



# A ratiometric dual luciferase reporter for quantitative monitoring of pre-mRNA splicing efficiency *in vivo*

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Precursor messenger RNA (pre-mRNA) splicing is critical for cell growth and development, and errors in RNA splicing frequently cause cellular dysfunction, abnormal gene expression, and a variety of human diseases. However, there is currently a lack of reliable systems to noninvasively monitor the mRNA splicing efficiency in cells and animals. Here, we described the design of a genetically engineered ratiometric dual luciferase reporter to continuously quantify the changes in mRNA splice variants *in vivo*. This reporter system is encoded within a single polypeptide but on separate exons, thus generating two distinct luciferase signals derived from spliced and unspliced mRNAs. With this reporter, the two kinds of luciferase in the same individual can minimize the influence of indirect factors on splicing, and the ratio of these two luciferase intensities represents the dynamic splicing efficiency of pre-mRNA. Our study offers a convenient and robust tool for the screening and identification of small molecules or trans-acting factors that affect the efficiency of specific splicing reactions.

Eukaryotic genes consist of multiple introns and exons with different sequence lengths (1, 2). Upon transcription, precursor messenger RNA (pre-mRNA) undergoes a splicing reaction to remove the noncoding intermediate sequences, that is, introns are precisely removed and adjacent exons are fused to produce translatable mRNA (3). The mechanism involves two transesterification reactions within the spliceosome: 1) cleavage at 5' splice site accompanied by lariat formation at the branch point adenosine and 2) cleavage at 3' splice site accompanied by exon ligation (4). Intron excision reactions are difficult to spontaneously occur in cells and require the participation of spliceosome (5). The spliceosome refers to a multicomponent complex mainly composed of small molecules of nuclear RNAs (snRNAs) and more than 100 proteins (6). These snRNAs do not translate any proteins, but play an important role in regulating gene activities (7). Indeed, up to 90% of all human genes undergo alternative splicing, and aberrations in the pre-mRNA splicing account for at least 15% of inherited diseases due to mutations (8, 9). Therefore, an in-depth study of splicing

activities is extremely important for gene regulation, disease mechanisms, and drug development.

Northern blot (10), ribonuclease protection assay (11), and real-time polymerase chain reaction (RT-PCR) (12) are the common strategies used to detect the specific mRNA transcripts. Although they are important and powerful approaches, they require lysis of cells and are not applicable to real-time measuring the efficiency of splicing of an intron in living cells. Moreover, these methods are relatively time-consuming and do not lend themselves to high-throughput screening of factors that affect mammalian splicing. Several reporter systems that encode luciferase (13–15) or fluorescence protein (16, 17) have also been developed by our group and other researchers to visualize pre-mRNA splicing in living cells and animals. The signals derived from these luciferase enzymatic activities or protein expression are supposed to be proportional to the quantity of mRNAs, which in turn depends on the fraction of the spliced pre-mRNA. However, these systems are single reporter function and vulnerable to variations among cell growth conditions, cell numbers, or transfection efficiency. In addition, some false-positive results might arise due to the steps in the rates of mRNA transcription, degradation, or translation of these single reporter genes (18). Therefore, it remains a challenge to develop a reliable system to realize the measurement and quantification of mRNA splicing variants *in vivo*.

To eliminate the convoluted variables in either mRNA transcription or transfection efficiency, we designed a ratiometric dual reporter system in this study by introducing an intron containing a translation stop codon between the two luciferase genes, which are translated into a single polypeptide. The signal from the upstream luciferase (Firefly luciferase, Fluc) reflects the unspliced and spliced RNA, whereas the downstream luciferase signal (Renilla luciferase, Rluc) represents the spliced RNA. Thus, the ratio of the two luciferase signals (Rluc/Fluc) indicates the splicing efficiency of pre-mRNA. We showed that there is a good correlation between the Rluc/Fluc ratio and the amount of RNA splicing. Our results demonstrate that this ratiometric reporter system enables real-time and quantitative monitoring of splicing activity in living cells and mice, as well as offering a potential tool for

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high-throughput screening of splicing regulatory compounds, which would facilitate the treatment of human diseases caused by splicing defects.

