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Mammalian gene circuits with biomolecule-responsive RNA devices

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Mammalian synthetic gene circuits are promising tools for both biological studies and therapeutic applications. These circuits enable autonomous cellular state-dependent regulation such as the killing of a target cell population. Biomolecule-responsive RNA devices are preferred components of mammalian synthetic gene circuits, because they enable cell-specific gene regulation with a low risk of insertional mutagenesis. In addition, because the output of one RNA device can be the input of another RNA device, synthetic biologists can construct layered gene circuits for more sophisticated regulation. In this review, we explain recent advancements of biomolecule-responsive mammalian gene circuits based on RNA devices.

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Introduction

Today, gene delivery is an important method not only for biological studies but also medical applications including gene and cell therapies. Compared with conventional small molecule drugs, vectors for gene and cell therapies can be readily designed to respond to the cellular state. Therefore, they are suitable for applications that target cell-specific gene expressions such as the selective killing of tumor cells in cancer gene therapies.

While transcriptional regulation by cell-specific promoters has been widely used for these applications [1,2], post-transcriptional regulation by biomolecule-responsive RNA devices is an alternative, safer approach. RNA devices are especially suitable for therapeutic applications, because they can be introduced into cells as DNA or synthetic RNA [3], the latter of which significantly lowers the risk of insertional mutagenesis. Protein-responsive and RNA-responsive RNA devices can be used for the repression [4–6] or activation [7] of protein-coding genes and the regulation of RNA processing [8]. In addition, because the output of one RNA device can be the input of another RNA device, synthetic biologists can design layered gene circuits with these RNA devices to enable more sophisticated cell-selective regulation.

In this review, we focus on recent advancements of biomolecule-responsive RNA devices and gene circuits that function in mammalian cells.

Protein-responsive RNA devices

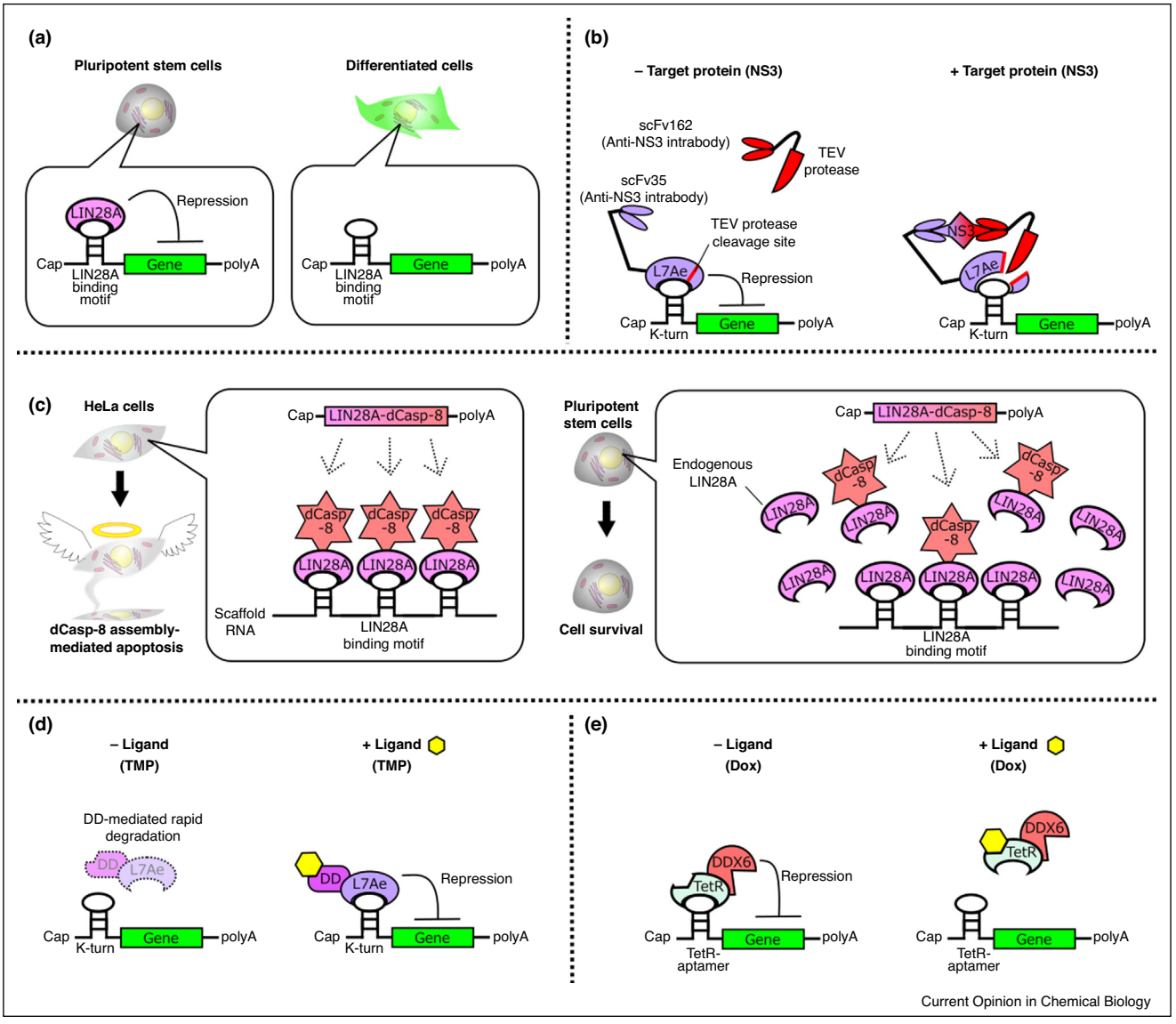
Cellular state-specific regulation by endogenous or pathogenic proteins

