Chemical Engineering Science 103 (2013) 36–41

Contents lists available at ScienceDirect

Chemical Engineering Science

journal homepage: www.elsevier.com/locate/ces

Synthetic regulatory RNAs as tools for engineering biological systems: Design and applications

Sang Woo Seo [a], Gyoo Yeol Jung [a,b,*]

[a] Department of Chemical Engineering, Pohang University of Science and Technology, San 31, Hyoja-dong, Nam-gu, Pohang, Gyeongbuk 790-784, Korea
[b] School of Interdisciplinary Bioscience and Bioengineering, Pohang University of Science and Technology, San 31, Hyoja-dong, Nam-gu, Pohang, Gyeongbuk 790-784, Korea

HIGHLIGHTS

- Synthetic regulatory RNAs can statically and dynamically control gene expression.
- We review current developments in the design of synthetic regulatory RNAs.
- We review applications of synthetic RNA tools in biological systems design.

ARTICLE INFO

Article history:
Received 7 July 2012
Received in revised form
14 December 2012
Accepted 10 January 2013
Available online 17 January 2013

Keywords:
Biological engineering
Biotechnology
Biochemical Engineering
Biomolecular Engineering
Synthetic biology
Synthetic RNA

ABSTRACT

The engineering of biological systems requires a set of molecular tools that can be predictably applied to the design of sophisticated genetic systems. Recent advances in the field of RNA synthetic biology, particularly in the design of synthetic regulatory RNAs for the static and dynamic control of gene expression, have increased our ability to efficiently build various biological systems capable of performing programmed cellular behaviors. Furthermore, implementing these synthetic regulatory RNAs in biological systems highlights the potential for designing synthetic cell systems for chemical synthesis, environmental, agricultural, and medical applications. In this paper, we review current developments in synthetic regulatory RNAs for the static and dynamic control of gene expression and the potential applications of these tools.

1. Introduction

The field of synthetic biology has fostered numerous efforts to redesign existing biological systems or synthesize new ones to serve specific purposes (Keasling, 2012; Lynch and Gill, 2012). Building a synthetic system requires optimization of genetic parts, devices, and systems as well as molecular tools for controlling gene expression in a predictable and quantitatively controllable manner (Yadav et al., 2012). Imbalances within systems can often lead to failed performance of the designed programs. Therefore, recent research in this area has focused on the development of new regulatory tools that can be utilized to design and optimize biological systems (Seo et al., 2012).

Natural RNA regulators that work in a variety of organisms through different mechanisms have been steadily discovered (David son and Ellington, 2005; Doudna and Cech, 2002; Gottesman, 2004; Kim et al., 2009; Serganov and Patel, 2007; Yao et al., 2007). The functional diversity of RNAs has accelerated the use of such molecules as versatile substrates for programming various functions inside cells (Chang et al., 2012; Michener et al., 2012). These functional RNAs are *cis*- and *trans*-acting regulatory elements that control gene expression without being affected by environmental/physiological changes (static) or by sensing those changes in conditions (dynamic). In this paper, we review recent developments in the design of synthetic regulatory RNAs as static and dynamic expression controllers for engineering biological systems, and discuss future perspectives in this area (Fig. 1).

2. Designing synthetic regulatory RNAs

2.1. Designing cis-acting regulatory RNAs to control gene expression

Several studies have shown that structural elements around the translation-initiation region (TIR) of mRNA determine the amount of protein produced from a particular mRNA sequence in

* Corresponding author at: Department of Chemical Engineering, Pohang University of Science and Technology, San 31, Hyoja-dong, Nam-gu, Pohang, Gyeong-buk 790-784, Korea, 790-784. Tel.: +82 54 279 2391; fax: +82 54 279 5528.
E-mail address: gyjung@postech.ac.kr (G.Y. Jung).

0009-2509/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ces.2013.01.017

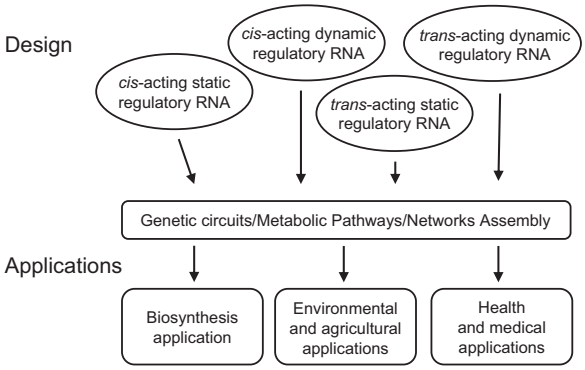

Fig. 1. Conceptual diagram of RNA synthetic biology. Various orthogonal RNA regulators, including *cis*-acting static regulatory RNA, *cis*-acting dynamic regulatory RNA, *trans*-acting static regulatory RNA and *trans*-acting dynamic regulatory RNA, can be designed and assembled to construct genetic circuits, metabolic pathways, and complex networks. These systems can be further utilized for biosynthesis, environmental/agricultural, and health/medical applications.

