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Novel riboswitches regulate AAV delivered transgene expression in mammals via small molecule inducers

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Abstract

As the field of gene therapy has progressed multiple elements of viral vectors have been optimized to increase potency, specificity and safety of these therapies, including the development of engineered promoters and transcriptional regulatory elements. However, until now the development of tight temporal control of gene therapies using oral small molecules has remained elusive, and protein-based switches have proved of little use due to low dynamic range, low levels of expression and potential for immunogenicity.

Here we present a potent gene regulation platform based on rationally designed synthetic riboswitches built in mammalian cells. These riboswitches drive a splicing-based expression platform engineered to create an 'on' switch in the presence of specific aptamer small molecule binding. Small molecule binding results in hairpin stabilization sequestering the splice site of an alternative exon. This platform regulates protein expression with high dynamic range and allows precise control of transgene expression levels. The extremely high dynamic range of this switch has allowed us to screen, identify and modify novel aptamers that bind and respond to novel small molecules. This platform is modular and adjustable and is optimized for each transgene to achieve the required expression level and dynamic range. This regulation platform has been demonstrated to work in multiple genes, multiple cell types *in vitro* and *in vivo*. When delivered through an AAV vector to the liver or the muscle in mice, the engineered riboswitches reversibly regulate transgene expression via an orally delivered small molecule inducer, providing precise control of transgene expression.

Thus, our potent gene regulation system provides a platform for using unique aptamer ligand pairs to regulate genes in mammals. This platform enables precise temporal and spatial control of gene expression expanding the range of possibilities for using gene based vectors as therapies.

Synthetic Riboswitch and Expression Platform Cassette

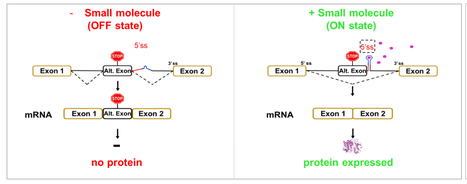

Figure 1. Schematics of synthetic riboswitch and expression platform cassette

Aptamer is inserted in the downstream intron of an Intron-AltExon-Intron cassette within the cDNA of transgene. The Alternative Exon contains a stop codon which when spliced into the mRNA drives nonsense mediated decay of the message with no protein produced. Riboswitch binding to small molecule results in the entire cassette being spliced out stable mRNA is produced and protein expressed.

In the absence of small molecule (left panel):
Alt. exon 5' ss is accessible
Alt. exon is included
No protein expressed
In the presence of small molecule (right panel):
Alt. exon 5' ss is sequestered
Alt. exon is skipped
Protein expressed