The expression level of endogenous proteins differs among cellular contexts including differentiation states or diseases. Therefore, protein-responsive devices are suitable for autonomous gene regulation dependent on the cellular context. Prokaryotic RNA-binding proteins (RBPs) such as L7Ae or MS2CP have been widely used for mammalian gene circuits because of their high affinity to target RNA motifs [3,5,7,9]. However, because these RBPs are not endogenous in mammalian cells, RNA devices that respond to them cannot be used to directly detect the cellular context.

For the direct detection of the differentiation state in mammalian cells, our group has developed an mRNA device that responds to a human endogenous protein, LIN28A [10•]. Because the expression of LIN28A is high in human induced pluripotent stem cells (hiPSCs) and low in differentiated cells, the translation of LIN28A-responsive mRNA device was selectively repressed in hiPSCs (Figure 1a). The responsiveness of 1st generation LIN28A-responsive mRNA was too low to distinguish pluripotent and differentiated cells but could be improved by stabilizing the LIN28A binding motif by increasing the GC content in the mRNA stem region. Using this 2nd generation LIN28A-responsive mRNA that coded a fluorescent protein, the monitoring of differentiation states in living cells was achieved.

A system to detect proteins that do not directly bind RNAs was also reported. Cella *et al.* developed a system composed of tobacco etch virus protease (TEVp), L7Ae with the TEVp-cleavage site (L7Ae-CS), and two

Figure 1



Protein-mediated regulation of gene expression and cell fate.
(a) Cell-specific translational regulation of the mRNA containing a binding motif that targets the cell-specific protein. In human pluripotent stem cells (hPSCs), LIN28A is highly expressed and binding of it to the binding motif represses translation (left). In contrast, in differentiated cells, the translation of the mRNA containing the binding motif is not inhibited because of the low expression of LIN28A (right). **(b)** Detection of the viral protein NS3 using intrabody-fused L7Ae and protease. L7Ae with the TEV protease (TEVp) cleavage site (L7Ae-CS) and TEVp are fused with the anti-NS3 intrabodies scFv35 and scFv162, respectively. In the absence of NS3, L7Ae-CS-scFv35 represses the translation of K-turn-embedded mRNA. The interaction of L7Ae-CS and TEVp mediated by NS3 and intrabodies enhances the cleavage of L7Ae-CS. Because of the cleavage, translation of the mRNA is released. **(c)** Defective Caspase-8 (dCasp-8) assembly mediated selective cell killing utilizing RNA-protein interactions. In HeLa cells, LIN28A-dCasp-8 fusion proteins are bound to LIN28A-binding motif of the scaffold RNA, which induces dCasp-8 assembly mediated apoptosis (left). In contrast, in pluripotent stem cells, endogenous LIN28A acts as a competitor for the scaffold RNA-binding, which represses dCasp-8 assembly mediated apoptosis (right). **(d)** Small molecule-controllable degradation of a translationally repressive protein. Destabilization domain-fused L7Ae (DD-L7Ae) is rapidly degraded in the absence of the ligand, trimethoprim (TMP) (left). Addition of the ligand inhibits destabilization domain-mediated rapid degradation, such that DD-L7Ae binds to K-turn, the target RNA motif of L7Ae. When the K-turn motif is embedded in the 5′ UTR of the mRNA, the binding of L7Ae to it represses translation (right). **(e)** Small molecule-controllable binding of a translationally repressive protein. Tetracycline-responsive repressor fused with dead box helicase 6 (TetR-DDX6) is bound to the TetR-aptamer in the absence of the ligand, doxycycline (Dox), and represses translation (left). Addition of the ligand inhibits the binding, which results in translation of the mRNA containing the TetR-aptamer (right).