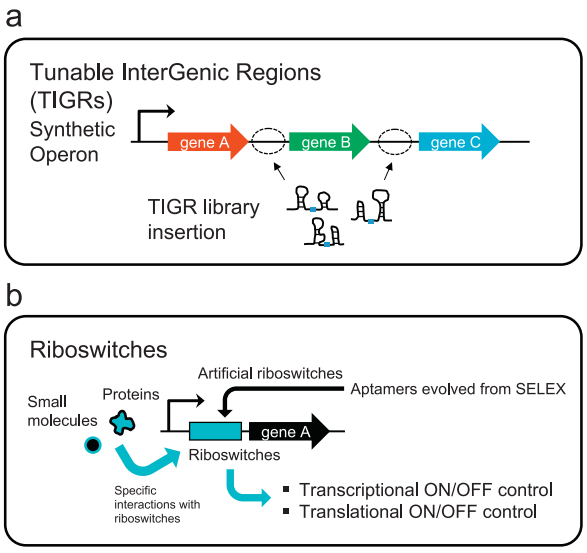

Fig. 2. Synthetic *cis*-acting regulatory RNAs. (a) Insertion of libraries of tunable intergenic regions (TIGRs) with various mRNA secondary structures and RNase cleavage sites (blue square) in a synthetic operon can lead to combinatorial engineering of static gene expression. (b) Riboswitches can be used for transcriptional/translational ON/OFF control of gene expression in response to various small molecules and proteins. Aptamers evolved from SELEX can be utilized to construct artificial riboswitches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

prokaryotes (Kudla et al., 2009; Park et al., 2007; Seo et al., 2009). Both the sequence itself and secondary structure within the TIR control the binding affinity as well as accessibility of the ribosome to the mRNA (de Smit and van Duin, 1990, 1994a, 1994b, 2003). When these structural elements do not respond to specific environmental/physiological signals, they can be used as *cis*-acting static controllers to regulate gene expression levels. The potential of controlling these regulatory elements in the post-transcriptional process was demonstrated by a combinatorial engineering approach, in which libraries of tunable intergenic regions (TIGRs) with various mRNA secondary structures and RNase cleavage sites were generated for static gene expression control in *Escherichia coli* (Fig. 2a) (Pfleger et al., 2006). However, since the regulatory ranges of these libraries are unpredictable, a method for designing structural elements with a defined regulatory repertoire is required to efficiently utilize these functional characteristics for static gene expression control.

In addition to *cis*-acting static controllers, RNA molecules can also serve as *cis*-acting dynamic controllers containing sensors that respond to intracellular metabolites (Liang et al., 2011). One example of such a *cis*-acting dynamic controller is the riboswitch that is discovered and characterized across numerous prokaryotes and eukaryotes (Fig. 2c). Riboswitches are noncoding, *cis*-acting regulatory elements found in the 5′-untranslated regions (5′-UTRs) of mRNAs that typically sense ligands such as small molecules through aptamer domains. Binding of a ligand to the aptamer domain of a riboswitch leads a conformational change in the mRNA that consequently control the expression of downstream genes through different types of regulatory mechanisms, including premature transcription termination, sequestration of ribosome-binding sites (RBS); a subset of these riboswitches is the self-cleaving ribozymes (that is, RNA enzymes for nucleotide splicing and phosphodiester bond cleavage and formation) (Breaker, 2011; Montange and Batey, 2008; Serganov and Patel, 2007). Besides the naturally existing riboswitches, artificial riboswitches have been designed and constructed by rational approaches for gene expression control (Beisel et al., 2011; Wieland et al., 2009; Win and Smolke, 2007). Moreover, several studies have shown that these types of regulatory mechanisms could be easily modulated to generate synthetic riboswitches with naturally existing aptamer domains using a high-throughput screening method (Muranaka et al., 2009; Nomura and Yokobayashi, 2007; Sharma et al., 2008). In addition, artificial aptamers specific for target chemicals can be constructed using the powerful SELEX (systematic evolution of ligands by exponential enrichment) technique (Stoltenburg et al., 2007). Although the application of SELEX to design new types of aptamers can be limited by the technology used to immobilize target molecules to a support resin, a recent study showed the potential of using grapheme oxide for immobilization-free screening of aptamers (Park et al., 2012). This approach would further increase our ability to rapidly and simply screen functional aptamers without the need for specialized instruments; such aptamers selected *in vitro* can be used to generate synthetic riboswitches that work *in vivo* using various high-throughput screening methods (Lynch and Gallivan, 2009; Muranaka et al., 2009; Sinha et al., 2010).

Although these *cis*-acting dynamic RNA controllers could be generated through either rational design or high-throughput screening methods as described above, the availability of quantitative models to design the functionality of these devices would allow the development of programmable engineering pathways and complex circuits (Benenson, 2009; Carothers et al., 2010; Holtz and Keasling, 2010). A recent report described the development of a design-driven approach using mechanistic modeling and kinetic RNA folding simulations to quantitatively program gene expression in two different manners: one is a dynamic, ligand-controlled regulation based on a aptazyme that fuses aptamers with ribozymes to create allosteric RNA enzymes and the other is a static regulation based on a ribozyme (Carothers et al., 2011). Advances in the computer-aided design (CAD) of RNA should make expanding the use of synthetic regulatory RNA devices that respond to various intracellular chemicals practicable, allowing more complex biological systems to be built.