Hairpin Riboswitch and Splicing Expression Platform Cassette Optimization

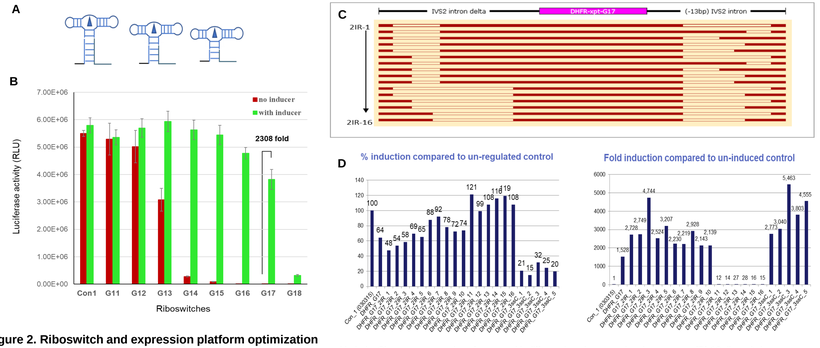

Figure 2. Riboswitch and expression platform optimization

(A) Riboswitch activity depends on the length of the stem connecting the aptamer and hairpin. Shown are examples of stems with different length connecting the aptamer. **(B)** 18 riboswitch constructs were generated with the difference in the length of the hairpin stem connecting the aptamer sequence. The activity of riboswitches were tested in HEK 293 cell for regulating luciferase expression in response to small molecule treatment to identify the hairpin length that results in the tightest regulation. G17 provides the highest level of expression with the lowest background resulting in 2308 fold dynamic range. **(C)** Expression platform activity is modulated by intron sequences and the 3'ss of alternative exon. Schematics of deletions in the introns and generation of riboswitches with deletions in both upstream and downstream intron sequences of the alternative mutant DHFR exon (constructs 2IR-1 to 16). 3'ss was mutated to generate construct 3ssC_1 to 5. The lower panel **(D)** shows for each of the deleted and mutated constructs the % induction relative to the unregulated control construct without the cassette (left graph) and the dynamic range / fold induction for each construct (right graph). When a cassette is optimized in the cDNA sequence of any gene the target 'on' expression level is 100%-120% of unregulated and a dynamic range of >1500 is targeted at the highest small molecule dose *in vitro*.

Regulation of gene expression with Hairpin Riboswitch and Splicing Expression Platform

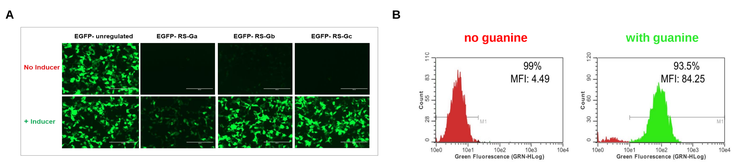

Figure 3. **(A)** HEK 293 cells were transfected with EGFP constructs containing riboswitches with different hairpin lengths driven by the guanine aptamer. Ga, Gb or Gc. The transfected cells were treated with either vehicle control = no inducer (top panel) or with small molecule inducer (bottom panel). The riboswitch sequence modulates both the 'on' level and the dynamic range of the switch. **(B)** EGFP expression from HEK 293 cells stably transfected with EGFP construct containing riboswitch G17 before and after guanine treatment. Red represents cells without GFP expression and green shows cells expressing GFP following induction with guanine.

Different Aptamer Sequences can be used interchangeably in the Splicing Expression Cassette

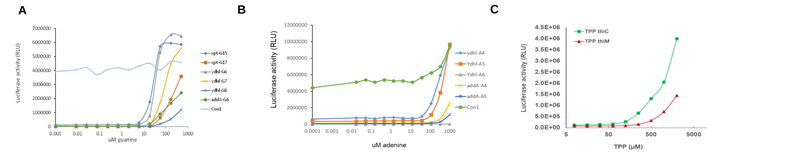

Figure 4. Aptamers that drive synthetic riboswitch in the splicing expression platform cassette are interchangeable. The switch can be driven by multiple aptamers interchangeably each of which binds specifically to its own target small molecule. Different aptamer small molecules drive different expression levels and dynamic ranges. HEK 293 cells were transfected with Luciferase constructs with riboswitches containing guanine aptamers (A), adenine aptamers (B) or TPP aptamers (C). Transfected cells were treated with the aptamer ligands at the indicated doses of guanine (A), adenine (B) or TPP (C). The graphs show the dose responses of the switch to different aptamer binders. In A and B Con 1 is the expression level of the control construct with no riboswitch cassette.

