Targeting the Deregulated Spliceosome Core Machinery in Cancer Cells Triggers mTOR Blockade and Autophagy

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Abstract

The spliceosome is a large ribonucleoprotein complex that guides pre-mRNA splicing in eukaryotic cells. Here, we determine whether the spliceosome could constitute an attractive therapeutic target in cancer. Analysis of gene expression arrays from lung, breast, and ovarian cancers datasets revealed that several genes encoding components of the core spliceosome composed of a heteroheptameric Sm complex were overexpressed in malignant disease as compared with benign lesions and could also define a subset of highly aggressive breast cancers. siRNA-mediated depletion of SmE (SNRPE) or SmD1 (SNRPD1) led to a marked reduction of cell viability in breast, lung, and melanoma cancer cell lines, whereas it had little effect on the survival of the nonmalignant MCF-10A breast epithelial cells. SNRPE or SNRPD1 depletion did not lead to apoptotic cell death but autophagy, another form of cell death. Indeed, induction of autophagy was revealed by cytoplasmic accumulation of autophagic vacuoles and by an increase in both LC3 (MAP1LC3A) protein conversion and the amount of acidic autophagic vacuoles. Knockdown of SNRPE dramatically decreased mTOR mRNA and protein levels and was accompanied by a deregulation of the mTOR pathway, which, in part, explains the SNRPE-dependent induction of autophagy. These findings provide a rational to develop new therapeutic agents targeting spliceosome core components in oncology. Cancer Res; 73(7); 2247–58. ©2013 AACR.

Introduction

Targeted therapies have been shown to improve outcome in breast cancers. Molecular targeted agents usually inhibit key oncogenic pathways involved in cancer progression or resistance to conventional treatments. Most of these new molecular therapies target kinase pathways and are usually highly effective in a specific molecular segment. In the recent years, accumulative evidence has been reported that beyond kinase and DNA repair defects, many pathways are crucial for cancer progression. For instance, metabolism, immune system, and epigenetics have been reported as targetable in oncology. Looking for novel pathways involved in cancer progression, we previously identified spliceosome assembly components as the most enriched pathway in breast cancer samples, when compared with benign lesions (1).

The aim of the present study was to determine whether the spliceosome could be a relevant therapeutic target in cancer. The spliceosome is a dynamic macromolecular ribonucleoprotein (RNP) complex that catalyses the splicing of nuclear pre-mRNA (precursor mRNA) into mRNAs. Nuclear pre-mRNA splicing is essential to remove internal noncoding regions of pre-mRNA (introns) and to join the remaining segments (exons) into mRNA before export to the cytoplasm and translation. The spliceosome does the 2 primary functions of splicing, that is, recognition of the intron/exon boundaries and catalysis of the cut-and-paste reactions that remove introns and join exons. The spliceosomal machinery complex is formed from 5 RNP subunits, termed uridine-rich (U-rich) small nuclear RNP (snRNP), transiently associated to a range of protein factors (2, 3). Each snRNP (U1, U2, U4/U6, and U5) consists of a uridine-rich small nuclear RNA (snRNA) complexed with a set of 7 proteins known as Sm or SNRP proteins. The Sm proteins (B/B', D1, D2, D3, E, F and G) form a 7-member ring core structure that encompasses RNA. All Sm proteins share a conserved Sm domain, consisting of 2 sets of conserved sequences (Sm1 and Sm2) that are responsible for the assembly of snRNAs in an ordered manner, to form the Sm core of the spliceosomal snRNPs (4, 5) and are thereby involved in pre-mRNA processing (6).

The splicing process has a crucial role in the control of the expression of many genes (such as those involved in cell cycle, signal transduction, angiogenesis, apoptosis, and invasion;
of 1 mL. For controls, cells were either exposed to Hiperfect reagent alone (mock) or transfected with a nontargeting control siRNA. Transfection medium was replaced by fresh culture medium every 48 hours until the end of experiment. After 96 hours of transfection, cells were harvested, counted using the trypan blue exclusion method or crystal violet staining, and processed for the various biochemical assays.

In the present study, we have evaluated whether components of the spliceosome machinery complex were overexpressed in malignant tumors and whether blocking spliceosome could lead to antitumor effects.

