# Chapter 5

# Conclusion

Robustly engineering cell fate via synthetic circuits offers the potential to direct developmental programs and intervene in aberrantly activated cell processes. Controlling cell fate will advance regenerative medicine by facilitating the *ex vivo* construction of replacement tissues. Further, synthetic circuits may be configured to recognize hyperactive programs that lead to disease and reinstitute the proper behavior or eliminate misbehaving cells on a cell-by-cell basis limiting side-effects of such therapies [1, 2]. However, the engineering of systems to regulate cell fate requires precise control over circuit performance as well as the ability to interface with decision-making pathways [3]. To facilitate the construction of synthetic circuits that regulate cellular behavior for a wide range of potential applications, we developed RNA-based controllers and systems that were applied to regulate signaling in a model MAPK pathway and direct cell fate.

Utilizing modular and tunable RNA-based controllers and network architectures, we constructed networks of RNA-based control systems that interface with the *Saccharomyces cerevisiae* mating pathway to dictate entry into one of three alternative fates dependent on environmental stimuli. In building these networks, we developed a readily translatable method for identifying control points within natural networks that enable the construction of a modular interface between synthetic circuitry and native networks. Additionally, we demonstrated the rational tuning of circuit performance via the exchange of well-defined parts to compose networks capable of actuating changes in cellular behavior in response to environmental cues. Further, we constructed network architectures which facilitate reduced interference from simultaneously integrated opposing programs and identified sensitive parameters for engineering robust circuit performance. This work provides a model for engineering systems that regulate signaling

and direct cells fate which may be applied to additional decision-making pathways to advance tissue engineering strategies, treat diseases, and study the behavior of natural regulatory networks.

**Immediate challenges for the development of RNA-based control systems that regulate signaling and dictate cell fate**

The constructed molecular network diverters robustly program fate in response to small-molecule input when integrated into cells independent of the opposing diverter. However, due to diverter antagonization, dual-fate routing is only permitted at elevated levels of small-molecule input supported by metabolic modulation.. Diverter antagonization occurs through subthreshold basal levels of expression from the opposing diverter reducing the actuated change in pathway activity induced by the triggered diverter, leading to weaker routing. There exist several potential avenues by which to reduce the basal expression levels from the diverters and minimize diverter antagonization. Reducing the strength of expression by exchanging the promoters that regulate the most antagonistic expression modules will reduce basal expression levels. However, this strategy necessarily reduces the expression induced from the module, potentially hindering diverter performance. Reducing module expression by modification of the plasmid system or integration of the expression modules offer the same limitations as promoter exchange. Nevertheless, integration of the expression modules may provide an advantage over promoter exchange, as integration has been demonstrated to reduce the variance of gene expression compared to plasmid-based systems [4]. While broadly reducing expression from the various modules may reduce the triggered levels of expression from the diverters, the reduction in diverter antagonism may more than

compensate for the reductions, potentially enhancing dual-fate routing performance in these systems.

Secondary layers of control provide an alternative to broadly reducing expression from the diverters. Trans-acting RNA elements have been demonstrated to reduce basal level leakage from circuits in the OFF state, enhancing circuit performance [5-7]. While these particular elements are not amenable to the regulation gene expression in yeast, trans-acting ribozymes offer a control tool for limiting the basal levels of expression in our system. We developed trans-acting ribozymes that target a sequence in the GFP transcript. Appending this GFP target sequence to the genes of pathway regulators may reduce basal expression when trans-ribozymes are coexpressed with the diverters. The development of additional trans-acting ribozymes and cognate target sequences could allow independent regulation of various genes. However, our results indicate that the developed trans-acting ribozymes efficiently regulate expression within a relatively narrow window of transcript expression, presenting limitations in the effective incorporation of these elements to our system. Further work is required to determine if the limited window of efficacy is specific to trans-acting ribozymes in general or to the particular target sequence to which the ribozyme was developed. Additionally, our results suggest that one of the key limitations to trans-ribozyme-mediated knockdown may be facilitating the intermolecular binding event between the trans-acting ribozymes and the target transcript. Co-expression of trans-ribozymes with target from the same locus or vector may overcome these limitations and improve the range of expression levels over which knockdown is observed by facilitating transcript colocalization.

