Rational Design of an Activatable Reporter for Quantitative Imaging of RNA Aberrant Splicing In Vivo

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Pre-mRNA splicing, the process of removing introns from pre-mRNA and the arrangement of exons to produce mature transcripts, is a crucial step in the expression of most eukaryote genes. However, the splicing kinetics remain poorly characterized in living cells, mainly because current methods cannot provide the dynamic information of splicing events. Here, we developed a genetically encoded bioluminescence reporter for real-time imaging of the pre-mRNA splicing process in living subjects. We showed that the bioluminescence reporter is able to visualize the pre-mRNA aberrant splicing process in living cells in a dose- and time-dependent manner. Moreover, this reporter could provide quantitative and longitudinal information of splicing activity in response to exogenous splicing inhibitors in living animals. Our data suggest that this activatable reporter could serve as a promising tool for the high-throughput screening of splicing modulators, which would facilitate the drug development for human diseases caused by the abnormal splicing of mRNA.

INTRODUCTION

Precursor messenger RNA (pre-mRNA) splicing is a pivotal step in gene expression, by which introns are removed from the initial transcribed sequence and flanking exons are ligated to form a continuous RNA molecule. Pre-mRNA splicing follows the GU-AG rule, which depends on the short conserved splice sites at the intron-exon junctions. A small nuclear ribonucleoprotein (snRNP) complex called spliceosome participates in the splicing process. The spliceosome is a large ribonucleic acid protein complex consisting of small nuclear RNAs (snRNAs) and over 100 proteins. Intron structure is very conserved in the evolution of life processes. It contains the 5' and 3' splicing boundaries, as well as branching point sequences. The mechanism of RNA splicing requires cis-acting elements and trans-acting factors by which to facilitate splicing or recruitment of the spliceosome. Genes undergoing aberrant splicing would alter the amino acid sequence of the polypeptide chain when expressed, thereby altering the structure and function of the protein and affecting various biological processes such as differentiation, development, and tumorigenesis.

Currently, detection of splicing at the mRNA level is primarily performed by reverse transcription polymerase chain reaction (RT-PCR), northern blot, or primer extension analysis. However, these methods are laborious and time consuming. Most importantly, they require cell lysis and thus cannot repeatedly visualize pre-mRNA splicing activity in living cells, which is critical to acquire the dynamic information of spliced or unspliced mRNA. One of the approaches for gene imaging at mRNA levels refers to radiolabeled antisense oligonucleotides (RASONs). RASONs are involved in pairing with the mRNAs of interest and therefore could target any specific sequences of mRNAs, providing a more direct means of mRNA imaging. However, their application was greatly hindered due to the high background activity, low specificity of localization, and limited delivery of probe. In contrast, the employment of reporter genes to quantify mRNA molecules shows several advantages: low background due to rapid clearance, highly specific localization through the local trapping of a unique enzyme of reporter product, and simplified reporter probe delivery due to higher cell membrane permeability. Several studies have designed fluorescent proteins or luciferase as splicing reporters to evaluate the mRNA that constitute splicing or alternative splicing events. Although these reporter systems were able to successfully detect that the pre-mRNA splicing process responded to extracellular splicing modulators, some of the reporters were negative systems in nature, and people could not distinguish whether the reduced signal is attributed to cell death or endogenous splicing induced by the splicing compound. In addition, most of the splicing reporters exhibited poor dynamic range or fail to distinguish changes in compound-affected splicing from changes in mRNA transcription or translation.

In the present study, to overcome the drawbacks, we designed an activatable bioluminescent reporter for imaging pre-mRNA aberrant splicing in living subjects. This reporter gene, Rluc-SMN, was designed by inserting a human survival motor neuron 2 (SMN2) mini-gene upstream of the renilla luciferase (Rluc) so that only the...
unspliced mRNA translates active luciferase protein. We proved that the dynamics of splicing activity was measured in real-time and quantitatively with this splicing reporter. Our study may offer a promising tool for in vivo visualizing of pre-mRNA splicing and the high-throughput screening of splicing regulators, which would benefit the treatment of human diseases induced by aberrant splicing of pre-mRNA.