Materials and Methods

Cell culture and siRNA transfection

All cell lines were supplied by American Type Culture Collection (ATCC, LGC Standards) between 2007 and 2011. Each ATCC cell line was first amplified to generate a cell master bank. All experiments were carried out from this cell bank. All the cell lines used were tested negative for mycoplasma contamination using VenorGeM Advance PCR Kit (Biovalley). Human mammary carcinoma cell lines SKBr-3 (ATCC HTB-30), MDA-MB 231, and breast carcinoma A549 (ATCC CCL-185), and malignant melanoma A375 (ATCC CRL-1619) were cultured as monolayers at 37°C with 5% CO2 and maintained by regular passage in complete medium consisting of RPMI-1640 GlutaMax or Dulbecco’s Modified Eagle’s Media (DMEM):F12 (Gibco BRL, Invitrogen Life Technology) supplemented with 10% heat-inactivated FBS (PAN Biotech, Dutscher), 1 mmol/L sodium pyruvate (Invitrogen), and 10 mM mmol/L HEPES (Invitrogen). MCF-7 (ATCC HTB-22) was maintained in completed DMEM/F12–GlutaMax medium supplemented with 0.01 mg/mL insulin (Invitrogen). Nontumoral epithelial cell line MCF-10A (ATCC CRL-10781) was maintained in DMEM/F12–GlutaMax medium supplemented with 20 ng/mL EGF (Gibco, Invitrogen), 100 ng/mL chola toxin (Sigma-Aldrich), 0.01 mg/mL insulin (Invitrogen), 500 ng/mL hydrocortisone (Sigma-Aldrich), and 5% heat-inactivated horse serum (Invitrogen). Forty-eight hours after plating, the cells were transfected with specific SNRPE or D1 validated siRNA (see Supplementary Table S1 for sequence information; Qiagen) or nontargeting control siRNA (ON-TARGETplus Non-targeting Pool, Dharmacon) using HiPerfect transfection agent (Qiagen) according to the manufacturer’s instructions. Briefly, cells were plated in 12-well plates in 4 replicates and grown to approximately 50% to 60% confluence. siRNA was diluted in OPTI-MEM minimal culture medium (Invitrogen) at a final concentration of 50 nmol/L and then Hiperfect was added to the diluted siRNA and the mixture was dropwise added to each well in a final volume of 1 mL.
necrotic cells; top right: 7-AAD+/PE−, late apoptotic or necrotic cells). Staurosporine (5 μmol/L)-treated SKBr-3 cells served as positive control of apoptosis induction.

**Autophagy analysis by transmission electron microscopy**

SKBr-3 cells were transfected with SNRPE-specific validated siRNA or nontargeting control siRNA as described above. Forty-eight hours after transfection, cells were fixed with 1.6% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at room temperature for 1 hour. Samples were rinsed in the same buffer then postfixed with osmium tetroxide and 1.5% potassium ferrocyanide in 0.1 mol/L cacodylate buffer (pH 7.4) for 1 hour at room temperature. Cells were rinsed with distilled water, dehydrated in increasing concentrations of ethanol, and embedded in epoxy resin. Embedded samples were then conventionally processed for thin sectioning (70 nm), contrasted by uranyl acetate and lead citrate, and observed with Zeiss 902 electron microscope in filtered zero loss mode using CDD megaviewIII camera and SIS system (Olympus). Autophagic cells were counted on 60 cells series per sample. The identification of autophagy was based on the observation of macro-autophagic vacuoles, that is, multiple membranes vesicles engulfing a portion of the cytoplasm.

**Detection and quantification of acidic vesicular organelles with acridine orange staining using flow cytometry**

To detect the presence of acidic vesicular organelles (AVO), tumor cells staining with acridine orange (Sigma-Aldrich) was conducted as described previously (25). Briefly, 96 hours after siRNA transfection, SKBr-3 cells were washed with PBS and stained with 1 μg/mL acridine orange in serum-free medium (Sigma Aldrich) for 20 minutes at 37°C. Acidine orange-stained cells were trypsinized, washed, and analyzed with flow cytometer. Green (510–530 nm, FL1-H channel) and red (>650 nm, FL3-H channel) fluorescence emissions from 10⁶ cells illuminated with blue (488 nm) excitation light are measured with a FACScanto from BD Biosciences using DIVA software. Autophagy was quantified as a ratio between geometric fluorescence intensity of red versus green fluorescence (FL3/FL1). Treated cells with nutrient-free medium EBSS (Invitrogen) for 24 hours before the addition of acridine orange served as positive controls.