single-chain fragment intrabodies that target different epitopes of the identical protein [11••]. One of these two intrabodies was fused with TEVp, and the other was fused with L7Ae-CS (Figure 1b). In the absence of the target protein, L7Ae-Cs repressed the translation of the mRNA containing the kink-turn (K-turn) motif, the target motif of L7Ae. In contrast, in the presence of the target protein, TEVp and L7Ae-CS were attached via the intrabodies and the target protein, such that TEVp cleaved L7Ae-CS. Because of the cleavage, the translational repression by L7Ae-CS was released. Using the system, they succeeded in detecting the hepatitis C virus protein NS3. Although this approach can be used only when two intrabodies targeting different epitopes of the identical protein are known, it does not need the direct binding of RNA devices and target proteins. Therefore, it will enable the detection of proteins lacking RNA-binding properties.

An alternative approach to regulating cell fate uses a nanostructured RNA device. Our group has succeeded in the cell-selective induction of apoptosis by regulating protein assembly through scaffold RNA [12••]. The system is composed of LIN28A protein fused with a death effector domain-defective Caspase-8 (LIN28A-dCasp-8) and scaffold RNA that contains nine copies of the LIN28A-binding motif. Caspase-8 is a protein that induces apoptosis when it oligomerizes. Because the death effector domain is necessary for the oligomerization of Caspase-8, dCasp-8 cannot induce apoptosis by itself. The fusion of LIN28A to dCasp-8 enables scaffold RNA-mediated assembling of the fusion protein. Therefore, in cells with low levels of endogenous LIN28A expression (e.g. HeLa cells), LIN28A-dCasp-8 fusion proteins are assembled, which induces apoptosis. In contrast, in cells with high levels of endogenous LIN28A expression e.g. (hiPSCs), endogenous LIN28A competes with LIN28A-dCasp-8 to bind to scaffold the RNA, which hinders the apoptosis induction (Figure 1c).

Post-transcriptional regulation by small molecule-responsive proteins

In general, a single administration of vectors for gene therapy has longer effects than that of small molecule drugs, because the vectors continuously express proteins, especially when long-lasting vectors such as RNA replicons [13] are used. Long-lasting therapeutic effects are one of the advantages of gene therapy, but for the same reason, it is difficult to stop gene expression even if any adverse effects are observed. Although cellular state-dependent gene regulation systems can lower the risk of such adverse effects, in actual therapy, unpredicted adverse effects may be observed. Therefore, a system that turns gene expressions on and off by an easily administrable small molecule should improve the safety of gene therapies.