## Results and discussion

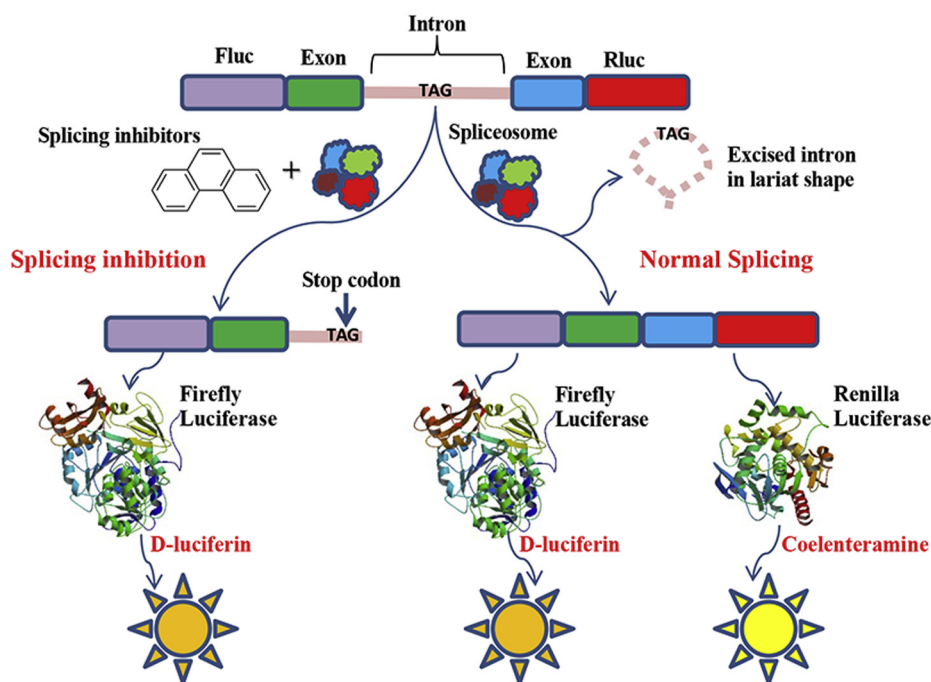
### Design of the ratiometric dual reporter system

To measure the splicing efficiency of pre-mRNA, we engineered a dual luciferase reporter system where two luciferase genes were fused with an exon–intron–exon minigene (Fig. 1). An in-frame termination codon (TAG) was introduced into the intronic region. Upon transfection of the dual luciferase reporters into mammalian cells, the pre-mRNA of this dual reporter would be processed in either of two ways. During normal splicing in cells, the internal stop codon would be removed, then the upstream reporter gene (Firefly luciferase, Fluc) and the downstream reporter gene (Renilla luciferase, Rluc) will be placed in the same reading frame to generate a fusion protein Fluc-Rluc. In the case that splicing is inhibited by splicing inhibitors, the stop codon in the intron would lead to the translation termination of the mRNA, thus producing the Fluc protein alone. Therefore, the Fluc gene is expressed regardless of whether splicing occurred, whereas the downstream Rluc gene can only be expressed after splicing. The ratio of Rluc to Fluc intensity after addition of respective substrate (D-luciferin or coelenteramine) represents the proportion of spliced transcripts, that is, the splicing efficiency of pre-mRNA.

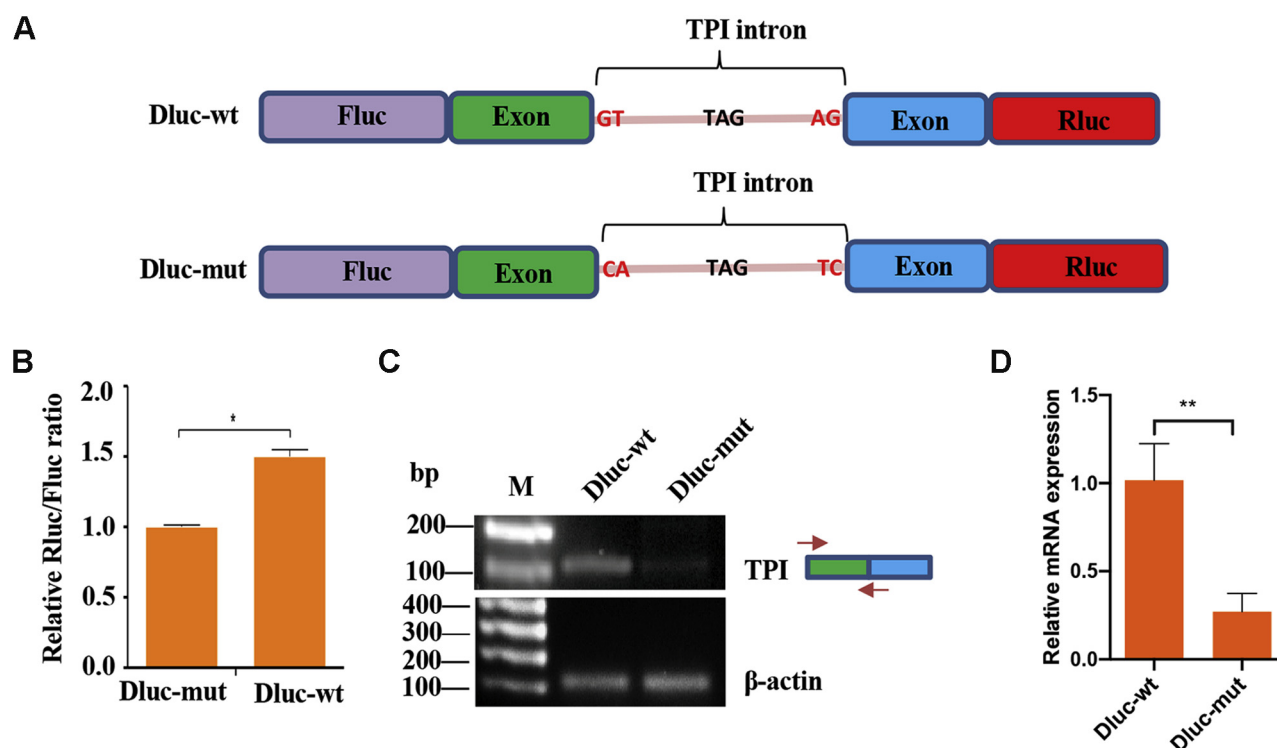
### Validation and characterization of the dual luciferase reporters

