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<tr>
<td>Citation</td>
<td>Nature Communications (2017), 8(1)</td>
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<tr>
<td>Issue Date</td>
<td>2017-09-14</td>
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<td>URL</td>
<td><a href="http://hdl.handle.net/2433/227162">http://hdl.handle.net/2433/227162</a></td>
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Protein-driven RNA nanostructured devices that function in vitro and control mammalian cell fate

Tomonori Shibata¹, Yoshihiko Fujita¹, Hirohisa Ohno¹, Yuki Suzuki²,³, Karin Hayashi¹, Kaoru R. Komatsu¹, Shunsuke Kawasaki¹, Kumi Hidaka², Shin Yonehara³, Hiroshi Sugiyama²,³, Masayuki Endo²,³ & Hirohide Saito¹

Nucleic acid nanotechnology has great potential for future therapeutic applications. However, the construction of nanostructured devices that control cell fate by detecting and amplifying protein signals has remained a challenge. Here we design and build protein-driven RNA-nanostructured devices that actuate in vitro by RNA-binding-protein-inducible conformational change and regulate mammalian cell fate by RNA-protein interaction-mediated protein assembly. The conformation and function of the RNA nanostructures are dynamically controlled by RNA-binding protein signals. The protein-responsive RNA nanodevices are constructed inside cells using RNA-only delivery, which may provide a safe tool for building functional RNA-protein nanostructures. Moreover, the designed RNA scaffolds that control the assembly and oligomerization of apoptosis-regulatory proteins on a nanometre scale selectively kill target cells via specific RNA-protein interactions. These findings suggest that synthetic RNA nanodevices could function as molecular robots that detect signals and localize target proteins, induce RNA conformational changes, and programme mammalian cellular behaviour.

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n the nucleic acid nanotechnology field, a variety of nanostructures have been designed and constructed to utilize the programmable features of nucleic acids and the defined size and periodicity of the double-helical structure1–3. From this field, the concept of ‘nanomachine’4 or ‘molecular robots’5 has been investigated, because nucleic acids have the potential to change their conformations and functions based on the principle of simple Watson–Crick base pairing. For example, dynamic DNA nanostructures, such as the DNA walker6, the DNA motor7 and the DNA nanomachine8–9, have been constructed using DNA–DNA interactions. For biological applications, it is important to develop functional nanodevices that detect various environmental signals (e.g., RNA or protein signals), induce structural changes and produce desired functions (e.g., control mammalian cell fate). Several DNA nanostructures have been generated for potential biomedical and biotechnology applications, such as target cell-surface detection10,11; imaging12–13, drug delivery14,15 and chemical reaction control16. For example, a DNA-based nanorobot has been designed to detect cancer cell-surface receptors and release a drug in target cells10. Stimuli-responsive DNA nanohydrogels with size-controllable properties17 and pH- or chloride-sensing DNA nanodevices have been constructed inside cells18,19. In addition to DNA, RNA has attracted the attention of bioengineers because of the structural diversity of RNA molecules (i.e., structured RNA uses both canonical Watson–Crick base pair and non-canonical RNA structural motifs to form various two-dimensional and three-dimensional (3D) structures)20,21. Several RNA nanostructures, such as triangles, squares, nanorings, three-way junctions and prisms, have been constructed in vitro22–25 and some have been used for cellular applications through the attachment of a functional molecule, such as RNA (e.g., siRNA or aptamer)25,26,27,28,32 or protein (e.g., cell-surface binder)26,27,31–34, on the designed RNA structures. Synthetic RNA scaffolds that control the assembly of enzymes for hydrogen production in bacteria have also been reported26. However, the construction of nanostructured devices that control mammalian cellular behaviour by detecting or accumulating intracellular protein signals has not yet been demonstrated.

Insid...
Supplementary Fig. 4). These results indicated that two L7Ae proteins bound to two K-turn motifs of the RNA nanostructures and induced the RNA conformational change, as expected.

To further analyse the structural change caused by the RNP interaction, we measured the distance between the two ends of 2Kt-33-Tri and 2Kt-28-Z (shown in red arrows in Fig. 2a) in the absence and presence of L7Ae (Fig. 2f, g). In the absence of L7Ae, the edge distance of 2Kt-33-Tri was widely distributed from 0 to 44 nm. In contrast, the addition of L7Ae narrowed the distribution of the distance (the major population was within 16 nm), because the two ends of 2Kt-33-Tri came close to each other due to the two L7Ae-K-turn interactions that bent the RNA at the two K-turn regions to give a Tri-like shape as the major population (Fig. 2f). Similarly, we observed a distribution change in the distance between the two ends of 2Kt-28-Z in the absence and presence of L7Ae (the average distance was ~21 nm; Fig. 2g), which was consistent with the expected distance from the 3D modelling of 2Kt-28-Z (~18 nm in the presence of L7Ae).

Conformational changes that regulate the distance between the two ends of structured RNAs support our concept, i.e., that we can control the distance and arrangement of RNA-conjugated molecules on the two edges through protein sensing as an input signal. To confirm our idea, we attached fluorescent Cy3 and Cy5 molecules to the edge of 2Kt-33-Tri and analysed the L7Ae-induced RNA conformational change using Förster resonance energy transfer (FRET; Supplementary Figs 5 and 6). We observed an increased fluorescence signal ($F_{560}/F_{660}$) of 2Kt-33-Tri in the presence of L7Ae, but we did not observe this signal for the defective RNA, 2dKt-33-Tri, suggesting that FRET occurred likely due to the structural transition of 2Kt-33-Tri induced by the L7Ae-K-turn interaction.