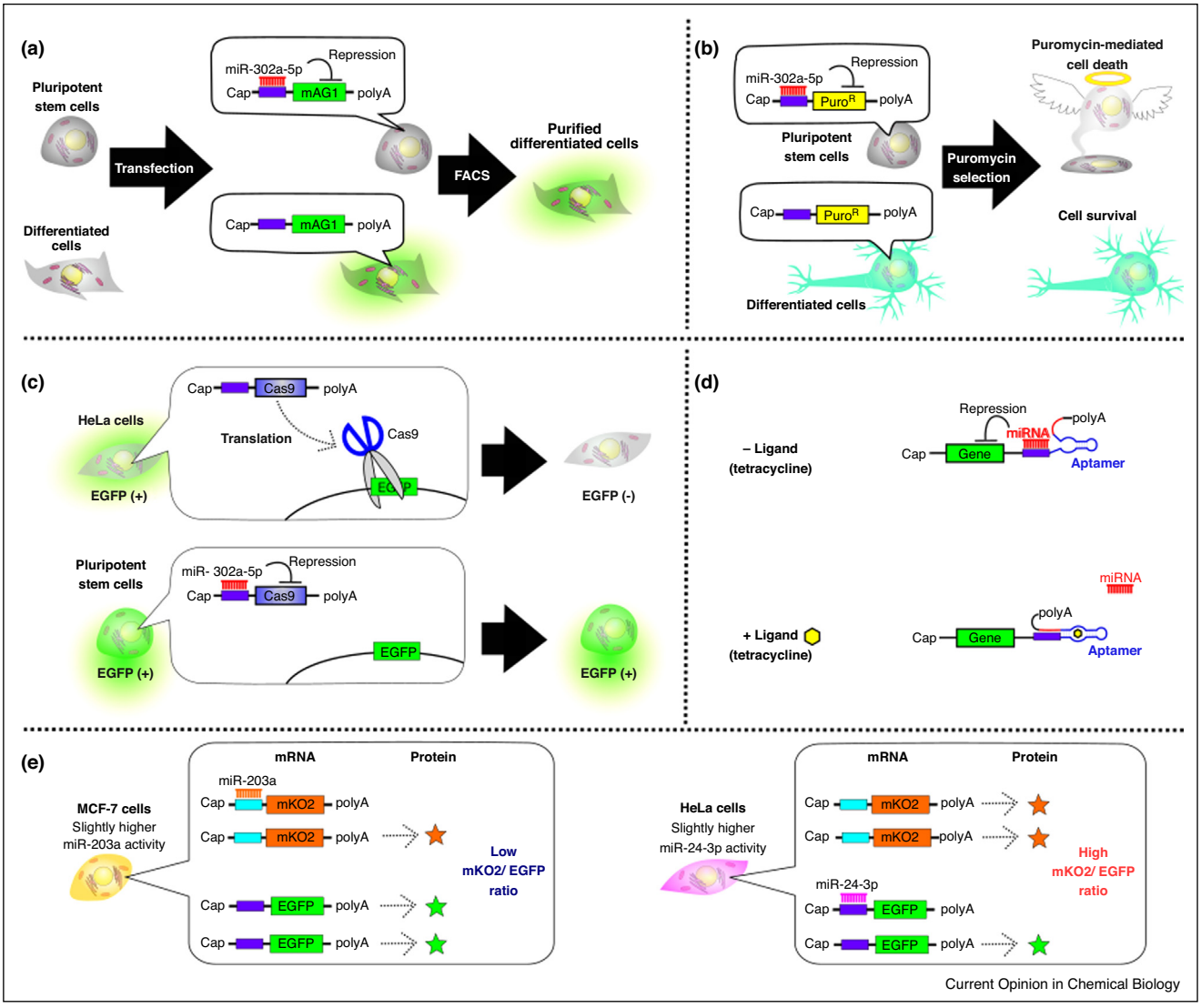
Accordingly, for small molecule-mediated gene regulation, Wagner *et al.* utilized small molecule-responsive proteins to regulate the translation of mRNAs [14•]. They fused trimethoprim (TMP)-responsive destabilization domain (DD) to L7Ae. Conventional L7Ae strongly represses the translation of mRNAs containing a K-turn motif in its 5′ UTR or ORF [4]. However, DD-fused L7Ae (DD-L7Ae) cannot efficiently repress translation due to DD-mediated rapid degradation. The addition of TMP stabilized DD-L7Ae and induced the repression of K-turn-embedded mRNAs through the interaction between DD-L7Ae and the K-turn motif (Figure 1d). The researchers also designed a fusion construct of the tetracycline-responsive repressor (TetR) for the translational regulation. TetR is a transcription factor that binds its target DNA sequence and also to TetR-targeting RNA aptamers. Importantly, the binding of TetR and the aptamer is inhibited by tetracycline and doxycycline. Although translational repression by TetR itself was weak, the fusion of TetR and dead box helicase 6 (DDX6), a protein that interacts with the RNA degradation-silencing complex, enabled efficient translational regulation by doxycycline (Figure 1e).