In this study, we developed two dual luciferase reporter genes to detect the splicing efficiency of pre-mRNA. One is the wild-type reporter Dluc-wt, the other is the mutant version of reporter Dluc-mut. The Dluc-mut is identical to the Dluc-wt, except that both the conserved 5' splice site GT and 3' splice site AG are mutated to CA and TC bases (Fig. 2A). To evaluate the effect of reporters on the cell viability, Dluc-wt and Dluc-mut were transfected into 239T cells at various concentrations of 0, 0.25, 0.5, 1, 2  $\mu\text{g}/\text{ml}$ , respectively. The effects of Dluc-wt and Dluc-mut on cell viability after 24 or 48 h transfection were determined by CKK-8 assay. Neither the Dluc-wt (Fig. S1, A and B) nor Dluc-mut (Fig. S1, C and D) showed obvious cytotoxicity after transfection for 24 or 48 h. We then explored the luciferase performance of the dual reporter by transfecting different concentrations of Dluc-wt into cells and measured the Fluc and Rluc intensity separately. With the increased concentration (0, 0.125, 0.25, 0.5, 1  $\mu\text{g}/\text{ml}$ ) of the Dluc-wt, both the Fluc (Fig. S2A) and Rluc (Fig. S2B) signal gradually increased in a dose-dependent manner, indicating a good proportional relationship between luciferase signal and reporter concentration.

In order to further verify these reporter constructs, we transfected Dluc-wt and Dluc-mut into 293T cells, respectively. Then the luciferase activities of Fluc and Rluc were measured and the ratio of Rluc to Fluc was calculated (Fig. 2B). The results showed that the relative Rluc/Fluc ratio in Dluc-mut-transfected cells was significantly lower than that in



**Figure 1. Schematic diagram of the dual reporter gene.** The dual luciferase reporter encodes a firefly luciferase (Fluc) and a Renilla luciferase (Rluc), which are fused in frame via an exon–intron–exon minigene. The minigene contains an in-frame translation stop codon TAG in the intronic region. Upon transfection of this reporter in cells, two different types of mRNAs would be produced. In the case of normal splicing, the stop codon would be spliced out, which results in the generation of Fluc-Rluc fusion protein. On the contrary, when the mRNA splicing is inhibited by splicing inhibitors, the stop codon would be reserved in the RNA product and leads to the expression of Fluc alone.



**Figure 2. Verification of the reporter genes.** A, schematic diagram of Dluc-wt and Dluc-mut reporters. GT and AG bases in the Dluc-wt indicate the conserved 5' splice site and 3' splice site, respectively. TAG indicates the translational stop codon in the intronic region. GT and TC in the Dluc-mut indicate the mutated splice sites. B, the same amount (1  $\mu$ g) of Dluc-wt and Dluc-mut plasmids was transfected into 293T cells, respectively. Twenty-four hours later, the dual luciferase assay was carried out to measure the luminescence intensity of Fluc and Rluc respectively, and then Rluc/Fluc ratio was calculated in each transfected group. The relative Rluc/Fluc ratio was shown. The Rluc/Fluc ratio in Dluc-mut group was 1. Data are expressed as mean  $\pm$  SD. \* $p$  < 0.05. C, RT-PCR was performed to analyze the total RNA extracted from the above transfected groups. The representative agarose gel picture indicated the spliced mRNA. M indicates DNA Marker. The arrows indicate the forward and reverse primer positions on the reporter construct. D, qPCR analysis of the spliced mRNA from the abovementioned transfection group as in (C).  $\beta$ -actin was used as the internal control.

