Chapter 6

Conclusions and Future Prospects

Design of ligand-responsive RNAi effectors

To extend riboswitch design to include RNAi, we rendered three RNAi effectors in mammalian cells – siRNAs, shRNAs, and miRNAs – ligand-responsive through the rational integration of an aptamer domain. Ligand binding induced a local or global conformational change, thereby modulating the extent of processing by the RNAi machinery and subsequent gene silencing. The resulting riboswitches can be broadly applied by identifying an appropriate guide strand sequence, selecting an aptamer against the molecule of choice that will control silencing, and following the prescribed design approach detailed for siRNAs (Chapter 2), shRNAs (Chapter 3), or miRNAs (Chapter 4). The benefit of each design is the relative ease in selecting new gene targets and recognized molecules based on the demonstrated modularity of the guide strand sequence and aptamer domain and the ability to tune riboswitch performance through strategies specific to each design. Additional benefits include the propensity for forward design using available computational tools and the ability to program multiple input-output functions through the rational integration of riboswitches that recognize different biomolecules and target different genes.

The experimentally validated designs all displayed ON behavior, where target gene expression levels increased in the presence of the recognized ligand. Of equal value would be the design of ligand-responsive RNAi effectors displaying OFF behavior. The

focus on ON behaving riboswitches in this work reflects the relative ease in physically disrupting rather than improving biological activity – in this case processing by the RNAi machinery. With the exception of the miRNA designs, the principles underlying ligand control of RNAi effectors could be readily extended to generate OFF-acting designs. The major caveat is the alternative behavior associated with a regulatory mechanism that relies on catalytic cleavage, such as irreversible processing by the RNAi machinery. As discussed in Chapter 5, the speed of the irreversible regulatory event can reduce riboswitch performance if it dominates over the rate of reversible events such as conformational switching or ligand binding and release. Designing riboswitches with biased conformational folding during transcription can relieve the reduced performance, although OFF-acting designs are insensitive to this approach. Therefore, the relative speed of the reversible events and processing by the RNAi machinery may restrict the accessible performance for OFF-acting designs.

At first glance, all three designs for ligand-responsive RNAi effectors may be interchangeably used. However, the redundancy is short-lived when considering the location within the RNAi pathway, the elucidated modes of tuning, and potential toxicity issues. Since the RNAi pathway spans the nucleus and the cytoplasm, localization of the recognized ligand would dictate the selected design. For instance, the miRNA design is better suited to recognize nuclear proteins while the siRNA design is better suited to recognize cytoplasmic proteins. In addition, the tuning strategies varied between designs, where the predominant strategy in the siRNA and shRNA designs was modifying the competing strand complementarity while the miRNA design relied on introducing different miRNA copies. Finally, recent insights into the cytotoxicity induced by RNAi

effectors suggests that the shRNA design may introduce bottlenecks in the RNAi pathway not associated with siRNAs or miRNAs, such that the shRNA designs may be less appropriate for therapeutic applications. These critical factors must be weighed when selecting the most appropriate design for ligand control of gene silencing.

Broad implementation of ligand-responsive RNAi effectors

The lack of superficial restrictions on the recognized ligand and targeted gene opens the described designs to diverse applications. For instance, riboswitches could be readily designed that respond to small molecules with ideal pharmacokinetic properties such as high solubility, tissue permeability, and serum stability, and low immunogenicity and cytotoxicity. RNAi-mediated targeting of any selected gene could then be controlled by adding or removing this small molecule either in cell culture or organisms with negligible side effects. Another application is the development of smart therapeutics – molecules that can decipher between diseased and healthy cells and only enact the therapeutic response in the diseased cells. Aptamers could be selected against known disease biomarkers, such as oncogenic, viral, or mutant proteins or perturbed metabolite levels. Selected aptamers could then be integrated into any of the ligand-regulated RNAi effectors that target essential genes or other therapeutically-relevant targets such that cell death will be induced only in the presence or absence of the biomarker. When the diseased state is typified by a quantitative increase or decrease in the marker, performance tuning will be essential to ensure that the typical perturbation in the disease marker induces a robust change in the therapeutic effect from modulated gene silencing.