RESULTS

Design of the Rluc-SMN Reporter

To obtain a reporter appropriate for monitoring pre-mRNA splicing, the Rluc-SMN construct was designed by fusing a SMN2 minigene upstream of the open reading frame of the Rluc gene. Meanwhile, all the in-frame stop codons in the intron were removed, and one base was inserted at the 3' end of exon 6 of SMN2 minigene (Figure 1). When the intron is spliced out from pre-mRNA under the mediation of spliceosome, the triplet codons are disrupted by the inserted base, which would result in frameshift mutation of Rluc. Thus, Rluc is inactivated and fails to produce bioluminescence signal. When splicing is inhibited by a compound such as isoginkgetin, Rluc is in frame in the unspliced mRNA, and the translation of Rluc initiates from the AUG start codon. The bioluminescence signal could be detected with the addition of the substrate coelenterazine.

Characterization of the Rluc-SMN Reporter

We constructed three reporters for the detection of the splicing pattern. The Rluc-SMN reporter consists of the human SMN2 exon 6-intron6/7-exon 8 cassette upstream of the Rluc gene. The conserved spliced sequences of pre-mRNA are GT at the 5' splice site and AG at the 3' splice site, which are at both ends of intron. We also created a mutational reporter (Rluc mutant), which is the same with the Rluc-SMN except for containing the mutated 5' and 3' splicing sites. An intronless Rluc gene with no SMN2 minigene but that can express Rluc continuously was also constructed, which is designated as the Rluc-control reporter (Figure 2A).

To characterize the splicing reporters, the three reporters were transfected into 293 cells. Then the luciferase expression was measured using a luciferase reporter assay. As shown in Figure 2B, the luciferase intensity from Rluc-SMN was remarkably lower than that from Rluc-control or Rluc mutant, suggesting that Rluc is out of frame and inactivated in the Rluc-SMN reporter. To further verify the natural splicing activity of these reporters, RT-PCR was conducted to examine the total RNA from the cells transfected with equal amounts of Rluc reporters. The RT-PCR analysis confirmed that pre-mRNA from Rluc-SMN were mostly spliced, whereas those from Rluc mutant were not (Figure 2C). Therefore, the constructed splicing reporters could be further utilized to monitor RNA splicing patterns.

Measuring Splicing Activity upon Extrinsic Stimuli

To exclude the effect of the splicing reporters on cell viability, different concentrations (0, 0.25, 0.5, 1, and 2 μg/mL) of Rluc-SMN or Rluc mutant constructs were transfected into 293 cells, and the cell viability was further examined by CCK-8 assay. With the concentration increasing, Rluc-SMN had no effect on cell viability after 12 h (Figure 3A) or 24 h transfection (Figure 3B). We observed a similar phenomenon in Rluc-mutant-transfected cells (Figures 3C and 3D). These results suggested that the splicing reporters had no influence on cell viability.

To further validate the efficiency of the Rluc-SMN reporter for sensing splicing change upon extrinsic stimulation, 293 cells were transfected with the three reporters and then were treated with...
40 µM ISO or DMSO. ISO was proved to be a general inhibitor of pre-mRNA splicing by blocking the recruitment of U4/U5/U6 snRNPs.\textsuperscript{20} As shown in Figure 4A, the luciferase intensity from Rluc-SMN group increased obviously after ISO treatment, whereas the luciferase signals from Rluc-control or Rluc mutant reporter had no obvious change. To validate that the luciferase change was caused by splicing inhibition, RT-PCR assay was conducted to measure the total RNA from cells treated with ISO or DMSO. Compared to DMSO treatment, the exposure to ISO resulted in a significant increase in Rluc-SMN pre-mRNA accumulation but not in the Rluc mutant group (Figure 4B). These results suggest that the Rluc-SMN reporter successfully responded to extrinsic splicing compounds.

**Quantitative and Real-Time Imaging of Splicing Activity with the Rluc-SMN Reporter**

To test whether Rluc-SMN responded to ISO stimulation in a concentration dependent manner, Rluc-SMN was transfected into cells, and then they were treated with an increased dose of ISO (10 µM, 20 µM, 30 µM).
As shown in Figure 5A, the luciferase intensity was increased according to the increased dose of ISO. Consistent with the luciferase assay results, the RT-PCR analysis demonstrated that ISO treatment led to an increased accumulation in unspliced pre-mRNA (Figure 5B). In contrast, ISO had little influence on the luciferase activity (Figure 5C) or pre-mRNA expression (Figure 5D) in Rluc mutant-transfected cells, as evidenced by luciferase assay or RT-PCR assay. To further assess the feasibility of the reporter genes for in vitro bioluminescence imaging, the Rluc-SMN or Rluc mutant-transfected cells were treated with different doses of ISO. The bioluminescence signals were observed to be increased with increased concentrations of ISO in Rluc-SMN-transfected cells (Figures 6A and 6B), but not in the Rluc-mutant-transfected group (Figures 6C and 6D).