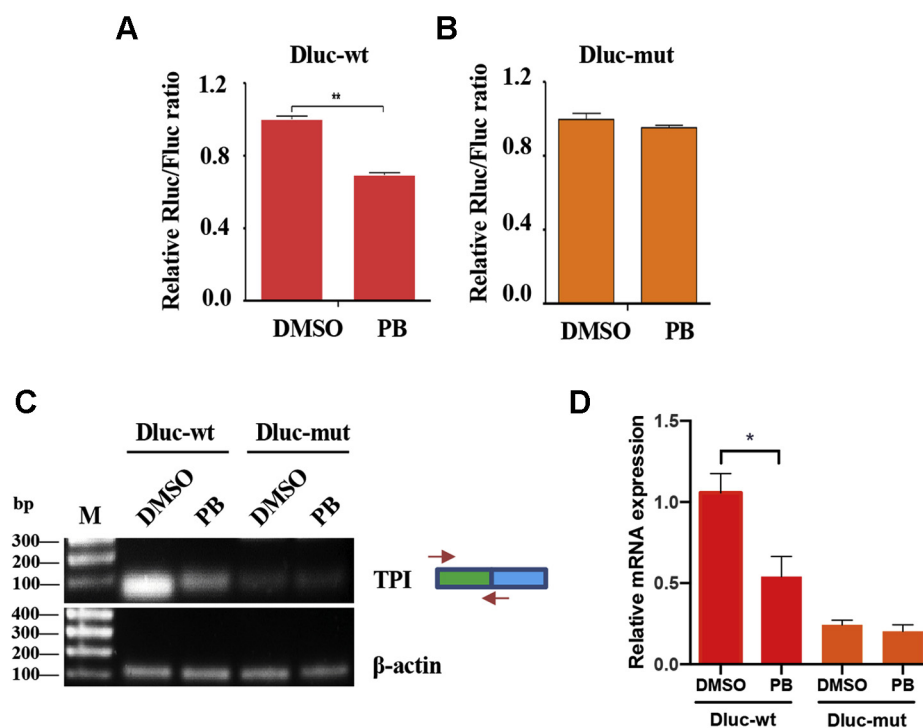
cells transfected with Dluc-wt, indicating that the mutated splice sites suppressed the mRNA splicing and led to the expression of Fluc alone. To further validate that this result was caused by splicing inhibition, RT-PCR and qPCR were performed to determine the amount of spliced RNA (Fig. 2, C and D). The RT-PCR and qPCR results agreed well with the luciferase reporter analysis, showing that most of pre-mRNA from Dluc-wt was spliced out but Dluc-mut splicing was inhibited. Therefore, the splicing efficiency could be reflected by the luciferase ratio of these reporters according to the luciferase intensity.

#### Detecting the splicing efficiency change stimulated by splicing inhibitors

To detect the splicing efficiency change upon the splicing is inhibited, Dluc-wt and Dluc-mut were transfected into 293T cells and followed by treatment with a natural compound pladienolide B (PB), which has been proved to inhibit pre-mRNA splicing by binding to SAP130 in the splicing factor SF3b complex (19). The results demonstrated that the Rluc/Fluc ratio in Dluc-wt transfected cells was significantly decreased after PB treatment (Fig. 3A), whereas the ratio in Dluc-mut reporter had no obvious change (Fig. 3B), indicating that PB could inhibit the splicing of Dluc-wt but not Dluc-mut

reporter. To verify that the Rluc/Fluc ratio change was due to splicing inhibition, RT-PCR and qPCR were performed to analyze the total RNA extracted from Dluc-wt or Dluc-mut transfected cells after PB treatment. As shown in Figure 3, C and D, the exposure to PB induced a decreased amount of spliced Dluc-wt mRNA. In contrast, there is no significant change of spliced mRNA products in Dluc-mut group before and after PB treatment. These results prove that the change in the luciferase intensity ratio was indeed due to the inhibition of splicing.

In order to verify whether this reporter works for other genes, we chose an endogenous gene Coilin with constitutive splicing between exon 2 and exon 3 (20). We replaced the TPI minigene with the coilin minigene containing exon 2–intron 2–exon 3 to generate a new reporter Dluc-coilin (Fig. S3A). We performed dual luciferase assay, RT-PCR and qPCR to validate this Dluc-coilin reporter after treatment with PB. The luciferase assay results demonstrated that the Rluc/Fluc ratio in Dluc-coilin transfected cells was significantly decreased after PB treatment (Fig. S3B). The RT-PCR (Fig. S3C) and qPCR (Fig. S3D) results showed that the exposure to PB induced an approximately 50% decreased amount of spliced Dluc-coilin mRNA. These results suggest that our reporter system can work for other endogenous genes.



**Figure 3. The effect of splicing inhibitors on splicing efficiency change.** A, Dluc-wt or B, Dluc-mut plasmids were transfected into 293 cells respectively. After 24 h, the transfected cells were treated with DMSO or pladienolide B (PB, 1000 nM) for 24 h. Then the luciferase intensity of Fluc and Rluc was measured and Rluc/Fluc ratio was calculated, respectively. The Rluc/Fluc ratio in DMSO group was 1. Data are expressed as mean  $\pm$  SD.  $**p < 0.01$ . C, the cells were transfected and treated as described in (A and B). RT-PCR was performed to detect the spliced RNA from the cells after DMSO and PB treatment. A representative agarose gel picture was shown. M indicates DNA Marker. D, the transfected cells were treated as in (C). qPCR was performed to detect the spliced RNA from the cells.  $\beta$ -actin was used as the internal control.