Current challenges in riboswitch design

Before realizing the full implementation of ligand-responsive RNAi effectors and other riboswitches, a few major challenges must be overcome in the design process that should be the immediate focus of future research. First, aptamer selection must be advanced to yield aptamers with in vivo binding capacity that function in the context of riboswitches. Most available aptamers were selected under buffer conditions that do not resemble the intracellular environment, where the binding capacity of these aptamers is severely compromised under intracellular conditions (Lorsch and Szostak, 1994). Furthermore, a significant fraction of aptamers do not fit the standard stem-bulge-loop structure and instead form pseudoknots and dangling ends (Kim and Jeong, 2003; Koizumi and Breaker, 2000; Tuerk *et al*, 1992). These nonstandard aptamer conformations are incompatible with current riboswitch designs and may require alternative design schemes to accommodate these structures.

Besides conducting aptamer selections under conditions that mimic the intracellular environment, aptamers could be selected in the context of riboswitches. The precedence was established in the context of allosteric ribozymes with the in vitro selection of aptamers against nucleoside 3',5'-cyclic monophosphate compounds (Koizumi *et al*, 1999). While transitioning to an in vivo selection scheme is confounded by the reduced screening capacity in cells, recent work has suggested an intermediate approach that combines in vitro and in vivo aptamer selections (Weigand *et al*, 2008). Continued efforts may yield standard procedures for the selection of aptamers that function in the context of riboswitches in vivo, thereby facilitating the process of design and implementation.

A second challenge is predicting how a particular riboswitch sequence will behave when expressed in cells. Understanding the associated sequence-function relationship would greatly improve efficacy and reduce the time investment in riboswitch design. One major contributor to sequence-function relationships is RNA folding, where recent computational advances have offered algorithms for the prediction of RNA secondary and tertiary structure with improving accuracy and resolution (Mathews *et al*, 2004; Parisien and Major, 2008; Sharma *et al*, 2008). As these algorithms progress toward atomistic models of RNA folding under equilibrium conditions, the next major area of development is investigating the dynamics of folding and activity. RNA is a naturally dynamic molecule that can transition between many different conformations rather than adopting a single energetically favorable structure. Furthermore, RNAs transcribed by cells are unlikely to reach thermodynamic equilibrium because of RNA processing and degradation. Chapter 5 provides a coarse investigation of the contribution of dynamics to riboswitch activity, which may serve as a foundation in which to further explore the in vivo events that occur in the lifetime of an RNA molecule. By accurately accounting for the energetics of RNA folding and cellular interactions, we can move toward the development of in silico frameworks that reliably predict the behavior of an RNA sequence in cells. Such frameworks would be integral to the design process in the construction of riboswitches and other noncoding RNAs.

As more riboswitch designs become available, a third research challenge will be implementing riboswitches toward different applications. While riboswitches can seemingly render any gene sensitive to any intracellular molecule, care must be taken when identifying and pursuing applications suitable for riboswitch design. Paramount

considerations include the intracellular concentration range of the recognized molecule, the swing in gene expression required to robustly induce the prescribed phenotype, and the performance limitations of the riboswitch. These quantitative parameters must be specified as design goals or restrictions, marking a progression beyond the simple designation of ON or OFF behavior associated with riboswitches.

The ensuing challenge is identifying riboswitch sequences that meet the identified performance needs of the selected application. Simple screening and selection strategies have been developed that could be applied to identify riboswitch sequences with appropriate activity (Fowler *et al*, 2008; Lynch and Gallivan, 2009; Muranaka *et al*, 2009; Nomura and Yokobayashi, 2007; Topp and Gallivan, 2008; Weigand *et al*, 2008; Wieland and Hartig, 2008). However, the progression to larger systems in higher organisms with more complex phenotypes excludes simple screening strategies. In contrast, elucidated sequence-function relationships that link riboswitch performance and systems-level behavior would provide valuable tools to screen riboswitch sequences in silico.

The future of RNA design

The recent construction of synthetic RNAs and the characterization of natural noncoding RNAs continue to reveal the rich and versatile nature of RNA. The myriad of abilities afforded to this ubiquitous biomolecule are ripe for further engineering efforts, raising the question where RNA-based components should be utilized in the design of more sophisticated biological systems. An excellent case study is the natural world. In examples ranging from bacterial small RNAs to miRNAs, RNA is predominantly utilized

in combination with protein regulators to orchestrate cellular processes (Shalgi *et al*, 2007; Shimoni *et al*, 2007). These observations argue against the construction of systems entirely composed of RNA or protein regulators. Toward the construction of hybrid systems, recent engineering efforts have combined protein and RNAi-based regulation with an improved dynamic range and logic capabilities (Deans *et al*, 2007; Greber *et al*, 2008; Rinaudo *et al*, 2007). More studies in the natural utilization of RNA are required to fully comprehend how and why nature utilizes this biomolecule. Insights can be immediately translated into design strategies as we continue engineering biological systems.

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