**Gene expression microarray**

Transcriptome analysis following SNRPE depletion was conducted using exon array (ExonHit Therapeutics). SKBr-3 cells were transfected with SNRPE-specific validated siRNA or nontargeting control siRNA as previously described. Total RNA was extracted using RNA isolation RNeasy micro Kit (Qiagen) at 96 hours after transfection. The concentration of RNA was measured using Nanodrop spectrophotometer. The quality of RNA preparations was assessed using Lab-on-a-Chip Bioanalyzer 2000 technology (Agilent Technologies). Aliquots of extracted total RNA from the samples were sent to ExonHit Therapeutics for array processing. ExonHit staff processed to a human Genome-Wide SpliceArray™ microarray on the Affymetrix platform using single color labeling. Processing included sample amplification and labeling, hybridization, array scanning, and data normalization.

**Reverse transcription and RT-quantitative PCR**

Total RNA was extracted using RNA isolation RNeasy micro Kit (Qiagen) at 96 hours after transfection. mTOR mRNA expression was analyzed by real-time quantitative PCR (RT-qPCR) in SKBr-3 cells transfected by SNRPE or control siRNA. One microgram of total RNA from each sample was reverse-transcribed by superscript II reverse transcriptase (Invitrogen) in the presence of random primers (Applied Biosystems, Life technologies). Quantitative PCR (qPCR) was carried out on an equivalent amount of 12.5 ng total RNA per tube in a final volume of 25 μL. Oligonucleotide primers and TaqMan probes were designed using the PrimerExpress computer software (Applied Biosystems) and purchased from MWG Biotech. The primers and probe sequences used for the qPCR for mTOR and housekeeping genes are detailed in Supplementary Table S2. The relative expression of mTOR mRNA was determined by using the Ct value and the 2−ΔΔCt method. The data are presented as the fold change in gene expression normalized using housekeeping genes (TBPB, RPLPO).

**Expression profiling of spliceosome-related genes**

Two unpublished datasets of gene expression arrays were used to determine a correlation between spliceosome expression and clinical characteristics as well as the proliferation biomarker MKI67. These two studies used Agilent 44K platform. The first study compared gene expression levels between ovarian adenocarcinomas (n = 21) and borderline (nonmalignant) lesions (n = 21). The second one evaluated gene expression in 148 breast cancer samples including 27 triple-negative breast cancer (TNBC). In this latter study, the reference for gene expression was normal breast (pool of 3 samples). In addition to these 2 studies, we used publically available gene expression data available from Landi and colleagues (26) and André and colleagues (1). Lung dataset from Landi and colleagues included 107 non–small cell lung carcinomas (n = 58) and nontumor tissues (n = 49). Breast dataset from André and colleagues included 165 samples (120 cancers and 45 benign lesions) obtained by fine-needle aspiration of breast lesion. Spliceosome complex components (from Biocarta database) were extracted from these datasets respectively. The log2 gene expression was analyzed and when one gene is represented by more than one probe set, the one with the highest variance was used. To illustrate the gene expression distribution of the spliceosome complex components, boxplot graphs depict this information according to sample types.

**Statistical analysis**

Effects of siRNA transfections on cell lines proliferation were estimated by 2-way ANOVA. The effects of transfection conditions were first analyzed by considering all cell lines as a first factor and the transfection conditions as a second factor (nontransfected cells, mock, unspecified siRNA). Here, and for each cell line, counting values were initially transformed into proportions of the maximum value in each corresponding cell line and then linearized with a logit link. Next, the effects of the
2 specific siRNA transfections were analyzed independently. For each cell line, both nonspecific and specific siRNA counting values were initially transformed into proportions of the corresponding mock counting value. After a logit transformation, cell lines and transfection (specific siRNA vs. nonspecific siRNA) were considered as the 2 factors in a 2-way ANOVA.