Given the success of their cis-acting counter parts as switches, the development of more potent trans-acting ribozymes would poise these elements as expression platforms by which to construct ligand-responsive trans-acting RNA-based controllers. Trans-acting RNA-based controllers that regulate gene expression in response to small molecule concentrations have been demonstrated in mammalian systems [8, 9] With these trans-acting switches, basal expression levels could be regulated in response to small-molecule concentrations by appending the target sequence to the desired genes. Conditionally reducing target expression via the presence or absence of the small molecule offers a mechanism for reducing basal expression levels from the various modules without a similar reduction in the triggered levels. Reduced diverter antagonism and greater fold changes in expression from the diverters may be achieved by the development and incorporation of ligand-responsive trans-acting RNA controllers.

In addition to layers of trans-acting controllers, the introduction of mutual inhibitory modules between the dual diverters may facilitate enhanced dual-routing performance by reducing diverter antagonization. Mutually inhibitory loops have been demonstrated to allow cells to toggle between two distinct phenotypes [10, 11] and may be constructed from transcriptional or post-transcriptional elements. Construction of transcriptional mutually inhibitory modules requires two orthogonal repressible promoters each regulating the expression of the other's repressor protein. Transcriptional mutual inhibitory modules are simply connected to the diverters by promoter exchange in the expression modules containing constitutive promoters. While the system is readily constructed, identifying repressible promoters of the requisite strength and tuning the mutual inhibitory module present a critical hurdle for enhancing diverter function via

transcriptional mutually inhibitory modules. Construction of repressible promoter libraries will increase the feasibility of constructing and connecting transcriptional mutual inhibitory modules to synthetic circuits. Alternatively, post-transcriptional controllers provide a promoter-independent mechanism for introducing mutual inhibitory loops into the diverter networks, providing greater design flexibility. Post-transcriptional mutually inhibitory loops may be constructed by directly modifying the constructed expression modules with target sites specific for a trans-acting RNA regulatory element. Imbedding cognate trans-acting RNA regulators in the expression modules of the opposing diverter establishes an inhibitory regulatory loop from one diverter to another. Addition of a secondary inhibitory loop with a different trans-acting RNA regulator and cognate target site results in a mutually inhibitory architecture. Further, mutual inhibition via trans-acting RNA regulators can be made ligand-dependent by the use of small molecule-responsive trans-acting RNA switches. The continued development of ligand-responsive switches from potent trans-acting ribozymes, microRNAs, and other trans-acting RNA-based regulators of gene expression will enable the construction of more complex control schemes within synthetic networks. While regulation via post-transcriptional control may be limited in stringency compared to transcriptional control, the added flexibility, tunability, and potential for ligand-dependent regulation position post-transcriptional mutually inhibitory loops to be important tools for the construction of synthetic circuits. The addition of mutually inhibitory modules that enhance differentiation between synthetic circuits' ON and OFF state outputs will enable robust control of cell fate, expanding the range of systems and applications to which synthetic circuits may be applied.

**Future directions for the development of RNA-based control systems to regulate signaling and dictate cell fate**

The *ex vivo* construction of tissues, the implementation of autonomous immune surveillance, and the development of 'smart' therapeutics will require flexible, tunable, and scalable synthetic networks that can interface with a range of native pathway via circuitry capable of extracting cellular information and actuating changes in cellular behavior. Our work presents a method for identifying control points in native pathways that is amenable across a wide array of systems. Further, the development of the molecular network diverters provides a framework for constructing and tuning synthetic networks that control cellular behavior that is readily transportable across pathways, organisms, and applications. Yet there remain several challenges to extending these systems to applications in health and medicine.

