Chapter 7

Conclusions

The development of cell-based therapies ranging from well-established practices such as bone marrow transplant to next-generation strategies such as adoptive T-cell therapy represents exciting progress in medical technology. The ability to harness the complex functionalities and adaptive responses of living cells as therapeutic agents promises to address critical diseases that have so far eluded effective treatment. However, with the intricate capabilities of living cells come myriad system parameters that must be fine-tuned and stringently controlled to achieve safe and effective therapies. To address this challenge, we have developed rationally designed, RNA-based regulatory systems capable of genetic control over functional outputs in mammalian lymphocytes.

We constructed ligand-responsive regulatory systems that employ *cis*-acting ribozyme switches and *trans*-acting miRNA constructs for the effective regulation of T-cell proliferation in primary human cell culture and in mouse models. We demonstrated unique properties of these synthetic control systems—including modular composition, tunable regulatory stringency, and rapid response to ligand input—that are critical for translations to clinical applications. This work provides a foundation upon which further system developments may be achieved, particularly in the incorporation of additional regulatory targets, the generation of novel sensor components to clinically suitable molecular inputs, and the development of engineered T cells with enhanced tumor-targeting capabilities for cancer treatment.

Immediate Challenges for the Clinical Translation of RNA-Based T-Cell Proliferation Control Systems

The regulatory systems developed in this work modulate the expression of upstream signaling molecules in the growth-stimulatory Interleukin (IL)-2/IL-15 cytokine signaling pathways. Although the autocrine signaling processes mediated by the cytokines and receptor chains that are the focus of our current system designs are critical to T-cell proliferation, several additional factors have profound impacts on the survival and expansion of T cells *in vivo*. For example, the tumor microenvironment is known to be immunosuppressive due to the presence of potent T-cell growth-inhibitory molecules such as tumor growth factor β (TGF-β)[1] and receptor programmed death ligand 1 (PD-L1)[2]. Furthermore, receptors such as the cytotoxic T-lymphocyte antigen 4 (CTLA4) on T-cell surfaces interact with ligands present in the tumor microenvironment to inactivate tumor-infiltrating lymphocytes[3]. Future regulatory system designs can incorporate the TGF-β receptor, PD-1 (the receptor to PD-L1), and CTLA4 as regulatory targets to mediate these intercellular, growth-inhibitory signaling events.

A second area of potential improvement is the generation of new RNA aptamers that will enable the construction of regulatory devices responsive to more clinically suitable molecular inputs. The constructs developed in this work have relied on well-characterized aptamers to theophylline and tetracycline, neither of which can be administered to human patients at the dosages required for regulatory system function due to cytotoxicity concerns. Our efforts in generating new RNA aptamers to better tolerated molecules such as folinic acid and vitamin B_{12} have not resulted in high-affinity aptamer sequences. The selection of aptamers to small-molecule ligands is particularly challenging due to a frequent lack of functional groups suitable for chemical conjugation to solid supports, which are required for the aptamer selection process. Furthermore,

clinical applications pose the additional requirements of low toxicity, high bioavailability, and efficient cell permeability for the target molecules. Ongoing efforts in the Smolke Laboratory aim to develop alternative aptamer selection methods—including *in vivo* selection and *in vitro* protocols utilizing alternative separation techniques such as capillary electrophoresis that do not require the use of solid supports—to facilitate the selection of new RNA aptamers. The experiences presented in this work suggest that not all small-molecule ligands are equally well suited to RNA aptamer selections. Therefore, the effective generation of novel aptamers will require the identification of a broad range of clinically applicable molecules and the development of high-throughput protocols that enable the simultaneous selection of aptamers toward a large panel of target molecules, a subset of which may yield high-affinity aptamer sequences.

The goal of T-cell proliferation control is to improve the safety and efficacy of adoptive T-cell therapy for cancer. Therefore, it is of great scientific interest to verify that effective control over T-cell growth *in vivo* can enhance the tumor-fighting capability of engineered T cells. Tumor-targeting T cells stably expressing RNA-based regulatory systems should be tested in tumor xenograft models to examine not only T-cell proliferation, but also the impact on tumor regression and eradication. Since relevant models for therapeutic applications will require systemic injections of input ligands, which must be well tolerated by the host organism, this investigation will be more effectively performed after the development of RNA aptamers to clinically suitable molecules. Beyond these immediate steps toward the development of a more complete and versatile T-cell proliferation control system, several areas of investigation have the

potential to further improve the functionalities of RNA-based regulatory systems and broaden their applications in health and medicine.

Future Directions for the Development of RNA-Based Regulatory Systems for Clinical Applications

Stringent control of the fates and functions of cells in therapeutic applications will likely require the simultaneous regulation of multiple targets in response to various molecular inputs. The regulatory systems developed in this work are adaptable to diverse input molecules and genetic targets, have compact footprints, do not require exogenous and potentially immunogenic protein components, and support the construction of integrated control networks incorporating both ribozyme- and miRNA-based regulatory mechanisms. However, several critical challenges remain in the development of RNA-based regulatory systems for clinical applications and present interesting opportunities for future investigations.