microRNA-mediated translational inhibition

While endogenous proteins can be used as markers of cellular states, designing RNA motifs that specifically bind to target proteins is difficult. Interestingly, one small noncoding RNA, microRNA (miRNA), can also be used as markers of cellular states, because the activities of individual miRNAs are different among cellular states. Importantly, different from protein-responsive mRNAs, miRNA-responsive mRNAs are easily designed. In fact, the simple insertion of complementary miRNA sequences into mRNA UTRs enables miRNA-mediated post-transcriptional repression of the mRNAs. Using mRNA devices containing reporter genes and the complementary sequence of cell-specific miRNAs, we can distinguish cell types based on the activities of endogenous miRNAs. Our group developed mRNA devices that respond to cell-specific miRNAs and enable purification of a target cell population. miRNA-responsive mRNA devices coding fluorescent proteins enabled fluorescence-activated cell sorting (FACS) of specific cells such as neuronal cells, cardiomyocytes, and insulin-producing cells differentiated from hiPSCs (Figure 2a) [15–17].

Because we can use any gene of interest as the coding region of the miRNA-responsive mRNA, the application is not limited to the visualization or purification of cell types by reporter genes. For example, by using an antibiotic-resistant gene as the coding region of the miRNA-responsive mRNA, we can eliminate specific miRNA-positive cells (Figure 2b). Indeed, we have succeeded in eliminating hiPSCs by using mRNA encoding puromycin-resistant gene and responsive to