#### Quantitative monitoring of splicing efficiency in living cells

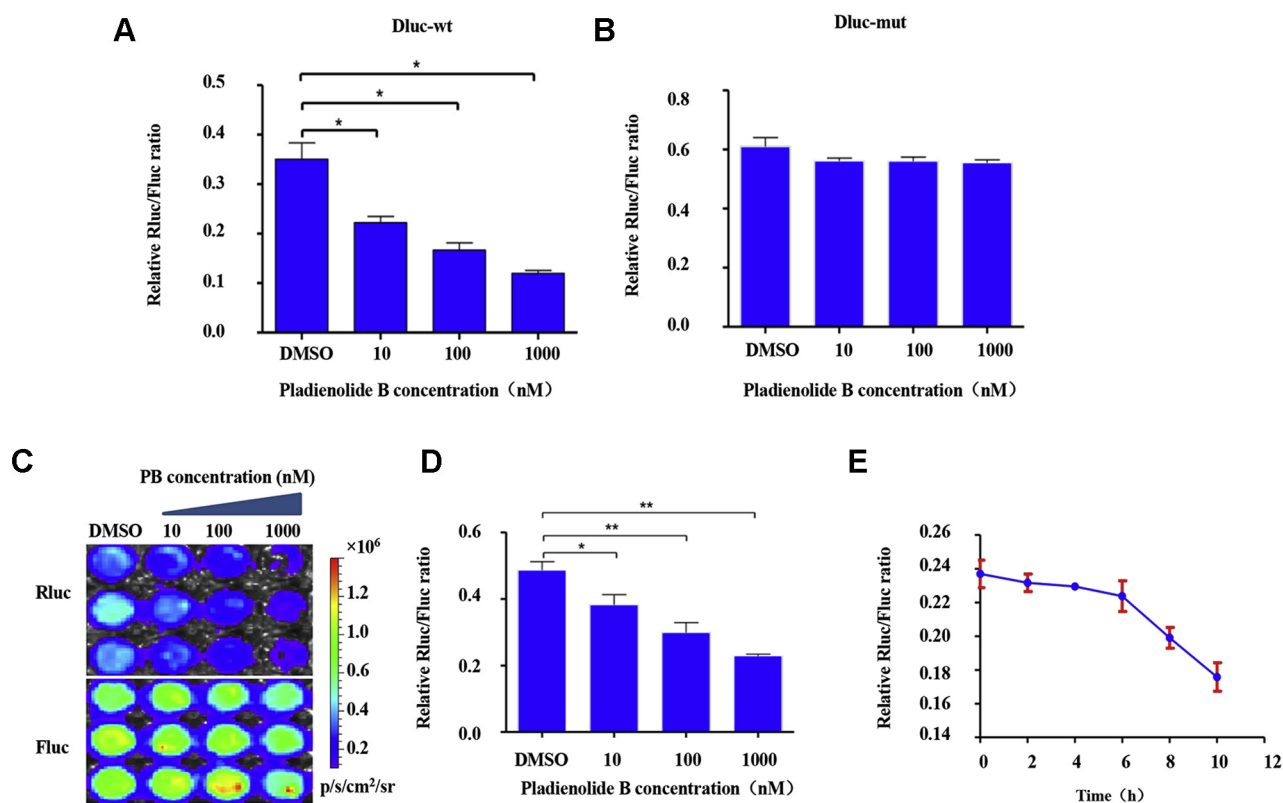
To further quantitatively monitor the splicing efficiency with the dual luciferase reporters, Dluc-wt and Dluc-mut were transfected in 293T cells and treated with different concentrations of PB (10, 100, 1000 nM). As the PB concentration increased, the ratio of Rluc/Fluc gradually decreased in Dluc-wt-transfected cells (Fig. 4A), indicating that the effect of splicing inhibition is related to the concentration of the splicing inhibitors. In contrast, PB had little influence on the Rluc/Fluc ratio of Dluc-mut (Fig. 4B). To further investigate the bioluminescence performance of reporters in the cells, the Dluc-wt transfected cells were treated with different dose of PB and assessed their Rluc and Fluc luminescence signals, respectively. The Rluc luminescence signal was observed to be decreased with the increase dose of PB, but the Fluc signal did not change significantly (Fig. 4C). The quantitative region of interest (ROI) analysis of Rluc and Fluc showed that the Rluc/Fluc ratio also decreased according to the increased dose (Fig. 4D), consistent with the luciferase assay results. To further quantify the splicing efficiency in real time with the reporter, Dluc-wt was transfected into the cells and treated with 1000 nM PB for different times (0, 2, 4, 8, 10 h). As shown in Figure 4E, the Rluc/Fluc ratio gradually decreased with the exposure time of treatment with PB. Taken together, these results suggest that Dluc-wt reporter enabled ratiometric sensing the splicing efficiency in a dose- and time-dependent manner.