First, compared to alternative regulatory strategies such as inducible promoter systems, RNA-based control devices have relatively high basal expression levels or "leakiness" in target-gene expression. Although we have demonstrated that miRNA switch systems are capable of greater gene expression knockdown compared to non-switch miRNAs expressed from inducible promoters (Chapter 5), neither miRNA switches nor ribozyme-based devices can completely suppress gene expression, as would be possible with transgenes placed directly under the control of inducible promoters. Patient safety demands that T-cell proliferation be fully and reliably terminated in the OFF state, and even a low level of leakiness in proliferative cytokine or cytokine receptor

chain expression may prove unacceptable for therapeutic applications. In addition to the various performance tuning strategies presented in this work, the regulatory stringency of RNA-based control systems may be improved by utilizing alternative ribozymes with optimal cleavage activities in mammalian hosts, by placing critical target genes under the simultaneous control of multiple types of control devices, and by carefully specifying the strengths of constitutive promoters from which transgenes such as proliferative cytokines and regulatory devices such as ribozyme and miRNA switches are expressed.

Second, the switch dynamic ranges of the RNA-based control devices presented in this work are relatively modest and leave significant room for improvement. We have demonstrated that by targeting upstream signaling events and taking advantage of signal amplification cascades, even systems with modest switch dynamic ranges can meaningfully regulate functional outputs in both mouse and human T cells. However, such signal amplification effects may not be available in all applications of interest, and the ability to generate a large output range in response to a defined input gradient will greatly enhance the applicability of RNA-based regulatory systems. Ligand toxicity has been an important constraint on the dynamic ranges of devices presented in this work. Specifically, the maximum ON-state expression level is often inaccessible due to the inability to administer ligand molecules at high concentrations, which frequently compromise cell survival. This challenge would be addressed by continuing efforts to generate high-affinity RNA aptamers to molecules with low toxicity and high cell permeability. Furthermore, the maximum dynamic range of RNA-based regulatory devices is subject to intrinsic thermodynamic and kinetic limitations[4]. Theoretical modeling and empirical studies have revealed several tuning strategies—including the

modulation of the rates of transcription, translation, and protein decay; the introduction of transcriptional pause sites to bias transcriptional folding; and the design of switch sequences to favor disrupted-aptamer conformations—that can be applied to increase the dynamic ranges of RNA-based control devices[4].

Third, the RNA-based regulatory systems presented here have been designed to generate gene-regulatory outputs in response to exogenously administered molecular inputs and have not been examined for their ability to respond to endogenous signals. The versatility of these control systems, particularly in the context of cancer treatment, can greatly benefit from the ability to detect endogenous molecular inputs. For example, a T-cell proliferation control system that promotes T-cell growth in the presence of a soluble tumor antigen would enable autonomous disease detections that lead to a therapeutic response. Alternatively, a system that detects changes in endogenous T-cell markers (e.g., surface receptors that indicate the transformation of a regulatory T cell into an effector T cell or vice versa) and subsequently eliminates the transformed cells would provide critical safeguards against unintended and potentially detrimental alterations in T-cell populations after their adoptive transfer to cancer patients. An RNA-based regulatory system capable of regulating alternative splicing patterns in response to endogenous signaling pathways and protein targets was recently reported[5], providing a novel example of RNA-based genetic control over critical cellular processes in response to endogenous input signals.

Finally, the stable integration of RNA-based regulatory systems into cellular hosts intended for therapeutic applications will likely require additional development and optimization. Although RNA-based regulatory systems have the distinct advantage of a

compact footprint compared to protein-based systems, genetically modifying cellular hosts such as primary human T cells to express any transgenic control system remains a non-trivial challenge. Safety considerations require that the cells used for immunotherapy be of sufficient genotypic and phenotypic consistency for adoptive transfer into human patients. Conversely, the race against tumor expansion and metastasis demands that such therapeutic cell populations be generated in a timely manner, possibly without allowance for a careful selection procedure to isolate optimally performing clones. We have demonstrated that the RNA-based regulatory systems presented in this work are sufficiently robust to produce the prescribed functional output without population refinement after stable integration into primary human T cells. Nevertheless, additional developments on efficient genetic modification techniques, such as site-specific gene insertions into mammalian genomes using engineered viral vectors, will greatly enhance the applicability of genetic control strategies in clinical settings.

The work presented here demonstrates the ability of RNA-based regulatory systems to control both gene expression and functional outputs in mammalian cells, and highlights the potential of synthetic biological designs to carry out complex functions with programmed precision. The continuing development of rationally designed biological systems promises to bring forth novel capabilities with diverse applications, particularly in the advancement of next-generation technologies in health and medicine.

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