Screen for Novel small molecule binding Aptamers within the Riboswitch Cassette

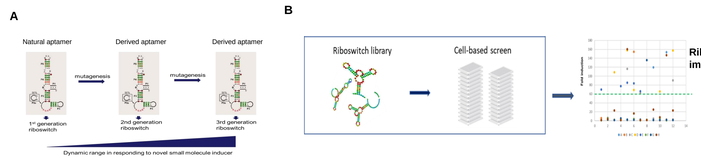

Figure 5. Development of riboswitches regulated by novel aptamer sequences binding to new small molecules

(A) The first generation riboswitch was built using a series of natural aptamers which respond to their natural target small molecule such as guanine, adenine or TPP. To create switches that respond specifically to novel small molecules, libraries of natural aptamers were mutated and screened against libraries of small molecules in HEK293 cells and novel small molecule aptamer pairs that drive switch 'on' of gene expression are selected. Next generation riboswitches contain aptamers derived from the natural aptamer through mutagenesis. **(B)** Schematics of cell-based high throughput screening to identify the riboswitches with enhanced activity as indicated by the relative level of small molecule induced-expression of luciferase reporter gene.

Evolution of aptamer sequence to improve specificity and potency of small molecule binding

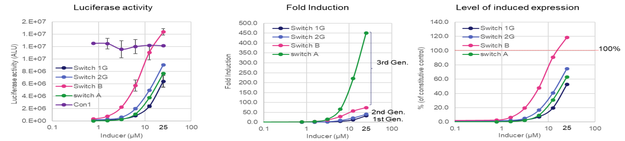

Figure 6. 2nd and 3rd generation riboswitches with further enhanced gene regulation activity

Constructs with luciferase gene containing the 1st (Switch 1G), 2nd (Switch 2G) and 3rd (Switch A) and Switch B) generation switches were transfected in AML12 cells, and transfected cells were treated with B1 analog at the indicated concentration. (A) luciferase expression at 20 hours post treatment. (B) the fold induction was calculated as the ratio of luciferase from inducer-treated cells divided by the luciferase from cells without inducer treatment.(C) the relative luciferase expression in comparison with the expression from luciferase construct without riboswitch (Con1).

Riboswitch functions in multiple cell types in addition to HEK 293

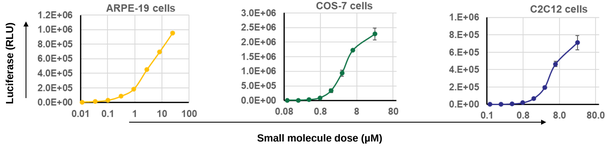

Figure 7. Riboswitch regulates gene expression in mammalian cells in response to novel small molecule. ARPE-19 cells (A), COS-7 cells (B), and mouse myoblast cell line C2C12 (C) were transfected with Luciferase constructs containing riboswitch. Transfected cells were treated with the novel synthetic small molecule at the indicated concentration, and luciferase activity was measured 20 hours after treatment.

Riboswitch Regulates Transgene Expression *in vivo*

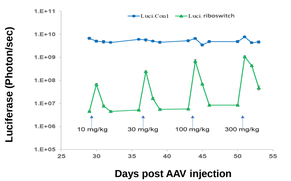

Figure 8. Riboswitch regulates luciferase gene expression in response to orally administered small molecule inducer. AAV8 containing luciferase transgene with riboswitch were injected intravenously into Balb/c mice (n=5) at 2.5E11 GC/mouse dose. 4 weeks after AAV delivery, mice were subjected to bioluminescence imaging before (0 hr) and 6 hr, 24 hr and 48 hr post a single oral small molecule dose at the indicated dose level. The luminescence signal escalating doses from 2.5E11 GC/mouse group is shown. Following each dose of oral small molecule activation of the transgene is seen in a dose responsive fashion.

Conclusions

- Rationally designed synthetic riboswitches activating expression via a splicing based expression cassette
- Novel synthetic riboswitches are highly dynamic in regulating gene expression in mammalian cells allowing precise activation of gene expression with a small molecule inducer
- Aptamers are interchangeable within the switch and multiple novel synthetic aptamer and novel small molecule pairs have been generated
- Multiple transgenes have been regulated in multiple cell types in vitro and in vivo.
- AAV delivered transgene expression *in vivo* via orally available small molecule inducer
- Our riboswitch gene regulation system provides an unprecedented platform for spatial and temporal control of gene therapy