Results

**Genes involved in spliceosome machinery are overexpressed in malignant disease and define aggressive phenotypes**

We evaluated the expression of 15 genes related to spliceosomal assembly, according to the BioCarta database, including U1snRNP-specific protein (SNRNP70 or U1-70K), constitutive splicing factors (U2AF1, U2AF2), splicing regulator (SFRS2), and core spliceosomal components (SNRP-A/A1, B/B2, C, D1/D2/D3, E, F, and G). Differential gene expression analysis between malignant and normal tissues was first conducted on (i) a microarray dataset from Landi and colleagues comparing gene expression in 107 non–small cell lung carcinomas (n = 58) and nontumor tissues (n = 49) and (ii) an unpublished microarray dataset comparing 148 breast cancers and normal breast samples. We found that genes related to spliceosome assembly components are overexpressed in lung adenocarcinoma (Fig. 1A) and breast cancer (Fig. 1B) as compared with nontumoral tissue. Next, we used an unpublished ovary microarray dataset to compare gene expression between 21 invasive ovarian cancers and 21 borderline lesions. The borderline lesions are defined as a subset of epithelial ovarian tumors of low malignant potential representing an intermediate stage between fully benign and fully malignant adenocarcinomas. Strikingly, spliceosome assembly components are overexpressed in ovarian cancer as compared with borderline tumors (Fig. 1C), suggesting that overexpression of spliceosomal machinery components was related to malignancy. No major correlation was found between the proliferation marker MKI67 and the spliceosome-related genes in the 3 datasets described (Supplementary Fig. S1).

We finally assessed whether overexpression of spliceosome assembly components defines a more aggressive disease. To do this, we looked at the correlation between the expression of the 15 genes and clinical characteristics. For this analysis, we used a public dataset of 120 breast cancer samples and 45 benign breast lesions (1). Interestingly, unsupervised clustering defined 2 populations, one of which overexpressed the spliceosome assembly components (Fig. 2). This spliceosome positive cluster was enriched in patients presenting with a TNBC (lack of estrogen receptor, progesterone receptor, and HER2) and Her2-overexpressing breast cancer (47% of the spliceosome-positive breast cancers). These findings suggested that overexpression of spliceosome assembly components were not only overexpressed in malignant diseases but also could define a subset of highly aggressive cancers.

**Knockdown of components of the spliceosome decreases viability of cancer cells, but not of nonmalignant cells**

To investigate the role of the spliceosome, we focused on the core spliceosomal components SNRPE and SNRPD1 that were consistently found to be among the top differentially expressed genes in the 3 series of samples. We first used siRNA-mediated depletion of SNRPE to knockdown SNRPE expression in the SKBr-3 and MDA-MB 468 breast cancer cell lines that were chosen because they are respectively HER2-amplified and triple-negative. We also used the nontumoral MCF-10A breast cell line to compare the effects of depleting a spliceosome component in cancer cells with nontumoral cells. As revealed by Western blot analysis, SNRPE protein level was efficiently reduced by siRNA in the 3 cell lines (Fig. 3A). SNRPE knockdown induced a major decrease of cell viability as compared with mock in the 2 tumor cell lines [MDA-MB 468 (88.6% vs. 102.1% in siControl cells) and SKBr-3 (58.1% vs. 104.9% in siControl cells)], whereas it had a much weaker effect (24.2% vs. 95.1% in siControl cells) in the nontumoral MCF-10A cells (Pinteraction = 0.00022).

To ascertain that this effect is due to spliceosome targeting, we knocked down the expression of another core spliceosomal component. We chose SNRPD1, another upregulated core spliceosomal protein in malignant tumors (Fig. 3B). Here, following SNRPD1 knockdown, we observed a major reduction of cell viability in the 2 breast tumoral cell lines MDA-MB 468 (78.4%) and SKBr-3 (57.7%) and a weaker effect (24.1%) in the nontumoral MCF-10A cells (Pinteraction = 0.00224).

Of note expression of the genes encoding the other core spliceosomal components (SNRP-A/A1, B2, C, D2/D3, F, and G) was assessed by RT-qPCR in SNRPE- or SNRPD1-depleted cells. No or only weak changes were observed in the expression of these genes (Supplementary Fig. S2), showing that the siRNAs used in this study and targeting SNRPE or SNRPD1 had no off-target effects on related components of the spliceosome.

We next evaluate whether spliceosome depletion impacts on cell proliferation of additional breast, melanoma, and lung tumoral cell lines. A significant decrease in cell viability was observed in all tested tumoral cell lines following SNRPE and SNRPD1 knockdown (Supplementary Fig. S3).

Altogether, these results revealed that all tumoral cell lines were sensitive to spliceosome depletion, independently of their proliferation rate (Supplementary Fig. S4). Moreover, a non-tumoral breast cell line (MCF-10A) was less sensitive to spliceosome depletion than tumoral cell lines, suggesting that the antiproliferative effect of spliceosome depletion was specific to malignant cells.