#### In vivo ratiometric imaging of splicing efficiency with Dluc-wt reporter

To ratiometric imaging of splicing efficiency with Dluc-wt reporter, the Dluc-wt transfected 4T1 mouse breast cancer cells were implanted in mice ( $n = 5$ ) to establish the xenograft mouse model. The Rluc and Fluc bioluminescence images were acquired before and after intraperitoneally administration with PB (100  $\mu$ g/kg) for 12 h. The Rluc bioluminescence signal was observed to decrease obviously after injection with PB (Fig. 5, A and B). In contrast, the Fluc signal did not change significantly before and after PB administration (Fig. 5, C and D). A quantitative analysis of the ROI in Rluc and Fluc revealed that the Rluc/Fluc ratio was remarkably lower in 12 time point group than that in 0 h group (Fig. 5E). Collectively, these results demonstrated that our Dluc-wt reporter is capable of monitoring the splicing efficiency *in vivo* upon stimulated by splicing regulators.

#### Conclusions

We have developed a genetically engineering ratiometric dual luciferase reporter to quantitatively monitor pre-mRNA splicing efficiency in living cells and animals. Different from the conventional approaches for measuring splicing efficiency, our reporter system contains two distinct luciferase signals that are derived from the translation of both unspliced and spliced mRNA in mammalian cells. Fluc is expressed regardless of splicing, downstream Rluc is expressed only after



**Figure 4. Measuring the splicing efficiency change after different doses of PB treatment.** A, Dluc-wt and B, Dluc-mut constructs were transfected into 293T cells, respectively. After 24 h, the cells were treated with different concentrations of PB (10, 100, 1000 nM) for 24 h. Then the luciferase intensities of Fluc and Rluc were measured and Rluc/Fluc ratio was calculated, respectively. Data are expressed as mean  $\pm$  SD. \* $p < 0.05$ . C, the Dluc-wt transfected cells were treated with pladienolide B at different concentrations for 24 h. Then *in vitro* bioluminescence imaging was performed to capture the Rluc and Fluc signals. D, quantitative analysis of bioluminescence intensity in the region of interest (ROI) was performed, and the Rluc/Fluc ratio was calculated. Data are expressed as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ . E, Dluc-wt was transfected into 293T cells and then treated with PB (1000 nM) for different times (0, 2, 4, 6, 8, 10 h). Then the bioluminescence intensity of Fluc and Rluc at different time point was measured, respectively. Then the Rluc/Fluc ratio was calculated. Data was presented as means  $\pm$  SD.

splicing and fusion with Fluc. We demonstrated that the ratio of Rluc/Fluc signal was well correlated with the proportion of spliced mRNA products. The data obtained from luciferase activity was also confirmed by traditional method RT-PCR and qPCR to further validate our dual reporter system. Our design may afford a potential ratiometric quantitative platform for determining mRNA splicing efficiency in living subjects, as well as a robust tool for high-throughput screening of chemicals that affect splicing activity.

## Experimental procedures

### Reporter plasmids construction

We firstly designed a wild-type dual luciferase reporter gene system (Dluc-wt), which contains reading frames for firefly luciferase (Fluc) and renilla luciferase (Rluc). These two luciferase genes were separated by an exon–intron–exon TPI minigene (14) containing a stop codon in the intron region. The minigene comprised an exon1 (88 nt), intron (128 nt), and exon2 (38 nt), all of which were inserted after the Fluc gene. In addition, we also designed a mutant reporter (Dluc-mut) by mutating both the 5' and 3' splice sites (GGT to AAA, GGC to AAA) through site-directed mutagenesis. We also designed a reporter gene (Dluc-coilin) to replace the TPI to coilin