To further examine the performance of Rluc-SMN in detecting splicing patterns in real time, Rluc-SMN-transfected cells were treated with 40 μM ISO at different time points (0, 2, 4, 6, 8, 10 h). The luciferase activity was consistently increased with the increase of exposure time to ISO (Figure 7A). To evaluate whether there exists a maximum signal after treatment with splicing inhibitors, the Rluc-SMN-transfected cells were stimulated with ISO for an extended period of time (0, 20, 24, 42, 48, 72 h). Unexpectedly, the luciferase

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**Figure 4. Detecting Splicing Activity Responded to Splicing Inhibitor**

(A) The Rluc-control, Rluc-SMN, and Rluc mutant plasmids were transfected into 293 cells. 24 h later, the cells were treated with ISO (40 μM) or DMSO for 24 h. Then the luciferase activities were measured. (B) RT-PCR was carried out to measure the reporter mRNAs from cells treated as in (A). Data are shown as means ± SD. ***p < 0.001.

**Figure 5. Measuring Splicing Activity after Different Doses of ISO Treatment**

(A) The Rluc-SMN transfected cells were treated with DMSO or different doses of ISO (10, 20, 40 μM) for 24 h. Then the luciferase activities were measured. (B) RT-PCR was performed to analyze the total RNA isolated from the Rluc-SMN-transfected cells, which were treated with the different doses of ISO. (C) The Rluc mutant transfected cells were treated as in (A). The luciferase activities were measured. (D) RT-PCR was performed to analyze the total RNA isolated from the Rluc-mutant-transfected cells, which were treated as in (B). Error bars represent the standard deviations for three independent experiments. ***p < 0.001.
signal reached a maximum at the 42-h time point and then gradually decreased (Figure 7B). Taken together, these results indicated that the Rluc-SMN reporter could successfully monitor the splicing activity in a dose- and time-dependent manner.

**In Vivo Imaging of Splicing with Rluc-SMN**

To assess the efficacy of the Rluc-SMN reporter for in vivo imaging of splicing activity, the Rluc-SMN-transfected SGC7901 cells were implanted in the right flank of nude mice (n = 5). The image was acquired at different time points (0, 4, 8, 12, 24 h) after intraperitoneal injection of 10 mg/kg ISO into mice. We observed that the bioluminescence signal gradually increased and reached the maximum at the 8-h time point (Figure 8A). Then the signal decreased afterward. A quantitative analysis of the region of interest (ROI) revealed that the luminescence intensity first increased, followed by a decreasing trend after 8 h (Figure 8B). Therefore, these data indicated that the Rluc-SMN reporter is capable of monitoring pre-mRNA splicing patterns in vivo upon stimulation with exogenous splicing regulators.

**DISCUSSION**

In the present study, we designed an activatable reporter system, Rluc-SMN, for continuous and noninvasive imaging of pre-mRNA splicing patterns in vivo. The principle of this reporter gene is that the aberrant splicing by exogenous splicing inhibitors would result in an easily detectable positive signal, such as the activation of luciferase readout in our case. We demonstrated the efficiency of Rluc-SMN reporter, and it was able to provide in vivo quantitative information of pre-mRNA splicing in real time and in a dose-dependent manner. The reporter system worked through transient transfection in our study. When this construct is integrated into a stable cell line, the reporter system is well fit for high-throughput screening of small molecules that modulate splicing.

**Figure 6. In Vitro Bioluminescence Imaging of Splicing Activity**

(A and C) The Rluc-SMN (A) and Rluc mutant (C) were transfected into 293 cells. 24 h later, the cells were treated with different concentrations of ISO (10, 20, 40 μM). Then an in vitro bioluminescence imaging assay was performed. (B and D) The quantitative analysis of bioluminescence intensities in the region of interest (ROI) for (B) Rluc-SMN or (D) Rluc-mutant-transfected cells were performed. Data are shown as means ± SD. *p < 0.05, **p < 0.01.