**SNRPE or SNRPD1 targeting mediates cancer cell death through autophagy**

To further investigate the cellular mechanism that led to a decrease in cell viability, we assessed whether the spliceosome regulated apoptosis or cell cycle. As revealed by fluorescence-activated cell-sorting (FACS) analysis, SNRPE knockdown induced a marked increase in the sub-G1 population in SNRPE-depleted cells (19.32%) as compared with control cells (2.62%), indicative of cell death occurrence (Fig. 4A). Apoptotic cell death was analyzed by using double cell labeling with Annexin V-PE and 7-AAD. SNRPE depletion did not lead to a significant increase in the amount of Annexin V–positive cells (31.44% as compared with 39.7% for control cells; Fig. 4B) and...
Figure 1. Genes involved in spliceosome assembly are overexpressed in malignant disease. Gene expression of spliceosome components related genes (from Biocarta database) using publicly available gene expression data sourced from Landi and colleagues (25) non–small cell lung carcinomas (n = 58) and nontumor tissues (n = 49). A, unpublished breast dataset from Gustave Roussy Cancer Institute of 148 samples including 27 TNBCs and 121 ER+ and/or HER2-overexpressing breast cancers B, unpublished ovarian dataset from Gustave Roussy Cancer Institute including 21 invasive ovarian cancers and 21 borderline tumors (C). In each panel, box plots represent gene expression (log2 values) between malignant and normal tissue [lung (A) and breast (B) datasets] or borderline tumors [ovarian (C) dataset].
therefore did not induce typical apoptosis. Similar effects were obtained in SNRPD1-depleted cells (Supplementary Fig. S5).

We next analyzed autophagy, another form of cell death, by using transmission electron microscopy (TEM) approach that allowed detecting the presence of autophagic vacuoles in cell cytoplasm. Autophagic features such as intracellular vacuolization and double-membrane structures referred as autophagosomes (early autophagic compartment) were observed in SNRPE-depleted cells (Fig. 5A). The amount of cells containing autophagosomes was 2-fold higher in SNRPE-depleted cells (9%) than in control cells (4%) at 48 hours posttransfection. The cytoplasmic accumulation of autophagosomes following SNRPE depletion is consistent with an induction of the autophagic pathway.

We next evaluated the expression of the microtubule-associated protein 1 light chain 3 (LC3), commonly used as a marker of the autophagic process that directly correlates with the extent of autophagosome formation (27, 28). LC3 protein exists in 2 forms: a cytosolic 18-kDa LC3-I and a 16-kDa LC3-II that binds to autophagosomes membranes during autophagy. SNRPE or SNRPD1 depletion led to an increase in the LC3-II form that indicated an autophagosome formation and an induction of autophagy (Fig. 5B).

To further reinforce the data concerning autophagy, we evaluated the presence of late (autolysosome) markers of autophagy using cell staining with acridine orange, a lysotropic dye that accumulates in acidic organelles formed during autophagy. The quantitative analysis of acridine orange-stained cells showed that knockdown of SNRPE or SNRPD1 protein expression in SKBr-3 cells induced a significant increase of orange red fluorescence as compared with control cells, indicating formation of acidic autophagolysosomal vacuoles following spliceosome depletion (Fig 5C).

Overall, these data indicated that targeting core spliceosomal component such as SNRPE or SNRPD1 could mediate cancer cell death through autophagy.

SNRPE knockdown leads to mTOR downregulation

To identify the molecular mechanisms leading to autophagy, we analyzed gene expression profiles in SNRPE-depleted SKBr-3 cell line. Transcriptome analysis on exon microarrays revealed that 51 genes were overexpressed and 41 genes were downregulated (Supplementary Fig. S1) following SNRPE depletion. Importantly, this decrease was robust as evidenced by the similar behavior of all the 142 probes covering the mTOR mRNA (Fig. 6A). This decrease (5-fold) in mTOR mRNA abundance in SNRPE-depleted cells was confirmed by RT-qPCR (Fig. 6B). Furthermore, a decrease in mTOR protein abundance was observed in SNRPE-depleted cells (Fig. 6C).

