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Synthetic RNA controllers for programming mammalian cell fate and function

Task 1: Develop RNA sensing-actuation platforms for designing tailored control systems in mammalian cells

The original objectives within Task 1 fell under several categories, including to: (i) extend the multi-input and multi-output control capabilities of RNA devices, (ii) develop multi-functional device platforms that support complex regulatory strategies, and (iii) develop an RNA device platform that controls posttranslational events. During the course of the DARPA contract we met the described goals of the first two objectives; however, the third objective, to develop an RNA device platform that controls posttranslational events, was not pursued after the first year of the agreement. This decision was based on progress made on this objective and the priorities of the program.

Under Task 1, we developed quantitative and experimental frameworks supporting the forward engineering of gene circuits that incorporate RNAi-based regulatory components in mammalian cells **(1)**. In particular, we developed a model that captures the quantitative relationship between miRNA and target gene expression levels as a function of parameters including mRNA half-life and miRNA target-site number. We extended a ligand-responsive miRNA platform to respond to protein ligands to enable the design of protein-responsive miRNA ON switches to numerous ligands including MS2, β-catenin, and NF-kB. We also optimized the spacer sequences between miRNAs to increase the efficiency of processing of transcripts incorporating miRNA switches. We then extended the model to synthetic circuits that incorporate protein-responsive miRNA switches. We utilized the model to design an optimized miRNA-based protein concentration detector circuit that noninvasively measures small changes in the nuclear concentration of β-catenin owing to induction of the Wnt signaling pathway.

We also extended the ribozyme-based device platform to respond to protein ligands **(2)**. This platform allows for both gene-ON and gene-OFF response upon sensing the protein ligand. As part of our design process, we developed an in vitro characterization pipeline for prescreening device designs to identify promising candidates for in vivo testing. The in vivo gene-regulatory activities in the two types of eukaryotic cells correlate with in vitro cleavage activities determined at different physiologically relevant magnesium concentrations. Localization studies with the ligand demonstrate that ribozyme switches respond to ligands present in the nucleus and/or cytoplasm, providing new insight into their mechanism of action.

We further extended the information processing capabilities of ligand-responsive RNAi-based regulatory components by developing a platform that combines a ligand-responsive ribozyme switch and synthetic miRNA regulators to create an OFF genetic control device **(3)**. We extended the computational framework to this system to highlight important design parameters in programming the quantitative performance of RNAi-based OFF control devices. By modifying the ribozyme switch integrated into the system, we demonstrated RNAi-based OFF control devices that respond to small molecule and protein ligands, including the oncogenic protein E2F1. We utilized the OFF control device platform to build a negative feedback control system that acts as a proportional controller and maintains target intracellular protein levels in response to increased in transcription rate.

Publications describing work under this task of the award:

1. Bloom RJ, Winkler SM, Smolke CD. 2014. A quantitative framework for the forward design of synthetic miRNA circuits. *Nat Methods*. 11: 1147-53.
2. Kennedy AB, Vowles JV, d'Espaux L, Smolke CD. 2014. Protein-responsive ribozyme switches in eukaryotic cells. *Nuc Acids Res*. 42: 12306-21.
3. Bloom RJ, Winkler SM, Smolke CD. 2015. Synthetic feedback control using an RNAi-based gene-regulatory device. *J Biol Eng*. 9: 5.

Task 2: Develop high-throughput methods for developing clinically-relevant sensing activities

The original objectives within Task 2 fell under several categories, including to develop: (i) a high-throughput, high-efficiency in vivo screening strategy, (ii) a high-throughput, solution-based in vitro selection strategy, and (iii) a streamlined high-throughput method for generating RNA devices responsive to new clinically-relevant inputs. During the course of the DARPA contract we met the described goals of the first two objectives. Substantial progress was made toward the third objective; however, work is ongoing to complete the third objective. We expect the third objective will be met over the course of the next six to twelve months. Progress on the third objective was delayed due to the incorporation of new methods into our in vitro selection strategy and thorough troubleshooting to streamline and integrate the first two objectives into a robust, integrated process that would better serve the broader community.

Under the first objective, we developed a high-efficiency, high-throughput and quantitative two-color fluorescence-activated cell sorting-based screening strategy to support the rapid generation of ribozyme-based control devices with user-specified regulatory activities **(1)**. The high-efficiency of this screening strategy enabled the isolation of a single functional sequence from a library of over 10^6 variants within two sorting cycles. We demonstrated the versatility of our approach by screening large libraries generated from randomizing individual components within the ribozyme device platform to efficiently isolate new device sequences that exhibit increased in vitro cleavage rates up to 10.5-fold and increased in vivo activation ratios up to 2-fold. We also identified a titratable window within which in vitro cleavage rates and in vivo gene-regulatory activities are correlated, supporting the importance of optimizing RNA device activity directly in the cellular environment. We subsequently took a number of the ribozyme devices that had been optimized through this yeast-based screening strategy and validated that their improved activities were observed in different mammalian cells **(3)**.

We further improved our two-color screening approach by leveraging next generation sequencing strategies to enable a high-throughput and massively-parallel assay approach **(4)**. We also developed an improved ribozyme device platform that does not require secondary structure rearrangement within the sequence. Specifically, we developed a framework for engineering RNA devices from preexisting aptamers that exhibit ligand-responsive ribozyme tertiary interactions. Our methodology utilizes cell sorting, high-throughput sequencing, and statistical data analyses (FACS-Seq) to enable parallel measurements of the activities of hundreds of thousands of sequences from RNA device libraries in the absence and presence of ligands. We demonstrated that tertiary-interaction RNA devices performed better in terms of gene silencing, activation ratio, and ligand sensitivity than optimized RNA devices that rely on secondary-

structure changes. We applied our method to build biosensors for diverse ligands and determine consensus sequences that enable ligand-responsive tertiary interactions.