2.2. *Designing trans-acting regulatory RNAs to control gene expression*

Small, non-coding RNA (ncRNA) molecules such as antisense RNA (in prokaryotes and eukaryotes), small interfering RNA (siRNA, in eukaryotes), and microRNA (miRNA, in eukaryotes) are responsible for various cellular functions. These functionalities arise from the ability of RNA to form complex structures (i) by interactions with DNA and other RNA through intramolecular Watson–Crick base-pairing and (ii) by interactions with proteins or small molecules through physical forces such as electrostatic,

hydrogen bonding, and van der Waals forces (Eddy, 2001; Sharp, 2009). These *trans*-acting regulatory RNAs control gene expression through a wide array of regulatory mechanisms, including static and dynamic *trans*-activation and *trans*-repression of both transcription and translation processes (Isaacs et al., 2006). These dynamic RNA functions have expanded our ability to construct and design various *trans*-acting regulatory RNAs for synthetic biology applications.

Trans-acting regulatory RNAs can function to activate or repress translation of mRNA molecules by ribosomes. Isaacs et al. (2004) designed artificial riboregulators that regulate gene expression in prokaryotes by *trans*-activating RNAs during the translation process (Fig. 3a) (Isaacs et al., 2004). Although previous studies have focused on achieving gene repression using antisense RNA or *trans*-acting ribozymes (Wagner and Flardh, 2002); this study designed *cis*-repressed and *trans*-activated RNA pairs to activate static gene expression by causing an alteration in the stem-loop structure only in the presence of *trans*-activating RNA molecules. Although this system controls gene expression in a static manner, placing *trans*-acting regulatory RNAs under the control of transcriptional sensors can result in indirect dynamic control just like natural Hfq-bnding small RNAs that gave an insight into this system (Valentin-Hansen et al., 2004). Another approach for dynamically controlling gene expression during the translation process employs a ligand-controlled *trans*-acting riboregulator, called an antiswitch (Fig. 3b) (Bayer and Smolke, 2005). These antiswitches were engineered to control translation in *Saccharomyces cerevisiae* through a conformational change that affects the ability of the antisense portion to bind a target region in the mRNA depending on the presence of small molecules. Two types of antiswitches – *trans*-activating and *trans*-repressing RNA molecules – successfully activated and repressed mRNA translation, respectively, in the presence of their cognate small molecules.

Trans-acting regulatory RNAs can also function to activate or repress transcription of DNA molecules. In eukaryotes, transcription factors comprising DNA-binding and activating/repressing domains first bind to specific sites on the DNA through their DNA-binding domain, and then initiate transcription through specific interactions with other localized proteins that are modulated by the activating/repressing domain. Buskirk et al. (2003) used a yeast three-hybrid system containing the *HIS3*/*lacZ* reporter under the control of promoters with LexA binding sites and a LexA–MS2 fusion protein that binds the RNA transcription activator to evolve RNA-based transcription activators (Fig. 3c). The evolved RNA showed up to a 53-fold higher activation ratio than a control system that used a Gal4 activation domain. In addition to a *trans*-acting RNA activator for static control, they created an artificial *trans*-acting RNA activator for dynamic control of gene expression by fusing a small molecule-binding aptamer to the evolved RNA and optimizing the linker region (Buskirk et al., 2004). Using a similar approach, Kehayova and Liu (2007) evolved a *trans*-acting RNA repressor with a potency comparable to that produced by binding of Sir1, a known silencing protein, to a promoter region. These results highlight the value of RNA molecules as tools for both static and dynamic regulation of the transcription process. Another recent study by Lucks et al. (2011b) reported the design of orthogonal variants of a natural antisense RNA/transcriptional attenuator pair that originated from the *Staphylococcus aureus* plasmid pT181 to simultaneously control the transcription of multiple genes in *E. coli* (Fig. 3d) (Lucks et al., 2011b). Furthermore, these authors showed that transcription attenuators could be placed in tandem on the same transcript to perform genetic logic, and demonstrated that an RNA-mediated transcriptional signal cascade was possible when these attenuators were engineered to regulate the transcription of antisense signaling RNAs.