We next hypothesized that NMD could mediate the mTOR loss induced by SNRPE-knockdown, as a recent study has shown that knockdown of SNRPB, another core SNRP spliceosomal protein, resulted in the inclusion of many PTC-containing alternative exons (29). These aberrant PTC-containing mRNAs are subsequently degraded by the NMD machinery (29). To determine whether NMD was involved in the disappearance of the mTOR mRNA upon SNRPE depletion, we...
disrupted NMD using cycloheximide in SNRPE-depleted cells. The translation inhibitor cycloheximide inhibits mRNA degradation by the NMD pathway, as this process requires ongoing translation. Following SNRPE depletion, NMD inhibition by cycloheximide resulted in an approximately 2.5-fold increase in the relative abundance of mTOR mRNA, as compared with 1.5-fold in control cells (Fig. 6D), suggesting that a substantial fraction of the mTOR pre-mRNA was aberrantly spliced into one or more mRNA isoforms that were degraded by NMD. Our results also showed that mRNA expression for 2 control genes (GUSB and RPLPO) was not increased upon cycloheximide treatment (Supplementary Fig. S7). These data indicated that the cycloheximide-dependent increase in mTOR mRNA expression was specific to SNRPE knockdown.

To next evaluate whether SNRPE depletion could affect downstream targets of the mTOR signaling pathway, we assessed the phosphorylation of 4E-BP1 that lies just downstream of mTOR. SNRPE depletion induced a dephosphorylation of 4E-BP1 (Fig. 6C) that indeed indicated a blockage of mTOR signaling. Overall, these data indicated a global SNRPE-dependent deregulation of mTOR signaling.
Discussion

In the present study, we report that components of the Sm core spliceosomal machinery are overexpressed in cancer, regulate mTOR, and impact on cell proliferation and autophagy (Fig. 6E).

Indeed, gene expression profiling using breast and lung datasets revealed that genes related to the core spliceosomal components are highly expressed in lung and breast cancer in contrast to nontumoral tissue. Moreover, gene expression analyses using 2 other datasets of ovarian and breast cancers indicated that overexpression of genes encoding spliceosome complex components could be associated with a more aggressive phenotype of cancers and could define a subset of highly malignant cancers. Among these genes involved in spliceosome complex, those encoding the core spliceosomal proteins SNRPE and SNRPD1 were consistently found to be among the top differentially expressed genes in the 4 series of samples.

Several studies have previously suggested that components of the splicing machinery are mutated or overexpressed in malignant diseases. Indeed, mutations in genes encoding the U2AF35, ZRSR2, SRSF2, and SF3B1 splicing factors have been found in hematologic malignancies (16). Sampath and colleagues reported that the splicing factor SPF45 was overexpressed in carcinoma from various origins including breast, lung, ovary, colon, and prostate (30). This group also reported that SPF45 overexpression was associated with resistance to cyclophosphamide (30). More recently, Niu and colleagues reported that proteins involved in splicing machinery were overexpressed in 8 samples of bladder cancer, as compared with normal tissue (31). The present study based on larger number of samples suggests that genes encoding the spliceosome core machinery are overexpressed in solid tumors. Overexpression of spliceosomal component is not only a hallmark of cancer but also could be a therapeutic target. Indeed, we have shown here that depletion of several components of the core spliceosome (i.e., SNRPE or SNRPD1) has a selective action on the proliferation of transformed cell lines but not of an immortalized nontumorigenic cell line (MCF-10A). In addition, SNRPE depletion

Figure 4. Cell-cycle analysis and apoptosis detection following SNRPE depletion. A, cell-cycle analysis was used to monitor changes in the DNA of SKBr-3 cells harvested at 72 hours posttransfection with 50 nmol/L of SNRPE targeting siRNA or nontargeting control siRNA as indicated. B, apoptosis detection was quantitated using PE Annexin V/7-AAD staining followed by flow cytometric analyses in cells at 72 hours posttransfection with 50 nmol/L SNRPE targeting siRNA or nontargeting control siRNA. The numbers show the percentages of cells in each quadrant (bottom left, 7-AAD−/PE−, intact cells; bottom right, 7-AAD+/PE−, early apoptotic cells; top left, 7-AAD−/PE+, necrotic cells; top right, 7-AAD+/PE+, late apoptotic or necrotic cells). Staurosporine (5 μmol/L)-treated SKBr-3 cells served as positive control of apoptosis induction.
leads to inhibition of mTOR expression. This effect may, in part, be the consequence of the production of aberrantly spliced mTOR mRNA isoforms degraded by the NMD pathway, as we have found here that NMD inhibition by cycloheximide results in an increase in the relative abundance of mTOR mRNA. Further-more, a recent study reports that regulation of mTOR alternative splicing leads to the production of a short transcript degraded by NMD during adipogenesis (32). Strikingly, by regulating mTOR, SNRPE impacts on one of the most often deregulated signaling pathways in cancer. mTOR is a key component that coordinately regulates the balance between growth and autophagy in response to cellular physiologic conditions and environmental stress (33). Consistent with this observation, we have shown here that targeting SNRPE or SNRPD1 leads to cell death through autophagy. Interestingly, a recent report using a short hairpin RNA screening procedure in Drosophila melanogaster identified the spliceosome machinery as one of the main pathway regulating the mTORC1 signaling pathway (34). This observation, together with our study, highlights the importance of the spliceosome–mTOR axis and reinforces the interest of the core spliceosome as an interesting target in the control of eukaryotic cell growth and death.