Under the second objective, we have developed an automated, solution-based platform for in vitro selection based on selections and counter-selections for ribozyme cleavage **(2, 6)**. The method has been optimized on a robotics platform so that greater than twenty cycles per day can be performed without human interruption. We have done control selections on naïve libraries for theophylline biosensors, and demonstrated that the method can be used to identify completely new sensors to this compound. We have been optimizing the steps of the selection to ensure that sufficient diversity is maintained in early rounds to support the generation of new sensors to a diversity of compounds. We expect that the method will be validated with a panel of new ligands and published on within the next year **(6)**.

Under the third objective, we will combine the methods in the first and second objectives to develop a streamlined approach to generating RNA sensors and controllers responsive to new ligands that work in vivo. In particular, the in vitro strategy will be used to enrich libraries to ~10^6 diversity. These enriched libraries will be then transformed into the cell line of interest (e.g., yeast or mammalian cell) and then every member in the enriched library will be characterized by Cleave-Seq and FACS-Seq analysis. From this large dataset, members with desired regulatory and binding properties will be selected and validated for downstream applications.

We have utilized the two-color screening strategy to generate ribozyme switches responsive to leucovorin **(5)**. These switches have been validated for activity in mammalian cells and have been used to develop cell death and cell proliferation control systems (see Task 3).

Publications describing work under this task of the award:

1. Liang JC, Chang AL, Kennedy AB, Smolke CD. 2012. A high-throughput, quantitative, cell-based screen for efficient tailoring of RNA device activity. *Nuc Acids Res*. 40: e154.
2. Kennedy AB, Liang JC, Smolke CD. 2013. A versatile cis-blocking and trans-activation strategy for ribozyme characterization. *Nuc Acids Res*. 41: e41.
3. Wei KY, Chen YY, Smolke CD. 2013. A yeast-based rapid prototype platform for gene-control elements in mammalian cells. *Biotechnol Bioeng*. 110: 1201-10.
4. Townshend B, Kennedy AB, Xiang JS, Smolke CD. 2015. High-throughput cellular RNA device engineering. *Nat Methods*. 12: 989-94.
5. McKeague M, Wang YH, Smolke CD. 2015. In vitro screening and in silico modeling of RNA-based gene expression control. *ACS Chem Biol*. *In press.*
6. Townshend B, Xiang JS, Smolke CD. 2016. An automated, high-throughput pipeline for generation of RNA sensors to small molecule ligands. *In preparation.*

Task 3. Build synthetic RNA-based control systems in mammalian cells for next-generation therapeutic strategies

The original objectives within Task 3 fell under several categories, including to develop: (i) a controllable cell death trigger system, (ii) conditional mammalian cell cycle control systems, and

(iii) rapid, conditional, and specific regulation of cell signaling pathways. The third objective of this task builds upon the third objective of Task 1, which, as described above, was not pursued after the first year of the agreement. In addition, as the activities under Task 3 represented applications of the foundational technologies developed in the earlier tasks, they were not started until later years in the agreement. Funding was reduced for the activities in Task 3 in later years of the agreement and thus less effort was directed to accomplishing the stated objectives. Even with this reduced effort much of the objectives were achieved through the agreement.

Specifically, we have engineered conditional control systems that trigger cell death and/or suspension of cell proliferation in mammalian cells **(3)**. This control system is based on the ligand-responsive miRNA switches developed in Task 1. We have also demonstrated a dual-acting control platform that allows for simultaneous control of endogenous genes and transgenes in mammalian cells by combing the ligand-responsive ribozyme and miRNA switches.

We also engineered mammalian cell cycle control systems based on small molecule-responsive ribozyme switches **(2)**. We identified key regulatory nodes that arrest mammalian cells in the G0/1 or G2/M phases of the cycle. We then optimized the most promising key regulators and showed that, when these optimized regulators are placed under the control of a ribozyme switch, we can inducibly and reversibly arrest up to ~80% of a cellular population in a chosen phase of the cell cycle. Characterization of the reliability of the final cell cycle controllers revealed that the G0/1 control device functions reproducibly over multiple experiments over several weeks.

During the DARPA Agreement we also entered into a collaboration with an industry performer (Novartis) who used our engineered ribozyme switches to control alphavirus-based replicon gene expression **(1)**. The ribozyme switches were placed in the replicon 3' UTR. Expression from DNA-launched and VRP-packaged replicons containing ribozyme switches were successfully regulated, achieving a 47-fold change in expression and modulation of the resulting type 1 interferon response. A novel control architecture where ribozyme switches were integrated into the 3' and 5' UTR of the subgenomic RNA region of the TC-83 virus, led to a 1160-fold regulation of viral replication. These studies demonstrate that the use of RNA regulators for control of RNA replicon expression and viral replication holds promise for development of novel and safer vaccination strategies. In addition, Sangamo has also entered into an agreement with us to test several of our RNA-based control systems for conditional control in mammalian cells for applications they are working on.

<u>Publications describing work under this task of the award:</u>

1. Bell CL, Yu D, Smolke CD, Geall AJ, Beard CW, Mason PW. 2015. Control of alphavirus-based replicon gene expression using engineered riboswitches. *Virology*. 483: 302-11.
2. Wei KY, Smolke CD. Engineering dynamic cell cycle control with synthetic small molecule-responsive RNA devices. *J Biol Eng. In press.*
3. Wong R, Smolke CD. 2016. A dual-acting transgene control and proliferation switch responsive to a clinically-relevant drug for genetically modified T cells. *In preparation.*