**Figure 7. In Vitro Real-Time Monitor Splicing Activity Induced by ISO**

(A) Luciferase activity for Rluc-SMN-transfected cells treated with 40 μM of ISO at different time points (0, 2, 4, 6, 8, 10 h). (B) Luciferase activity for Rluc-SMN-transfected cells treated with 40 μM of ISO for prolonged time points (0, 20, 24, 42, 48, 72 h).
RNA splicing is traditionally measured by RT-PCR, northern blot, or primer extension analysis. These biochemical methods could not reflect the dynamic changes of pre-mRNA splicing during cellular processes. Some reporter systems were described to monitor normal splicing-related drug development and gene therapy by regulation of splicing with chemical compounds. The detection of the splicing process has attracted great interest because it involves many important biological processes. pre-mRNA splicing is a key mechanism of transcriptome modification, and its dysregulation is the root cause of many human diseases. However, it remains a challenge to noninvasively monitor a splicing event in living subjects. The open reading frame of a gene with a frameshift mutation can result in a genomic abnormality, in which the triplet codon is disrupted by the inserted base or deleted base. This may affect pre-mRNA splicing, resulting in a protein frameshift mutation. Therefore, we constructed the Rluc-SMN luciferase reporter in which a single base was inserted at the 3' end of exon 6 of SMN2 minigene. Since the intron is normally spliced out from pre-mRNA, the luciferase is out of frame in the spliced transcript and thus inactivated by a single-nucleotide insertion. Only the intron retained transcript can generate a luminescence signal. With this reporter, the splicing patterns under physiological conditions or external stimuli were visualized noninvasively and repeatedly in living cells and animals.

Figure 8. In Vivo Imaging of Splicing Stimulated by ISO
(A) The nude mice were implanted with Rluc-SMN-transfected SGC7901 cells and intraperitoneally injected with 10 mg/kg ISO. The bioluminescence imaging was performed before ISO treatment or at different time points after treatment with ISO (0, 4, 8, 12, 24 h). (B) The quantification of the bioluminescence signals from the region of interest in mice at different time point. Data are shown as means ± SD.

The detection of the splicing process has attracted great interest because it involves many important biological processes. pre-mRNA splicing is a key mechanism of transcriptome modification, and its dysregulation is the root cause of many human diseases. However, it remains a challenge to noninvasively monitor a splicing event in living subjects. The open reading frame of a gene with a frameshift mutation can result in a genomic abnormality, in which the triplet codon is disrupted by the inserted base or deleted base. This may affect pre-mRNA splicing, resulting in a protein frameshift mutation. Therefore, we constructed the Rluc-SMN luciferase reporter in which a single base was inserted at the 3' end of exon 6 of SMN2 minigene. Since the intron is normally spliced out from pre-mRNA, the luciferase is out of frame in the spliced transcript and thus inactivated by a single-nucleotide insertion. Only the intron retained transcript can generate a luminescence signal. With this reporter, the splicing patterns under physiological conditions or external stimuli were visualized noninvasively and repeatedly in living cells and animals.

RNA splicing is traditionally measured by RT-PCR, northern blot, or primer extension analysis. These biochemical methods could not reflect the dynamic changes of pre-mRNA splicing during cellular processes. Some reporter systems were described to monitor normal or alternative splicing in cells via fluorescent or luciferase protein activity readout. For instance, Nasim et al. developed a dual reporter system consisting of a luciferase and a β-galactosidase gene for detecting the ratio change in spliced and unspliced mRNA in mammalian cells. A two-color fluorescent reporter was developed by Peter Stoilov et al. to monitor the alternative splicing process and screen the compounds that affect the splicing of microtubule-associated protein tau (MAPT) exon 10. These reporter-based minigene systems, however, can detect only normally spliced products but not aberrant splicing products, which are usually generated by frameshift mutation or cryptic splice-site activation. Comparing it with the aforementioned reporter systems, our Rluc-SMN reporter was designed by placing a SMN2 minigene upstream of the Rluc gene. Due to the insertion of a single nucleotide into the exon, only the aberrant splicing transcript can yield luminescence signal. By employing the Rluc-SMN reporter, we observed that the Rluc intensity was responsive to the treatment of splicing inhibitors. The in vivo bioluminescence signals were acquired repeatedly in one mouse for as long as 24 h, providing the dynamic information of the splicing process. With the splicing inhibitor-responsive Rluc-SMN reporter, aberrant splicing associated with frameshift mutation was quantitatively visualized by measuring the luciferase activity without protein blotting. We believe that such a signal-on reporter can be also utilized to high-throughput screening of small compounds that regulate pre-mRNA splicing.

CONCLUSIONS
In summary, we have established a cell-based reporter, Rluc-SMN, for in vivo real-time monitoring of the aberrant pre-mRNA splicing patterns upon extrinsic stimulation. In principle, this reporter can be potentially applied in the noninvasive visualization of splicing-dependent processes and the high-throughput screening of splicing modulators in cells. This would be a critical advancement in the fields of splicing-related drug development and gene therapy by regulation of splicing with chemical compounds.

