). In addition, several SF2/ASF-upregulated miRNAs (e.g., miR-221 and miR-222) have been implicated in tumorigenesis (Sun et al.,...
It is possible that these miRNAs may contribute to SF2/ASF-driven tumorigenesis. One attractive scenario is that splicing regulation and miRNA-mediated gene repression may be broadly coordinated in posttranscriptional gene regulatory networks, a possibility that deserves systematic characterization.

**EXPERIMENTAL PROCEDURES**

**MicroRNA Deep Sequencing**

Cells were harvested at the indicated time points after SF2/ASF induction. Total RNAs were isolated using mirVana miRNA isolation kit (Ambion). miRNA sequencing libraries were constructed as described (Lau et al., 2001) with several minor modifications. Detailed sections covering the library construction and data analysis are presented in the Supplemental Data.

**Plasmids**

T7-tagged SF2/ASF, SC35, and 9G8 were cloned into the pCG7 vector (Caceres et al., 1997). To construct the hnRNPK minigene reporter, an EGFP cDNA fragment was first cloned into the pTag2A vector (Stratagene). A genomic hnRNPK fragment, which corresponds to the last intron and its neighboring exons, was amplified and cloned in-frame with the EGFP gene with the primers 5’-CATGAGTCGGGAGGAGCTTC-3’ and 5’-TGCAGACTCTTCAGTGCTTCCTCCCTCT-3’. For the pCG-miR-7 construct, we first cloned EGFP into the pcDNA3.1+ vector (Invitrogen). MiR-7-1 precursor sequence was then cloned downstream of the EGFP gene. All clones were verified by sequencing.

**Cell Culture and Transient Transfection**

HeLa and HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS). To generate the stable cell line, HeLa Tet-off cells were transfected with STP retroviral vectors containing a human SF2/ASF cDNA; stable transductants were selected with puromycin (2 μg/ml). Transient transfection was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions.

**Luciferase Assay**

HEK293T cells were grown to ~50% confluence in 24-well plates. For each transfection, two luciferase reporters, pRL-SV40 (Renilla luciferase, Promega) and pcDNA-Luc with or without the 3′ UTR region of SFRS1 (Firefly luciferase, see above), were mixed at a 1:2.5 molar ratio. Synthetic miR-7 precursors or control RNAs (Applied Biosystems) were cotransfected at a final concentration of 25 nM. Firefly and Renilla luciferase activities were analyzed by a Dual-Glo control RNAs (Applied Biosystems) were cotransfected at a final concentration of 25 nM. Firefly and Renilla luciferase activities were analyzed by a Dual-Glo Luciferase Assay (Promega) following the manufacturer’s instructions.

**Crosslinking and Immunoprecipitation**

CLIP analysis of SF2/ASF was performed as described (Ule et al., 2003) with a few minor modifications. Briefly, HeLa cells were cultured in 10 cm dishes; UV crosslinking was carried out at 50 mJ/cm². Crosslinked cells were collected and lysed in RIPA buffer. After DNase treatment, the cell lysate was treated with RNase A (Promega) for 10 min at a final dilution of 1:1,000,000. The reaction was stopped by adding 200 U RNase inhibitor (Invitrogen). Immunoprecipitation was carried out at 4°C for 4 hr with protein A/G PLUS-agarose beads (Santa Cruz) coupled with SF2/ASF monoclonal antibody AK96. After extensive washing, SF2/ASF-bound RNAs were released by Proteinase K treatment, followed by phenol extraction and ethanol precipitation. The resulting RNAs were treated with DNase I and reverse transcribed with Superscript II and random hexamers. Quantitative PCR was then performed with radiolabeled primer pairs specific for EGFP and the miR-7 stem loop.

**Northern Blotting**

Total RNAs were resolved on a 15% polyacrylamide TBE-Urea gel and blotted onto a Hybond-N+ membrane (Amersham). An LNA probe (5′-A+C+ A+AT+C+A+CT+GGTCT+CCA-3′; +N stands for LNA base), which is anti-sense to mature miR-7, was labeled with 32P using T4 polynucleotide kinase (Invitrogen). After hybridization, the resulting membrane was exposed to a phosphorimaging screen (Amersham) for 1 hr to detect ectopically expressed miR-7, or overnight to visualize the endogenous miR-7. The signal was analyzed by ImageQuant (Amersham). For the hnRNPK/EGFP minigene experiments, the levels of minigene mRNA and mature miR-7 were first normalized to that of GAPDH mRNA and U6 snRNA, respectively. To normalize the transcription efficiency, gels were reloaded by keeping the minigene expression relatively constant, such that the differences in miR-7 levels can be better visualized.

**Semiquantitative PCR**

Fifteen to twenty cycles of radiolabeled PCR were carried out to ensure that the amplifications were in the linear range. The resulting PCR products were resolved by 8% TBE-PAGE gel and detected with a Storm 840 PhosphorImager. All primers used are listed in the Supplemental Data.

**Western Blotting**

Proteins were separated on 8% SDS-PAGE gels and blotted with monoclonal antibody against β-catenin (Sigma), α-tubulin (DM1A; Upstate), T7 epitope tag (Novagen), or SF2/ASF (AK96; Hanamura et al., 1998). The signal was detected with Alexa Fluor 488 goat anti-mouse IgG (H+L) antibody (Invitrogen) and quantified with a Storm 840 PhosphorImager.

**RNA Affinity Purification**

DNA templates for in vitro transcription were prepared by PCR from the pcG-miR-7 and its SF2/ASF-binding site mutant with the primers 5′-TATAACGACT CACTATAGGGTAGAAGATTCATTGGATGTTGG-3′ and 5′-TTGTCCCTGTAAG GCCATG-3′. In vitro transcription was carried out with T7 RNA polymerase (NEB) following the manufacturer’s protocol. After gel purification, the resulting RNAs were coupled to agarose beads (Sigma), and affinity purification of miRNA binding factors from HeLa extract was performed as described (Caputi et al., 1999). Protein factors associated with the immobilized RNAs were analyzed by western blotting.

**RNA Interference**

Dicer substrate small interfering RNA (DsiRNA) against SFRS1 (forward 5′-CCAAAGGACAUUGAGCGUUGCUUA-3′; reverse 5′-UAGAACACGUCC UCAUGGCUUGGGU-3′) was custom designed and synthesized (IDT). Ten μl HeLa cells were transfected with 100 pmol DsiRNA duplex using TriFECTin transfection reagent (IDT). Cells were harvested 48 hr after transfection. The knockdown efficiency was determined by RT-PCR and western blotting.

**In Vitro Pri-miRNA Processing Assay**

In vitro processing was performed as described (Michlewski et al., 2008). Briefly, each reaction (30 μl) contained 20% (v/v) wild-type or depleted HeLa cell extract, 0.5 mM ATP, 20 mM creatine phosphate (Sigma), 3.2 mM MgCl₂, and 200,000 cpm (~100 fmol) of in vitro-transcribed pri-miRNAs. The reactions were assembled on ice followed by incubation at 30°C for 30 min. After phenol/chloroform extraction and ethanol precipitation, the RNA samples were resolved by 10% (w/v) TBE-Urea gel electrophoresis and exposed overnight on X-ray film at ~80°C.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and Supplemental Experimental Procedures, and can be found with this article at doi:10.1016/j.molcel.2010.02.021.

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