Figure 2

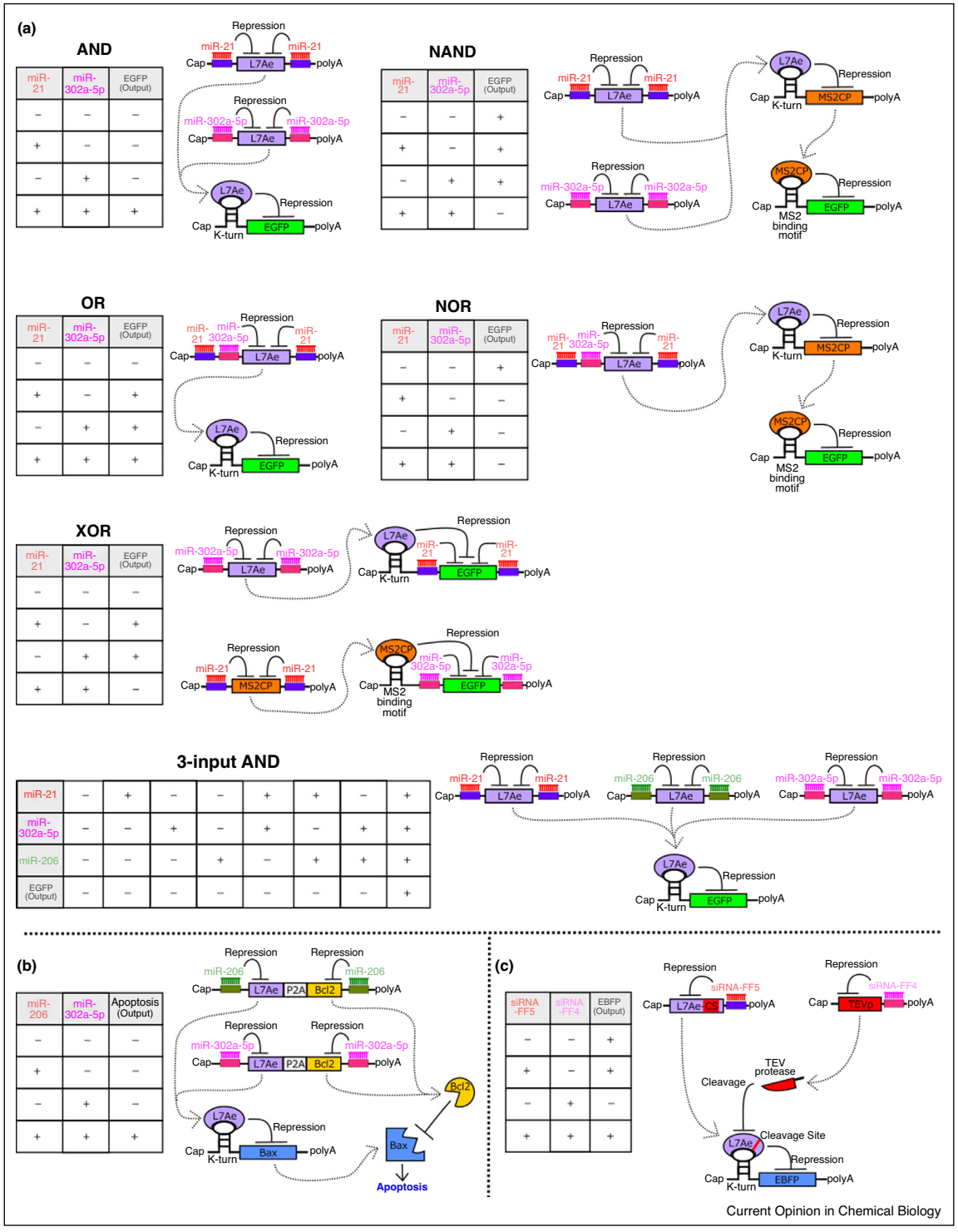


miRNA-mediated, cell-specific translational regulation.
The binding of miRNAs to their complementary sequences in the UTRs of mRNAs induces translational repression. **(a)** The purification of differentiated cells by fluorescence activated cell sorting (FACS). hPSCs and differentiated cells are distinguished based on the translational repression of a fluorescent protein (e.g. monomeric Azami Green (mAG1)). For example, Because miR-302a-5p activity is high in human pluripotent stem cells (hPSCs) and low in differentiated cells, mRNA containing the complementary sequence of miR-302a-5p is selectively repressed in hPSCs. **(b)** Selective killing of hPSCs. hPSCs are selectively killed by puromycin because puromycin resistance gene-encoding mRNA is repressed in these cells. **(c)** Cell-selective gene knock-out. Gene knock-out is selectively inhibited in hPSCs due to the repression of Cas9 mRNA. **(d)** An mRNA device that responds to both small molecules and miRNAs. In the absence of the small molecule ligand, the miRNA hybridizes its target site in the mRNA and represses translation (top). Addition of the ligand (tetracycline) induces a structural rearrangement, such that the hybridization of miRNA and its target site is inhibited by the competing strand (bottom). **(e)** The distinction of cells based on multiple miRNA activities. miRNA-responsive mRNAs containing different reporter genes (e.g. monomeric Kusabira-Orange (mKO2) and EGFP) and miRNA target sites are simultaneously transfected into the cells. The cell types are distinguished based on the expression ratio of the reporter genes.

miR-302a-5p, which is highly expressed in hiPSCs [17]. This approach may facilitate hiPSC-derived cell therapies, because the removal of any residual hiPSCs from the cell population is required before a cell transplantation. Cell-specific gene knock-out was also achieved by regulating the translation of Cas9 [18] (Figure 2c).

As in the case of protein-responsive RNA devices, control of miRNA-responsive devices by small molecules can further improve the safety of the devices. Mou *et al.* designed mRNA containing a miRNA target site and a tetracycline-binding aptamer flanked with the 'competing strand', which has the complementary sequence of the miRNA

Figure 3



Layered gene circuits composed of multiple RNA devices.
(a) Examples of layered gene circuits composed of miRNA-responsive mRNA, RNA binding proteins and their target mRNAs. **(b)** Input 1 AND input 2 gene circuit for selective cell killing. A pro-apoptotic protein, Bax, induces apoptosis only when both miR-206 (input 1) and miR-302a-5p (input 2) are active. In other cases, L7Ae and anti-apoptotic protein Bcl2 connected by a self-cleaving peptide, P2A, are expressed, and Bax is translationally and post-translationally repressed by L7Ae and Bcl2, respectively. **(c)** Input 1 OR (NOT input 2) gene circuit composed of miRNA-responsive mRNA, L7Ae-responsive mRNA, TEV protease (TEVp), and L7Ae with the TEVp cleavage site (L7Ae-CS). In the presence of siRNA-FF5 (input 1) or the absence of siRNA-FF4 (input 2), EBFP translation is not repressed by L7Ae-CS, because L7Ae-CS is translationally repressed by siRNA-FF5 or cleaved by TEVp. EBFP translation is repressed only when siRNA-FF5 is absent and siRNA-FF4 is present.

target site [19]. In the absence of tetracycline, the miRNA bound its target site and repressed the translation of the mRNA. The addition of tetracycline stabilized the aptamer structure and induced structural rearrangements that eventually resulted in the hybridization of the miRNA target site and the competing strand. Because the hybridization prevented the binding of the miRNA and its target site, the mRNA was translated in the presence of tetracycline (Figure 2d).