MATERIALS AND METHODS

Plasmid Construction
In order to generate the reporter gene Rluc-SMN, the exon 6–intron 6/7-exon 8 of the human SMN2 minigene was inserted into the upstream of the Rluc of a psiCHECK-1 vector (Promega). All the stop codons in the intron were removed, and a base was added at the 3’ end of exon 6 using the site-directed mutagenesis technology. We also designed a mutant construct (Rluc mutant) by introducing a mutation at both the 5’ and 3’ splice sites of SMN2 minigene. The empty vector psiCHECK-1, which has no SMN2 gene, was used as a control reporter (Rluc-control).

Cells Culture and Transfection
Human gastric cancer cell line SGC7901 and human HEK293 cells were cultured in DMEM medium (HyClone) supplemented with penicillin/streptomycin antibiotics (100 U/ml, penicillin, 100 g/mL streptomycin, Gibco) and fetal bovine serum (FBS; 10% v/v, Gibco) at 37°C in an incubator with 5% CO2. Cell transfection with the plasmids was performed using Lipofectamine 2000 (Invitrogen, Carlsbad,
CA, USA) following the manufacturer’s instructions. 4–6 h after transfection, the medium was changed, and the cells were incubated for another 24 h before analysis.

**Luciferase Assay**

For drug intervention experiment, the transfected cells were treated with different doses of ISO (Chembest, China). 24 h later, the cells were collected and lysed, and then the luciferase activity was measured using Glomax-20/20 Luminometer (Promega).

**RT-PCR**

The total RNA was isolated using Trizol reagents (Invitrogen) according to the manufacturer’s instructions. Then cDNA synthesis was performed using a DNA synthesis kit (Thermo Fisher) from 2 μg of total RNA. The cDNA product was then used for PCR reaction. The primer sequences are as follows: Rluc-SMN forward, 5'-GAT TCT CTT GAT GAT GCT GA-3'; Rluc-SMN reverse, 5'-TCT TTA GTG GTG TCA TTT AGT-3'. The primers are located at both ends of the exon of the minigene and the expected size of the PCR product is 166 bp.

**CCK-8 Assay**

The cells were transfected with Rluc-SMN and Rluc mutant plasmids in 96-well plates. After incubating at 37°C for 12 h or 24 h, the cells were added with CCK-8 reagents (10 μL/well, Beyotime Biotech, China) and were incubated at 37°C for another 4 h. The absorbance values at 450 nm were measured for each sample. All experiments were performed in triplicate.

**In Vitro Bioluminescence Imaging Assay**

The Rluc-SMN or Rluc mutant plasmids were transfected into 293 cells. After transfection for 24 h, the cells were washed with PBS, and coelenterazine substrate (Shanghai YEASEN, China) was added. Then the bioluminescence signals from cells were acquired by a Xenogen Lumina II system (Caliper Life Sciences). The bioluminescence intensity in the ROI was quantified and shown as average values (p/s/cm²/sr).

**In Vivo Bioluminescence Imaging in Nude Mice**

The animal experiments were conducted according to the Guidance for the Care and Use of Laboratory Animals approved by Xidian University. SGC7901 cells were transfected with Rluc-SMN plasmids. 24 h later, the cells (1 × 10⁶) were counted and resuspended in PBS (100 μL). Then the cells were subcutaneously implanted into the right flank of nude mice (6 weeks, n = 5, SPF Biotechnology, Beijing, China). ISO (10 mg/kg) was injected intraperitoneally (i.p.) into the mouse model. In vivo imaging system Lumina II (Xenogen) was employed to capture the bioluminescence signals. At 0, 4, 8, 12, and 24 h after ISO treatment, 2% isoflurane was used to anesthetize the mice, and coelenterazine (0.1 mg) was i.p. injected into mice to get the bioluminescence images. By using the living imaging software 4.1 (Xenogen), the luminescence intensity in the ROI was analyzed, and the luminescence intensity values were shown as p/s/cm²/sr.

**Statistical Analysis**

All data are shown as means ± SD from triple biological replicates. The Student’s t test was used to assess p values. p values under 0.05 were considered statistically significant.

**AUTHOR CONTRIBUTIONS**

J.X. performed most of the experiments. H.Z. analyzed the data. S.C., X.S., and W.M. helped to perform the experiments. J.X. and H.Z. wrote the manuscript draft. F.W. conceived and designed the experiments, analyzed the data, and finalized the manuscript.

**CONFLICTS OF INTEREST**

The authors declare no competing interests.

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