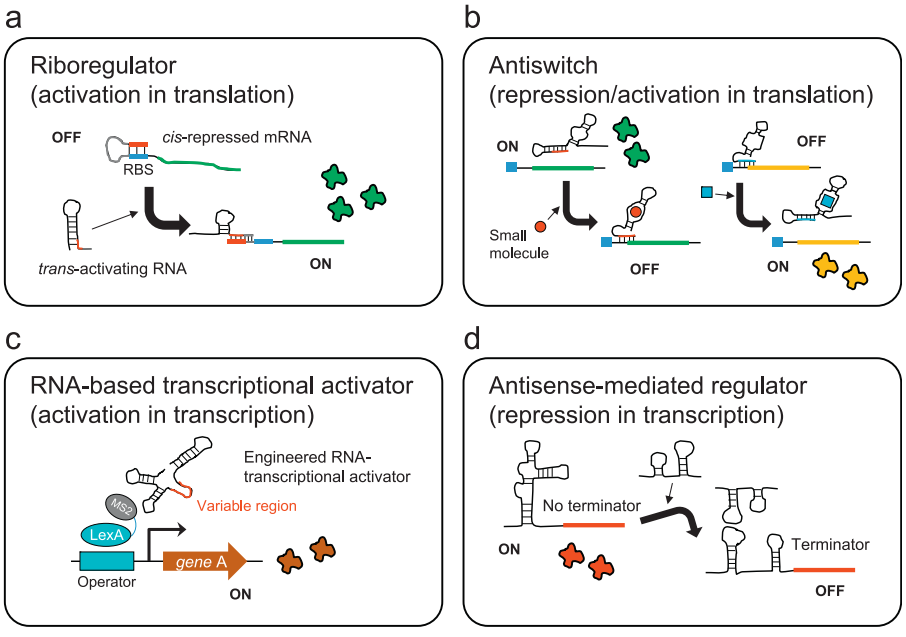

Fig. 3. Synthetic *trans*-acting regulatory RNAs. (a) *Cis*-repressed mRNA can specifically interact with *trans*-activating RNA through subsequent RNA–RNA linear loop interactions, a structural rearrangement that exposes the sequestered RBS to enable translation. (b) In the case of an "off" antiswitch, binding of a small molecule (red circle) exposes the antisense domain, allowing it to interact with its mRNA target and repress translation. Conversely, in the case of and "on" antiswitch, binding of a small molecule (yellow triangle) causes sequestration of the antisense domain, thereby activating translation. (c) Engineered RNA-based transcription activators can initiate transcription by recruiting transcription machinery. Variable regions can be engineered to control the activation ratio and confer dynamic control over the system by small molecules. (d) The absence of antisense RNA sequesters the intrinsic terminator, allowing transcription of the downstream gene. The presence of antisense RNA exposes an intrinsic transcription terminator, causing repression of transcription. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Recently published studies have further demonstrated the potential of designing RNA-based regulatory tools to modulate gene expression and construct higher-order biological systems. Qi et al. (2012) demonstrated a set of design principles for engineering naturally occurring *trans*-acting ncRNAs to respond to molecular signals such as small molecules and proteins by fusing ncRNAs to RNA aptamers, allowing the activity of ncRNAs to be modulated in a ligand-inducible manner. They showed that these engineered *trans*-acting dynamic regulators, which have architecture similar to that of a natural riboswitch, could regulate both transcription (pT181 ncRNA) and translation (IS10 ncRNA) in *E. coli* in response to their cognate molecular signals. This approach further expands our ability to engineer ligand-sensing regulatory system modules for use at molecular and network levels. Mutalik et al. (2012) rationally designed families of highly specific and non-cross-reacting (orthogonal) *trans*-acting RNA regulators of translation using sequence-activity modeling based on experimental data. They designed antisense-mediated translation system variants based on IS10 ncRNA to quantify more than 500 interactions in *E. coli*, and developed a model representing the key determinants of RNA-RNA-interaction specificity based on the data set to design other orthogonal RNA regulators. This approach would facilitate the use of orthogonal *trans*-acting RNA regulators in next-generation synthetic biology applications, such as controlling the expression of multiple genes in operons, executing different modes of RNA computation to establish a hierarchical order of regulation, and understanding the network architecture of natural small RNAs and the evolutionary aspects underlying their architectures (Benenson, 2009; Nandagopal and Elowitz, 2011; Smolke and Keasling, 2002).

As the work done by Carothers et al. (2011) that can design *cis*-acting regulatory RNAs (ribozyme- or aptazyme-regulated), the earlier work done by Beisel et al. (2008) described the development of a method to design of ligand-regulated RNA interference (RNAi) for programmable control of gene expression by a combined experimental and mathematical modeling approach. They could design *trans*-acting regulatory RNAs which is a ligand-controlled small hairpin RNA (shRNA) for multi-input control with an optimized dynamic range. Since the RNAi works in eukaryotes, development of a predictive design method can provide san advanced component for the construction of complex biological systems that can facilitate the usage of RNAi in disease therapeutics.

3. Implementing synthetic regulatory RNAs to engineer biological systems

3.1. Biosynthesis applications

A variety of synthetic regulatory RNA types have been implemented to manipulate and optimize biological systems in biosynthesis applications (Table 1). As discussed above, libraries of *cis*-acting synthetic regulatory RNAs (TIGRs) have been incorporated between the coding sequences of a polycistronic operon encoding enzymes in the mevalonate pathway (Pfleger et al., 2006). By balancing the expression levels of each gene in the operon, the resulting engineered strain was capable of producing mevalonate with a 7-fold higher titer than that yielded by the wild-type strain. Recently, Collins and colleagues (Callura et al., 2012) reported using *cis*-repressed mRNA/*trans*-activating mRNA pairs to construct a genetic switchboard that independently controls the expression of multiple genes. By building a metabolism switchboard that regulates four metabolic genes in central carbon metabolism, these authors successfully shunted carbon flow in glucose-utilization pathways (Embden–Meyerhof, Entner–Doudoroff, and pentose phosphate). These results illustrate that synthetic regulatory RNAs can be used to construct higher-order systems with a broad range of practical applications in biosynthesis including biofuels and chemicals (Nakagawa et al., 2011; Peralta-Yahya et al., 2011).