Application of the molecular diverter strategy for biomedical purposes will require the identification of titratable pathway regulators in the decision-making pathways in mammalian cells. While our results with the yeast MAPK pathways illuminate potential candidates within the homologous mammalian pathways, titration studies must be performed in these systems to validate potential pathway regulators' ability to route cells to alternative fates. Further, examination of the pathway response curve to varying regulator expression will facilitate identification of the transitory range over which fate diverges and enable rational design of molecule network diverters. Titration studies to identify pathway regulators may be performed in mammalian cells using the well-documented tetracycline-inducible promoter [12]. Already similar work has been performed using the tetracycline-inducible system to facilitate the programming

of induced pluripotent stem cells (iPSCs) via the control of key transcription factors [13]. In addition to the requisite pathway regulators, our work highlights the utility of pathway-responsive promoters for the construction of feedback loops that amplify and attenuate pathway activity. Identification of relevant pathway-responsive promoters will facilitate the construction of molecular network diverters within new pathways. In the ERK signaling pathway which is homologous to the yeast mating pathway and responsible for controlling cell growth, several pathway-responsive promoters have been characterized and reporter constructs with these elements and a host of other signaling pathway are commercially available [14]. Tuning promoter expression to achieve the desired strength may be accomplished by the construction of promoter libraries or by rational design [15, 16].

Beyond pathway regulators and promoters, improvements in the stringency and regulatory range of RNA-based switches as well as the development of new switch sensor domains will expand the applicability of molecular network diverters. The incorporation of RNA switches exhibiting low basal levels and large dynamic ranges may increase between the range of circuit output, enhancing differentiation between triggered and non-triggered cells. However, computational models of RNA switches have indicated a tradeoff in tuning the stringency of a switch and its sensitivity to the input ligand, ultimately impacting the dynamic range [17]. Nevertheless, selection of switches with high stringency and large dynamic ranges is important particularly for the development of switches responsive to new ligands, an important step in translating these systems to clinical applications. In this study, we incorporated switches responsive to theophylline and tetracycline in our designs. While these molecules are suitable for the regulation of

yeast gene expression, cytotoxicity restricts the concentrations that may be safely administered in mammalian systems thus reducing the range of expression from these elements in a therapeutically relevant context [2]. The selection of new aptamer sequences to FDA-approved molecules will facilitate the construction of switches that may be applied as therapies. Additionally, the development of new aptamer sequences to cellular molecules of interest offers the potential to connect information from endogenous networks to synthetic control systems. The construction of circuits responsive to endogenous molecules will facilitate autonomous cell-based therapies. Synthetic RNA aptamers have been generated *de novo* to various small-molecule and protein targets through *in vitro* selection or SELEX strategies [18, 19]. The Smolke laboratory has generated RNA aptamers that exhibit varying specificities to benzylisoquinoline alkaloids [20] and folinic acid derivates, and is developing high-throughput strategies for the direct selection and characterization of new protein- and small-molecule-responsive aptamers. The development of switches responsive to new molecules will provide additional independent channels of regulation by which to construct orthogonal control of multiple genes within complex networks.

Finally, connecting additional synthetic circuitry to the molecular network diverters may facilitate more complex and sophisticated cellular programming [3]. Complex functions such as the synchronization of genetic clocks and edge detection have been demonstrated via the rational coupling of communication circuits to oscillators and light-responsive logical circuits, respectively, [21, 22]. Coupling synthetic circuits that control cellular signaling to communication circuits may facilitate robust spatial patterning of cell fate, enable tissue homeostasis, and allow the coordination of distributed tasks, requisite achievements for the construction, maintenance, and function

of complex tissues [23-25]. Further, advances in iPSC technology may enable the construction of immunologically compatible tissues from cells synthetically programmed for precise spatial and temporal differentiation on designer cell scaffolds [26-28].

In this work we have demonstrated the construction of RNA-based control systems that route cells to divergent cell fates in response to exogenously applied triggers. These systems highlight the potential to develop synthetic networks that spatially and temporally program cell fate advancing the fields of tissue engineering and molecular medicine. As the systems biology and synthetic biology tool box expands, the future development of these systems promises the construction of larger, more complex networks engineered to control a range of systems from cells to organs to whole organisms for a wide array of biotechnological and medical applications.

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