In some cases, the difference of in miRNA activity is too small to distinguish the target cell type by using one miRNA-responsive mRNA. To further increase the resolution of the cell separation, our group has developed a method to distinguish cells using multiple miRNA-responsive mRNAs [20]. In this method, cells are distinguished based on the ratio of miRNA activities (Figure 2e). The method enabled clear separation of different cell types (e.g. simultaneous separation of HeLa, MCF-7, and 293FT cells) that have only less than twofold differences in miRNA activities.

Layered gene circuits composed of multiple devices

There are cases that a single biomolecule is insufficient for cell-specific transgene expression or repression. For example, some genes that are highly expressed in cancer cells are also expressed in stem cells [21–23]. In such cases, the integration of multiple inputs to make decisions for output protein expression is useful. Because both the inputs and outputs of biomolecule-responsive RNA devices are nucleic acids or proteins, these devices are suitable components of layered gene circuits that integrate multiple input signals. Our group has reported various layered gene circuits composed of both protein-responsive and miRNA-responsive mRNAs [24]. For example, the AND gate gene circuit expresses the output protein only when two miRNAs are active, whereas the OR gate circuit expresses the output protein when at least one of the two miRNAs is active (Figure 3a). Because we can use not only reporter proteins but also any other proteins as outputs, these logic gates can be used for cell fate-decision such as selective cell killing (Figure 3b).

In the same study mentioned above, Cella *et al.* developed logic gates that can integrate the information of multiple siRNAs to regulate the expression of the output protein [11••]. They combined the TEVp/L7Ae-CS system and siRNA-responsive mRNAs to construct a gene circuit that expresses the output protein in the presence of one siRNA or the absence of the other siRNA (Figure 3c).

Future directions

Although the number of RNA devices and their applications has been increasing, they are still a small minority compared with DNA-based systems. To expand the applicability of RNA devices in both biological studies and therapeutic applications, their further development is needed.

For example, there are only few RNA devices that can detect endogenous or pathogenic proteins. Increasing the number of detectable proteins will make RNA devices important tools for monitoring changes in cellular states such as differentiation or viral infections. As shown in the case of LIN28A [10•], the stabilization of protein-binding motifs may help the detection of motif-specific RBPs with high sensitivity. For the detection of proteins without RNA-binding properties, the method utilizing intrabody-fused L7Ae-CS and protease [11••] could be considered.

Cell fate regulation is one of the primary applications of mammalian synthetic biology. Although relatively simple cell fate regulation such as cell type-specific apoptosis induction has been already achieved, the development of RNA devices that enable more sophisticated cell fate regulation such as the induction of differentiation is desirable. For applications that need continuous transgene expression, gene circuits composed of RNA replicons [3,14•] or Sendai virus vectors [25] are suitable.

RNA devices are safe and effective tools for biological studies and therapeutic applications. Their development and improvement will help clarify biological phenomena and enable safe and effective gene and cell therapies.

Conflict of interest statement

Nothing declared.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Chen J-S *et al.*: **Cancer-specific activation of the survivin promoter and its potential use in gene therapy**. *Cancer Gene Ther* 2004, **11**:740-747.
2. Weeratna RD, Wu T, Efler SM, Zhang L, Davis HL: **Designing gene therapy vectors: avoiding immune responses by using tissue-specific promoters**. *Gene Ther* 2001, **8**:1872-1878.
3. Wroblewska L *et al.*: **Mammalian synthetic circuits with RNA binding proteins for RNA-only delivery**. *Nat Biotechnol* 2015, **33**:839-841.
4. Saito H *et al.*: **Synthetic translational regulation by an L7Ae-kink-turn RNP switch**. *Nat Chem Biol* 2010, **6**:71-78.
5. Saito H, Fujita Y, Kashida S, Hayashi K, Inoue T: **Synthetic human cell fate regulation by protein-driven RNA switches**. *Nat Commun* 2011, **2**:160.