Cis-acting dynamic controllers, such as RNA switches, have been utilized to monitor metabolite accumulation within cells. Win and Smolke (2007) developed a modular (that is, portably switchable across systems) and extensible (that is, universally applicable to a wide array of molecules) RNA-based system as a noninvasive sensor of metabolite accumulation and controller for optimizing flux and product yield in yeast. In this example, a xanthine-responsive, ribozyme-based device was constructed and used to regulate a reporter gene to monitor the conversion of xanthosine to xanthine. Recently, Michener and Smolke (2012) utilized these kinds of controllers for a high-throughput enzyme evolution system in *S. cerevisiae*. Using this system, they could quantitatively screen large enzyme libraries of caffeine demethylase and obtain a variant whose activity was increased 33-fold. Since various selection strategies can modify RNA switches to recognize new small molecules, these RNA-based screening systems can be applied to the evolution of a broad range of enzymes and metabolic pathways.

Table 1
Implementation of synthetic regulatory RNAs to engineer biological systems.

Application areas	RNA devices	Host cells	Programmed behaviors	Reference
Biosynthesis application	*cis*-regulatory RNA (TIGRs)	*E. coli*	Mevalonate pathway optimization	Pfleger et al. (2006)
	cis-repressed mRNA/*trans*-activating RNA	*E. coli*	Genetic/metabolism switchboard	Callura et al. (2012)
	Ribozyme-based *cis*-regulatory RNA	*S. cerevisiae*	Noninvasive sensors for metabolite production	Win and Smolke (2007)
	Ribozyme-based *cis*-regulatory RNA	*S. cerevisiae*	High-throughput enzyme evolution	Michener and Smolke (2012)
Environmental and agricultural applications	*cis*-regulatory RNA (Riboswitch)	*E. coli*	Sensing and destroying atrazine	Sinha et al. (2010)
	cis-repressed mRNA/*trans*-activating RNA	*E. coli*	Programming kill switch	Callura et al. (2010)
Health and medical application	Ribozyme-based alternative RNA splicing	HEK293	Cell-fate decision depending on disease marker	Culler et al. (2010)
	Protein-responsive RNA switch	HeLa	L7Ae-dependent apoptosis	Saito et al. (2011)
	Drug-responsive *cis*-regulatory RNA	CTLL-2, primary human T_{CM}	T-cell proliferation control	Chen et al. (2010)
	Synthetic RNA-based regulatory circuit (classifier)	DAOY, HEK293, HeLa, MCF-7, SH-SY5Y, SKBR3, T47D	miRNA profile-dependent apoptosis of cancer cells	Xie et al. (2011)

3.2. Environmental and agricultural applications

Environmental remediation of pollutants is a critical aspect of sustainability. Recently, Sinha et al. (2010) designed a *cis*-acting synthetic RNA device (riboswitch) that is responsive to the toxic environmental pollutant, atrazine, and used this device to express the *cheZ* gene and thereby control motility of *E. coli* toward atrazine. The presence of atrazine allowed cells to move along the pollutant gradient to its source and convert atrazine into the less harmful product, hydroxyatrazine. This riboswitch approach will accelerate the development of reprogrammed microorganisms that can sensitively detect other environmental pollutants and directly metabolize them into non-toxic materials. However, a critical factor in considering the environmental release of engineered organisms is managing microbial physiology for the control of cellular growth. In this context, the use of *cis*-repressed mRNA/*trans*-activating mRNA pairs can be expanded to enable tight regulation of toxic protein expression and program a "kill switch" in bacteria (Callura et al., 2010). Rewiring additional inputs in a logic circuit that produces a cell lysis output would provide the safety mechanism necessary to allow the controlled release of engineered organisms into natural environments.

3.3. Health and medical applications

Health and medical applications are among the most intriguing implementations of RNA synthetic biology, holding the promise of new therapies for the treatment of infectious diseases and cancer (Ruder et al., 2011). Because RNA molecules detect various small molecules as well as proteins as part of their complex functions in nature, designing RNA-based therapeutic system that respond to a specific subset of such signals can lead to specific therapeutic activities, eradication of disease phenotypes, and elimination of diseased cells. A recent study reported the development of an RNA control device that can regulate cell-fate decisions by detecting an increased abundance of particular proteins and regulating the expression of a target gene through alternative RNA splicing (Culler et al., 2010). In response to the disease-associated markers NF-κB and β-catenin, this synthetic RNA device controls the expression of a suicide gene encoding herpes simplex virus-thymidine kinase (HSV-TK), which confers sensitivity to the apoptosis-inducing actions of the pro-drug ganciclovir. Similarly, Saito et al. (2011) designed protein-responsive RNA switches for ON/OFF translational control of two distinct apoptosis pathways in target human cells in response to the heterologously expressed ribosomal protein, L7Ae. Also, Chen et al. (2010) devised a drug-responsive, ribozyme-based synthetic regulatory RNA linked to growth-related cytokine targets to control mouse and human T-cell proliferation. Because a major challenge associated with T-cell therapy is ensuring sufficient proliferation of T cells in the host to eradicate the diseased cells, this RNA-based regulatory system might advance cellular therapies and other applications in health and medicine. Additionally, a scalable transcriptional/posttranscriptional synthetic regulatory circuit, called a classifier, was developed to sense miRNA profiles indicative of a disease state and activate cell death (Xie et al., 2011). In this application, an engineered HeLa cancer cell classifier selectively identified HeLa cells and triggered apoptosis without affecting non-HeLa cell types, demonstrating the ability of a customized classifier to selectively kill cancer cells.