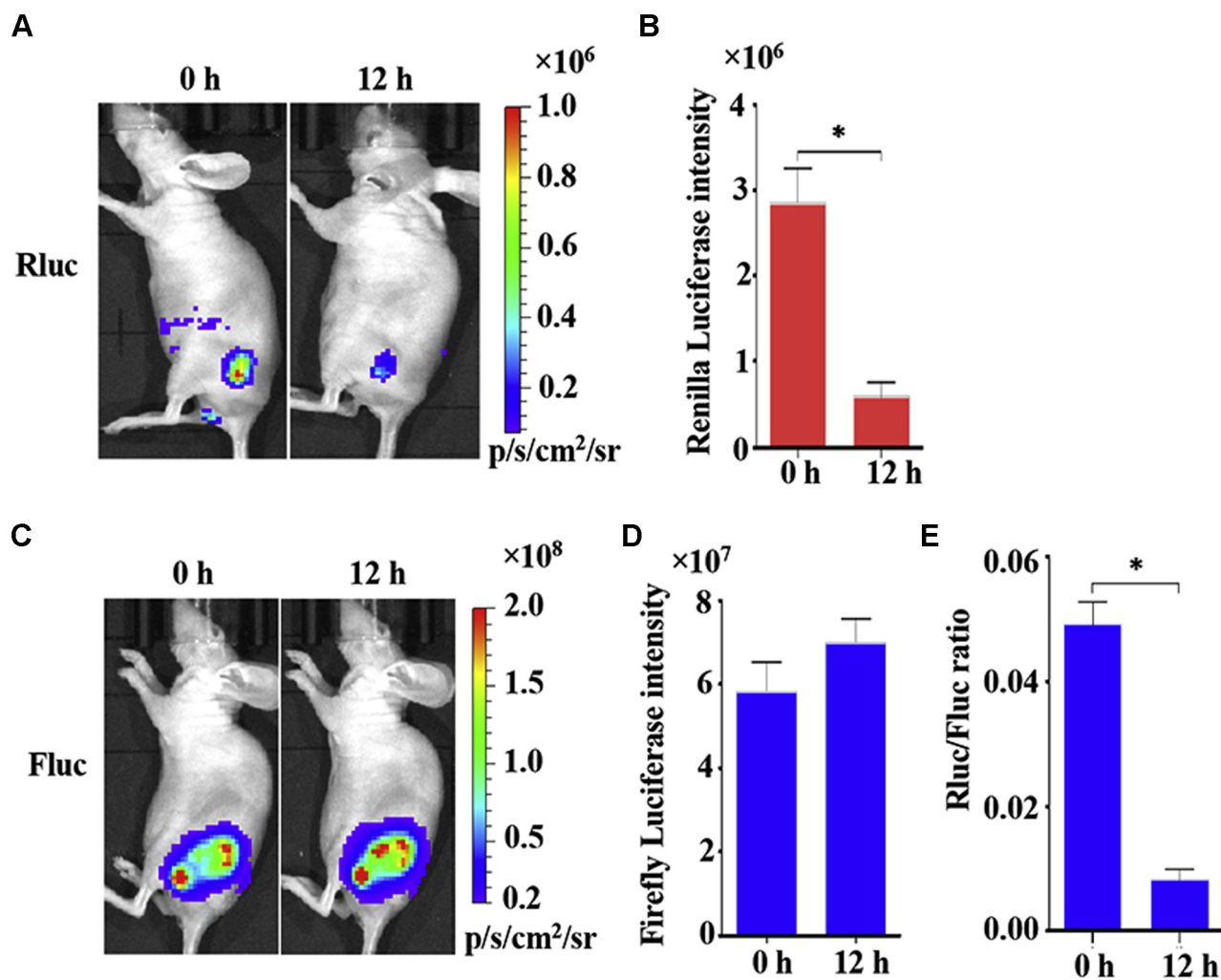
minigene to verify the system. The coilin minigene consists of exon 2 (87 nt), intron (124 nt including stop codon), and exon 3 (87 nt). The above sequences were synthesized (Shanghai Generay Biotech Co, Ltd) and inserted into the KpnI/XhoI site of pcDNA3.1<sup>(+)</sup> construct (Invitrogen). Finally, both the Dluc-wt and Dluc-mut reporters were sequenced to ensure the correctness of the construction.

### Cell culture and transfection

HEK239T cells (human embryonic kidney cells) were cultured in high-glucose DMEM medium (Hyclone) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin solution (Hyclone). 4T1 cells (mouse breast cancer cells) were cultured in RPMI1640 medium (Source Leaf) with 1% penicillin-streptomycin solution (Hyclone) and 10% FBS. All the cells were grown at 37 °C under 5% CO<sub>2</sub> humidity. The cells were transfected with Lipofectamine 2000 (Invitrogen), and the medium was changed 6 h after transfection according to the manufacturer's instructions.

### Dual luciferase assay

In order to detect the luciferase activity of Fluc and Rluc, cells were seeded in 24-well plates in a 5% CO<sub>2</sub> incubator. Next



**Figure 5.** *In vivo* imaging of splicing efficiency in xenograft mouse model. A–D, Dluc-wt transfected 4T1 cells were implanted on the left side of the mice (n = 5) to establish the xenograft mouse model. The (A) Rluc and (C) Fluc bioluminescence images were taken at designated time points (0, 12 h) before and after the intraperitoneal injection of PB (100 μg/kg) into mice. The quantification of the (B) Rluc and (D) Fluc bioluminescence signals from the region of interest (ROI) in mice at 0 or 12 h time point. E, the Rluc/Fluc ratio was calculated at different time point based on the ROI bioluminescence signals of Fluc and Rluc in mice. Data was presented as means ± SD. \*p < 0.05.

day, the different reporter plasmids were transfected into cells and cultured for 24 h. Then the cells were harvested and lysed in cell lysis buffer (CLB, TransGen Biotech). Finally, the dual luciferase assay was performed with dual-luciferase reporter assay system (Cat. No. E1910, Promega) using a Glomax-20/20 luminometer (Promega). For drug treatment experiments, pladienolide B (Cayman Chemical Company) was dissolved in DMSO. The cells were then treated with pladienolide B at different concentrations. After the harvested cells were lysed, the luciferase assay was also performed as above.