6. Endo K, Stapleton JA, Hayashi K, Saito H, Inoue T: **Quantitative and simultaneous translational control of distinct mammalian mRNAs**. *Nucleic Acids Res* 2013, **41**:1-12.

7. Endo K, Hayashi K, Inoue T, Saito H: **A versatile cis-acting inverter module for synthetic translational switches**. *Nat Commun* 2013, **4**:2393.

8. Kashida S, Inoue T, Saito H: **Three-dimensionally designed protein-responsive RNA devices for cell signaling regulation**. *Nucleic Acids Res* 2012, **40**:9369-9378.

9. Stapleton JA *et al.*: **Feedback control of protein expression in mammalian cells by tunable synthetic translational inhibition**. *ACS Synth Biol* 2012, **1**:83-88.

10. • Kawasaki S, Fujita Y, Nagaike T, Tomita K, Saito H: **Synthetic mRNA devices that detect endogenous proteins and distinguish mammalian cells**. *Nucleic Acids Res* 2017, **45**:1-13.

This study showed not only the detection of a cell-specific endogenous protein by an mRNA device but also the strategy to enhance the sensitivity of protein-responsive mRNA devices by binding motif engineering.

11. •• Cella F, Wroblewska L, Weiss R, Siciliano V: **Engineering protein-protein devices for multilayered regulation of mRNA translation using orthogonal proteases in mammalian cells**. *Nat Commun* 2018, **9**.

By combining a protein-responsive mRNA device and intrabodies, the authors developed the method to detect a protein that has no RNA binding property.

12. •• Shibata T *et al.*: **Protein-driven RNA nanostructured devices that function in vitro and control mammalian cell fate**. *Nat Commun* 2017, **8**:1-12.

This study demonstrated scaffold RNA-mediated protein assembly to regulate cell fate, which is a different approach from mRNA-based translational regulation systems.

13. Petrakova O *et al.*: **Noncytopathic replication of venezuelan equine encephalitis virus and eastern equine encephalitis virus replicons in mammalian cells**. *J Virol* 2005, **79**:7597-7608.

14. • Wagner TE *et al.*: **Small-molecule-based regulation of RNA-delivered circuits in mammalian cells**. *Nat Chem Biol* 2018, **14**:1043-1050.

Utilizing the small-molecule responsive proteins, the authors succeeded in the regulation of RNA-based gene circuits by small molecules.

15. Miki K *et al.*: **Efficient detection and purification of cell populations using synthetic microRNA switches**. *Cell Stem Cell* 2015, **16**:699-711.

16. Nakanishi H *et al.*: **Monitoring and visualizing microRNA dynamics during live cell differentiation using microRNA-responsive non-viral reporter vectors**. *Biomaterials* 2017, **128**:121-135.

17. Parr CJC *et al.*: **MicroRNA-302 switch to identify and eliminate undifferentiated human pluripotent stem cells**. *Sci Rep* 2016, **6**:32532.

18. Hirosawa M *et al.*: **Cell-type-specific genome editing with a microRNA-responsive CRISPR–Cas9 switch**. *Nucleic Acids Res* 2017, **45**:e118.

19. Mou H *et al.*: **Conditional regulation of gene expression by ligand-induced occlusion of a microRNA target sequence**. *Mol Ther* 2018, **26**:1277-1286.

20. Endo K, Hayashi K, Saito H: **High-resolution identification and separation of living cell types by multiple microRNA-responsive synthetic mRNAs**. *Sci Rep* 2016, **6**:21991.

21. Parisi S *et al.*: **Lin28 is induced in primed embryonic stem cells and regulates let-7-independent events**. *FASEB J* 2017, **31**:1046-1058.

22. Cao D *et al.*: **RNA-binding protein LIN28 is a marker for testicular germ cell tumors**. *Hum Pathol* 2011, **42**:710-718.

23. Santagata S, Ligon KL, Hornick JL: **Embryonic stem cell transcription factor signatures in the diagnosis of primary and metastatic germ cell tumors**. *Am J Surg Pathol* 2007, **31**:836-845.

24. Matsuura S *et al.*: **Synthetic RNA-based logic computation in mammalian cells**. *Nat Commun* 2018, **9**:4847.

25. Sano M *et al.*: **Sensitive and long-term monitoring of intracellular microRNAs using a non-integrating cytoplasmic RNA vector**. *Sci Rep* 2017, **7**:1-13.