4. Future directions in RNA synthetic biology

As discussed above, there has been an enormous increase in our options for both static and dynamic control of gene expression using *cis*- and *trans*-acting synthetic regulatory RNAs. Also, as mentioned earlier, layering control elements can modulate the mode of action of regulatory RNAs, for example, static control by *trans*-acting RNAs can be placed under the control of transcriptional sensors to result in indirect dynamic control and further increase the complexity of the constructed biological system. The elucidation of a wide array of RNA functions has enabled us to engineer these RNA molecules for applications in diverse biological systems. However, significant challenges remain before next-generation, RNA synthetic biology-based programmable biological systems can be realized.

Enlarging the diversity of existing functional RNAs will require high-throughput *in vitro* selection methods coupled to *in vivo* screening/selection methods to effectively examine large sequence spaces for novel activities. These experimental data might, in turn, yield novel insights that provide additional design principles. In addition, because detailed information about structures and mechanisms of these RNA-based regulators *in vivo* is invaluable, further advances in high-throughput RNA structure characterization methods such as SHAPE-Seq, which combines selective 2′-hydroxyl acylation analyzed by primer extension (SHAPE) chemistry with multiplexed, hierarchical bar coding and a deep sequencing strategy to characterize *in vivo* target structures, would facilitate design procedures by identifying flexible and/or rigid regions for intramolecular interaction (Aviran et al., 2011; Lucks et al., 2011a; Tran and Disney, 2012). Although there have been successful examples for rational design of RNA regulators, computational models and CAD tools will be further required to build more sophisticated systems based on various types of orthogonal RNA devices so that an automated system can convert the desired biological systems into DNA sequences. When this point is reached, synthetic regulatory RNAs can be utilized to design biological systems with novel functions through expanded signal integration and the creation of advanced logic gates, global regulation of many targets in response to customizable cellular signals at the gene-network level, and transmission of signals between networks to execute more complicated programmed regulations. Thus, future advances in RNA synthetic biology will expand our ability to build synthetic biological systems for biosynthesis, environmental, agricultural, health, and medical applications.

Acknowledgments

This work was supported by the Ministry of Education, Science and Technology through the Advanced Biomass R&D Center (ABC) (Grant no. ABC-2010–0029800) of the National Research Foundation, and by the Ministry of Land, Transport and Maritime Affairs through a Marine Biomaterials Research Center grant from the Marine Biotechnology Program, the Manpower Development Program, and the Gyeongbuk Sea Grant Program.

References

Aviran, S., Trapnell, C., Lucks, J.B., Mortimer, S.A., Luo, S., Schroth, G.P., Doudna, J.A., Arkin, A.P., Pachter, L., 2011. Modeling and automation of sequencing-based characterization of RNA structure. Proc. Natl. Acad. Sci. U.S.A. 108, 11069–11074.

Bayer, T.S., Smolke, C.D., 2005. Programmable ligand-controlled riboregulators of eukaryotic gene expression. Nat. Biotechnol. 23, 337–343.

Beisel, C.L., Bayer, T.S., Hoff, K.G., Smolke, C.D., 2008. Model-guided design of ligand-regulated RNAi for programmable control of gene expression. Mol. Syst. Biol. 4, 224.

Beisel, C.L., Chen, Y.Y., Culler, S.J., Hoff, K.G., Smolke, C.D., 2011. Design of small molecule-responsive microRNAs based on structural requirements for Drosha processing. Nucleic Acids Res. 39, 2981–2994.

Benenson, Y., 2009. RNA-based computation in live cells. Curr. Opin. Biotechnol. 20, 471–478.

Breaker, R.R., 2011. Prospects for riboswitch discovery and analysis. Mol. Cell 43, 867–879.