#### Reverse transcription–polymerase chain reaction (RT-PCR) and quantitative real-time PCR (QPCR)

Total RNA was isolated using Trizol (Invitrogen) followed by RT-PCR using the RevertAid First Strand cDNA Synthesis Kit (ThermoFisher) according to the manufacturer’s instructions. Specifically, 1 μg of total RNA was incubated with a component mixture containing oligo (dT) primer, M-MLV enzyme, dNTP mixture, RNase inhibitor, and reaction buffer

for 60 min at 42 °C. The reaction was then terminated by heating at 70 °C for 5 min. The product of the first cDNA was then used for PCR in a total volume of 25 μl. The PCR procedure included 95 °C for 5 min, 30 cycles for 95 °C 30 s, 56 °C for 30 s, and 72 °C for 30 s, and a final extension 5 min at 72 °C. The qPCR experiments were conducted with 7300 real-time PCR system (Applied Biosystems). In total, 10 μl of 2 × miRNA qPCR Premix (including SYBR) (10 μM) was added to the 20 μl system containing 0.4 μl each of the two primers, 2 μl cDNA product. The PCR program includes 94 °C for 2 min and 40 cycles (94 °C for 20 s, 60 °C for 30 s). All primer sequences are shown in Table S1.

#### Cytotoxicity analysis

To determine the cytotoxicity after transfection of two reporter constructs, the Cell Counting Kit-8 (CKK-8, Dojindo Laboratories) was used to detect the cytotoxicity after transfection of two reporter constructs at different concentrations for 24 h and 48 h. CCK-8 reagents (10 μl/well) were added to

the plates and incubated in the dark at 37 °C for 4 h. The obtained absorbance values were measured at 450 nm for each sample. All cell experiments were performed in triplicates.

### In vitro luminescence imaging assay

293T cells were transfected with different reporter plasmids. After 24 h, the transfected cells were washed with phosphate buffered saline (PBS, pH 7.4), and D-luciferin substrate (Shanghai Yeasen Company) was added. Luminescence signals for Fluc were captured using the Xenogen Lumina II system (Caliper Life Sciences). Then the coelenterazine substrate (Shanghai Yeasen Company) was added into the same plate, and the luminescence signal for Rluc was also detected using the same method. The ROI of luminescence intensity was quantified and presented as average values (p/s/cm<sup>2</sup>/sr).

### In vivo bioluminescence imaging

All animal experiments were performed in accordance with the Guidance for the Care and Use of Laboratory Animal approved by Xidian University. The Dluc-wt reporter was transfected into 4T1 cells. Twenty-four hours after transfection, 1x 10<sup>8</sup> cells were harvested and resuspended in 100 µl of PBS. These cells were then implanted subcutaneously into the left flank of nude mice (6 weeks of age, n = 5) obtained from SPF (Beijing) Biotechnology Co, Ltd. At a tumor volume of approximately 100 mm<sup>3</sup>, the xenograft mouse model was injected intraperitoneally (ip) with pladienolide B (100 µg/kg). For bioluminescence imaging of nude mice, mice were anesthetized with 2% isoflurane. At 0 and 12 h time point after pladienolide B treatment, 0.01 mg of coelenterazine was injected intraperitoneally into mice, and the bioluminescence imaging was performed using the *in vivo* imaging system Xenogen Lumina II. After Rluc luminescence was completely quenched, 3 mg of D-Luciferin was injected intraperitoneally into the same mice to obtain the Fluc luminescence image using the above method. Plot the ROI and analyze the luminescence intensity as p/s/cm<sup>2</sup>/sr using Living Imaging Software 4.1 (Xenogen).

### Statistical analysis

All data are shown as the mean ± SD of three biological replicates. Student's *t* test is used to evaluate the *p*-value. *p* value below 0.05 was considered statistically significant.

### Data availability

The data used to support the findings of this study are contained within the manuscript.

**Supporting information**—This article contains [supporting information](#).

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**Author contributions**—F. W. conceptualization; B. G., Z. M., M. J., and C. T. data curation; B. G. and Z. M. formal analysis; F. W. investigation; F. W. project administration; X. S. resources; X. S., M. J., and C. T. software; F. W. supervision; X. S. and C. T. validation; B. G. writing—original draft; F. W. writing—review and editing.

**Conflict of interest**—No potential conflicts of interest were disclosed.

**Abbreviations**—The abbreviations used are: CLB, cell lysis buffer; FBS, fetal bovine serum; Fluc, firefly luciferase; pre-mRNA, precursor messenger RNA; Rluc, renilla luciferase; ROI, region of interest; RT-PCR, reverse transcription–polymerase chain reaction; snRNAs, small molecules of nuclear RNA.

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**ACCELERATED COMMUNICATION:** Reporter for monitoring of splicing efficiency

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