These findings open up new avenues for drug development. Several drugs have already been developed to target the
Figure 6. Knockdown of SNRPE alters mTOR expression and downstream signaling pathway. A, analysis of mTOR expression by exon arrays in SNRPE-depleted or control (nontargeting siRNA) SKBr-3 cells. mTOR expression was analyzed by exon arrays. mTOR expression is represented in a green–red heat color coding from low expression (light green) to high expression (red color) for transfected (△) and control (●) cells separately. The x-axis corresponds to the rank of mTOR probes in the control cells sorted by their expression in an increasing order. B, mTOR mRNA expression was analyzed by RT-qPCR in SNRPE-depleted or control (nontargeting siRNA) SKBr-3 cells. The graph represents mTOR mRNA level (fold change) normalized using housekeeping genes. C, expression of SNRPE and different mTOR downstream proteins was analyzed by Western blotting in SNRPE-depleted or control (nontargeting siRNA) SKBr-3 cells. D, for NMD inhibition experiments, cells were transfected with specific SNRPE siRNA or nontargeting control siRNA. Three days later, cells were treated for 8 hours with 100 μg/mL cycloheximide (CHX) or with an equivalent amount of DMSO as a control. mTOR RNA expression was detected by RT-PCR. Quantifications of the RT-PCR signals were calculated from RT-PCR assays carried out on samples from 2 independent transfections. The graph represents the ratio of mTOR mRNA expression in cells treated with cycloheximide as compared with untreated cells and shows that NMD inhibition resulted in an approximately 2.5-fold increase in the relative abundance of mTOR mRNA in SNRPE-depleted cells as compared with 1.5-fold increase in control cells. E, schematic representation of the sequence of processes leading from targeting SNRPE and SNRPD1 by specific siRNAs to the inhibition of mTOR survival pathway. The spliceosomal machinery complex is formed from 5 RNP subunits, termed uridine-rich (U-rich) small snRNPs. Each snRNP (U1, U2, U4, U5, and U6) consists of a uridine-rich snRNA complexed with a set of Sm proteins (B/B2, D1, D2, E, F, and G) forming a ring core structure that encompasses RNA. The colored circles refer to the most significantly overexpressed Sm core proteins in the studied datasets (see Materials and Methods). Pink, breast cancer; blue, lung cancer; and green, ovarian cancer.
spliceosome machinery. Also, as they are SR splicing factors [e.g., SF3b] or constitutive splicing factors [e.g., SRSF/serine-arginine-rich proteins] drugs target splicing regulators [i.e., SRSF/serine-arginine-rich proteins] or constitutive splicing factors [e.g., SF3b] but not the core spliceosomal machinery. As also, as they are derived from natural compounds, they do not specifically target spliceosome machinery. On the basis of the present study, there is a rationale to design chemical compounds that specifically target SNRPE and SNRPDI. Other therapeutic strategies are possible to inhibit spliceosome activity. It has been reported that spliceosome activity is regulated by kinases, including SRPK1 (39). This could provide a rationale to develop kinase inhibitors that would eventually lead to the inhibition of spliceosome activity.

Overall, our results suggest that core spliceosome machinery could be an attractive therapeutic target in malignant solid tumors. These findings provide a rationale to design new drugs targeting SNRPE, SNRPDI, and to develop spliceostatin A and the pladienolide derivatives for patients with SNRPE/SNRPDI-overexpressing cancers.

Disclosure of Potential Conflicts of Interest

The sponsors did not participate in the design and conduct of the study; collection, management, analysis, and interpretation of the data; and preparation, review, or approval of the manuscript. Its contents are solely the responsibility of the authors. No potential conflicts of interest were disclosed.


typeSpliceosome as a Target for Anticancer Treatment

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