Buskirk, A.R., Kehayova, P.D., Landrigan, A., Liu, D.R., 2003. *In vivo* evolution of an RNA-based transcriptional activator. Chem. Biol. 10, 533–540.
Buskirk, A.R., Landrigan, A., Liu, D.R., 2004. Engineering a ligand-dependent RNA transcriptional activator. Chem. Biol. 11, 1157–1163.
Callura, J.M., Cantor, C.R., Collins, J.J., 2012. Genetic switchboard for synthetic biology applications. Proc. Natl. Acad. Sci. U.S.A. 109, 5850–5855.
Callura, J.M., Dwyer, D.J., Isaacs, F.J., Cantor, C.R., Collins, J.J., 2010. Tracking, tuning, and terminating microbial physiology using synthetic riboregulators. Proc. Natl. Acad. Sci. U.S.A. 107, 15898–15903.
Carothers, J.M., Goler, J.A., Juminaga, D., Keasling, J.D., 2011. Model-driven engineering of RNA devices to quantitatively program gene expression. Science 334, 1716–1719.
Carothers, J.M., Goler, J.A., Kapoor, Y., Lara, L., Keasling, J.D., 2010. Selecting RNA aptamers for synthetic biology: investigating magnesium dependence and predicting binding affinity. Nucleic Acids Res. 38, 2736–2747.
Chang, A.L., Wolf, J.J., Smolke, C.D., 2012. Synthetic RNA switches as a tool for temporal and spatial control over gene expression. Curr. Opin. Biotechnol. 23, 679–688.
Chen, Y.Y., Jensen, M.C., Smolke, C.D., 2010. Genetic control of mammalian T-cell proliferation with synthetic RNA regulatory systems. Proc. Natl. Acad. Sci. U.S.A. 107, 8531–8536.
Culler, S.J., Hoff, K.G., Smolke, C.D., 2010. Reprogramming cellular behavior with RNA controllers responsive to endogenous proteins. Science 330, 1251–1255.
Davidson, E.A., Ellington, A.D., 2005. Engineering regulatory RNAs. Trends Biotech. 23, 109–112.
de Smit, M.H., van Duin, J., 1990. Secondary structure of the ribosome binding site determines translational efficiency: a quantitative analysis. Proc. Natl. Acad. Sci. U.S.A. 87, 7668–7672.
de Smit, M.H., van Duin, J., 1994a. Control of translation by mRNA secondary structure in *Escherichia coli*. A quantitative analysis of literature data. J. Mol. Biol. 244, 144–150.
de Smit, M.H., van Duin, J., 1994b. Translational initiation on structured messengers. Another role for the Shine–Dalgarno interaction. J. Mol. Biol. 235, 173–184.
de Smit, M.H., van Duin, J., 2003. Translational standby sites: how ribosomes may deal with the rapid folding kinetics of mRNA. J. Mol. Biol. 331, 737–743.
Doudna, J.A., Cech, T.R., 2002. The chemical repertoire of natural ribozymes. Nature 418, 222–228.
Eddy, S.R., 2001. Non-coding RNA genes and the modern RNA world. Nat. Rev. Genet. 2, 919–929.
Gottesman, S., 2004. The small RNA regulators of *Escherichia coli*: roles and mechanisms*. Annu. Rev. Microbiol. 58, 303–328.
Holtz, W.J., Keasling, J.D., 2010. Engineering static and dynamic control of synthetic pathways. Cell 140, 19–23.
Isaacs, F.J., Dwyer, D.J., Collins, J.J., 2006. RNA synthetic biology. Nat. Biotechnol. 24, 545–554.
Isaacs, F.J., Dwyer, D.J., Ding, C., Pervouchine, D.D., Cantor, C.R., Collins, J.J., 2004. Engineered riboregulators enable post-transcriptional control of gene expression. Nat. Biotechnol. 22, 841–847.
Keasling, J.D., 2012. Synthetic biology and the development of tools for metabolic engineering. Metab. Eng. 14, 189–195.
Kehayova, P.D., Liu, D.R., 2007. *In vivo* evolution of an RNA-based transcriptional silencing domain in *S. cerevisiae*. Chem. Biol. 14, 65–74.
Kim, V.N., Han, J., Siomi, M.C., 2009. Biogenesis of small RNAs in animals. Nat. Rev. Mol. Cell Biol. 10, 126–139.
Kudla, G., Murray, A.W., Tollervey, D., Plotkin, J.B., 2009. Coding-sequence determinants of gene expression in *Escherichia coli*. Science 324, 255–258.
Liang, J.C., Bloom, R.J., Smolke, C.D., 2011. Engineering biological systems with synthetic RNA molecules. Mol. Cell 43, 915–926.
Lucks, J.B., Mortimer, S.A., Trapnell, C., Luo, S., Aviran, S., Schroth, G.P., Pachter, L., Doudna, J.A., Arkin, A.P., 2011a. Multiplexed RNA structure characterization with selective 2′-hydroxyl acylation analyzed by primer extension sequencing (SHAPE-Seq). Proc. Natl. Acad. Sci. U.S.A. 108, 11063–11068.
Lucks, J.B., Qi, L., Mutalik, V.K., Wang, D., Arkin, A.P., 2011b. Versatile RNA-sensing transcriptional regulators for engineering genetic networks. Proc. Natl. Acad. Sci. U.S.A. 108, 8617–8622.
Lynch, S.A., Gallivan, J.P., 2009. A flow cytometry-based screen for synthetic riboswitches. Nucleic Acids Res. 37, 184–192.
Lynch, S.A., Gill, R.T., 2012. Synthetic biology: new strategies for directing design. Metab. Eng. 14, 205–211.
Michener, J.K., Smolke, C.D., 2012. High-throughput enzyme evolution in *Saccharomyces cerevisiae* using a synthetic RNA switch. Metab. Eng. 14, 306–316.
Michener, J.K., Thodey, K., Liang, J.C., Smolke, C.D., 2012. Applications of genetically-encoded biosensors for the construction and control of biosynthetic pathways. Metab. Eng. 14, 212–222.
Montange, R.K., Batey, R.T., 2008. Riboswitches: emerging themes in RNA structure and function. Annu. Rev. Biophys. 37, 117–133.
Muranaka, N., Sharma, V., Nomura, Y., Yokobayashi, Y., 2009. An efficient platform for genetic selection and screening of gene switches in *Escherichia coli*. Nucleic Acids Res. 37, e39.
Mutalik, V.K., Qi, L., Guimaraes, J.C., Lucks, J.B., Arkin, A.P., 2012. Rationally designed families of orthogonal RNA regulators of translation. Nat. Chem. Biol. 8, 447–454.
Nakagawa, A., Minami, H., Kim, J.S., Koyanagi, T., Katayama, T., Sato, F., Kumagai, H., 2011. A bacterial platform for fermentative production of plant alkaloids. Nat. Commun. 2, 326.
Nandagopal, N., Elowitz, M.B., 2011. Synthetic biology: integrated gene circuits. Science 333, 1244–1248.
Nomura, Y., Yokobayashi, Y., 2007. Reengineering a natural riboswitch by dual genetic selection. J. Am. Chem. Soc. 129, 13814–13815.
Park, J.W., Tatavarty, R., Kim, D.W., Jung, H.T., Gu, M.B., 2012. Immobilization-free screening of aptamers assisted by graphene oxide. Chem. Commun. 48, 2071–2073.
Park, Y.S., Seo, S.W., Hwang, S., Chu, H.S., Ahn, J.H., Kim, T.W., Kim, D.M., Jung, G.Y., 2007. Design of 5′-untranslated region variants for tunable expression in *Escherichia coli*. Biochem. Biophys. Res. Commun. 356, 136–141.
Peralta-Yahya, P.P., Ouellet, M., Chan, R., Mukhopadhyay, A., Keasling, J.D., Lee, T.S., 2011. Identification and microbial production of a terpene-based advanced biofuel. Nat. Commun. 2, 483.
Pfleger, B.F., Pitera, D.J., Smolke, C.D., Keasling, J.D., 2006. Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. Nat. Biotechnol. 24, 1027–1032.
Qi, L., Lucks, J.B., Liu, C.C., Mutalik, V.K., Arkin, A.P., 2012. Engineering naturally occurring trans-acting non-coding RNAs to sense molecular signals. Nucleic Acids Res. 40, 5775–5786.
Ruder, W.C., Lu, T., Collins, J.J., 2011. Synthetic biology moving into the clinic. Science 333, 1248–1252.
Saito, H., Fujita, Y., Kashida, S., Hayashi, K., Inoue, T., 2011. Synthetic human cell fate regulation by protein-driven RNA switches. Nat. Commun. 2, 160.
Seo, S.W., Kim, S.C., Jung, G.Y., 2012. Synthetic regulatory tools for microbial engineering. Biotechnol. Bioprocess. Eng. 17, 1–7.
Seo, S.W., Yang, J., Jung, G.Y., 2009. Quantitative correlation between mRNA secondary structure around the region downstream of the initiation codon and translational efficiency in *Escherichia coli*. Biotechnol. Bioeng. 104, 611–616.
Serganov, A., Patel, D.J., 2007. Ribozymes, riboswitches and beyond: regulation of gene expression without proteins. Nat. Rev. Genet. 8, 776–790.
Sharma, V., Nomura, Y., Yokobayashi, Y., 2008. Engineering complex riboswitch regulation by dual genetic selection. J. Am. Chem. Soc. 130, 16310–16315.
Sharp, P.A., 2009. The centrality of RNA. Cell 136, 577–580.
Sinha, J., Reyes, S.J., Gallivan, J.P., 2010. Reprogramming bacteria to seek and destroy an herbicide. Nat. Chem. Biol. 6, 464–470.
Smolke, C.D., Keasling, J.D., 2002. Effect of copy number and mRNA processing and stabilization on transcript and protein levels from an engineered dual-gene operon. Biotechnol. Bioeng. 78, 412–424.
Stoltenburg, R., Reinemann, C., Strehlitz, B., 2007. SELEX—a (r)evolutionary method to generate high-affinity nucleic acid ligands. Biomol. Eng. 24, 381–403.
Tran, T., Disney, M.D., 2012. Identifying the preferred RNA motifs and chemotypes that interact by probing millions of combinations. Nat. Commun. 3, 1125.
Valentin-Hansen, P., Eriksen, M., Udesen, C., 2004. The bacterial Sm-like protein Hfq: a key player in RNA transactions. Mol. Microbiol. 51, 1525–1533.
Wagner, E.G., Flardh, K., 2002. Antisense RNAs everywhere? Trends Genet. 18, 223–226.
Wieland, M., Benz, A., Klauser, B., Hartig, J.S., 2009. Artificial ribozyme switches containing natural riboswitch aptamer domains. Angew. Chem. Int. Ed. 48, 2715–2718.
Win, M.N., Smolke, C.D., 2007. A modular and extensible RNA-based gene-regulatory platform for engineering cellular function. Proc. Natl. Acad. Sci. U.S.A. 104, 14283–14288.
Xie, Z., Wroblewska, L., Prochazka, L., Weiss, R., Benenson, Y., 2011. Multi-input RNAi-based logic circuit for identification of specific cancer cells. Science 333, 1307–1311.
Yadav, V.G., De Mey, M., Lim, C.G., Ajikumar, P.K., Stephanopoulos, G., 2012. The future of metabolic engineering and synthetic biology: towards a systematic practice. Metab. Eng. 14, 233–241.
Yao, Z., Barrick, J., Weinberg, Z., Neph, S., Breaker, R., Tompa, M., Ruzzo, W.L., 2007. A computational pipeline for high- throughput discovery of cis-regulatory noncoding RNA in prokaryotes. PLoS Comput. Biol. 3, e126.