ON/OFF switching of RNA function by changing the RNA shapes. Next, we aimed to control the function of the RNA aptamer (i.e., ON and OFF) using the L7Ae-induced RNA structural change. We chose the split RNA aptamer (binary malachite green aptamer, biMGA)39 and conjugated each split sequence to the 5′- and 3′-termini of the S-strand in 2Kt-33-Tri or 2Kt-28-Z (Supplementary Figs 7 and 8). In the presence of L7Ae, activated (ON) and repressed (OFF) states of the aptamer should be formed by the assembly and disassembly of two split RNA fragments through the formation of Tri-shape and Z-shape RNPs, respectively (Fig. 2h).

To modulate the function of biMGA, we optimized the length of the linker region between the RNA devices and biMGA, and the stem sequence of biMGA (Supplementary Figs 9–15 and Supplementary Note 1). We analysed the function of biMGA on the designed RNA nanostructures with malachite green (MG) in the presence and absence of L7Ae by measuring fluorescence spectra. The interaction between L7Ae and the two optimized RNA devices (Tri-MGA-ON and Z-MGA-OFF) controlled the formation of functional RNA aptamers in the ON state and OFF state, respectively (Fig. 2i). AFM analyses of Tri-MGA-ON and Z-MGA-OFF confirmed that the modulation of biMGA activity was indeed due to conformational changes in the designed RNA devices (Fig. 2i) and Supplementary Fig. 16). Thus, we are able to control both the functions (the activation and repression of the aptamer) and structures (Tri-shape and Z-shape) of the RNA nanostructures through detection of the target protein signal.

Actuation of protein-driven RNA nanodevices in living cells. In cell-signalling pathways, proteins are central to processing information and controlling cell behaviour. We next aimed to...
Fig. 2 Actuation of protein-driven RNA nanodevices in vitro. **a** Schematic representation of structural changes in 2Kt-33-Tri (left) and 2Kt-28-Z (right) caused by the induced-fit of RNA in response to L7Ae binding. The distance between the two ends of the RNA is indicated by a red double-headed arrows. The in silico predicted lengths of each side of 2Kt-33-Tri and 2Kt-28-Z were 8.7 nm and 7.4 nm, respectively. **b, c** EMSA confirmed the interaction between 2Kt-33-Tri (b) and 2Kt-28-Z (c) RNA nanostructures with L7Ae. Higher order bands (black arrowheads) indicate heterogeneous oligomers composed of L- and S-RNA strands. Concentrations of long and short RNAs: each 50 nM. **d, e** AFM images of the 2Kt-33-Tri (d) and 2Kt-28-Z (e) RNA nanostructures in the absence (left) and presence (right) of L7Ae. Scale bars, 20 nm. **f, g** Statistical distribution of the distance between the two ends of RNA, indicated by the double-headed arrows in **a**, for 2Kt-33-Tri (f) and 2Kt-28-Z RNA (g) nanostructures in the absence (black) and presence (red) of L7Ae (N number of nanostructures). **h** Schematic illustration of the ON/OFF switching of biMGA activity caused by structural changes in RNA nanodevices in response to L7Ae binding. Fluorescence emission of Tri-MGA-ON is caused by the formation of an active biMGA that occurs with a L7Ae-induced RNA conformational change that places two split aptamers close to each other (left). Fluorescence quenching of Z-MGA-OFF is caused by disassembly of the biMGA that occurs with a L7Ae-induced conformational change that separates the two split aptamers (right). **i** Fluorescence spectra of Tri-MGA-ON (left) and Z-MGA-OFF (right) in the absence (black) and presence (green) of L7Ae. **j** Fraction of open (black) and closed (red) nanostructures before and after RNP formation (N number of nanostructures). Tri-MGA-ON: Tri-MGA-ON-stem B (Supplementary Fig. 10). Z-MGA-OFF: Z-MGA-OFF-stem D (Supplementary Fig. 11)
An RNA device with a Cy3- and Cy5-labelled short RNA duplex at the termini should produce an increase in the interaction-mediated conformational change in cells (Fig. 3a).

We engineered 2Kt-17-Z by introducing Watson–Crick base pairs (from 4 to 8 bp) at the 5′- and 3′-ends of the RNA device (2Kt-17-Z-4, 5, 6, 7 and 8; 2Kt-17-Z-9). The effects of the RNA nanostructures, we also constructed larger Z-shape RNP nanostructures, 2Kt-28-Z, with short RNA duplexes (from 5 to 7 bp) and conformed that changes in the fluorescence signal were similar to 2Kt-17-Z (Supplementary Figs 18 and 19).

Thus, we chose 2Kt-17-Z-7 for the following cellular experiments.

To deliver and construct protein-responsive RNA nanostructures inside mammalian cells, we chose the synthetic RNA delivery approach. This approach may provide a safe tool to...
manipulate cells without random genomic integration\textsuperscript{40}, and in vitro transcribed RNAs (mRNA that codes a target protein and small RNAs to make RNA devices) are directly used to construct RNP nanostructures in cells. Synthetic RNA devices (2Kt-17-Z-7) and chemically modified mRNA (modRNA; the use of modRNA was expected to reduce the interferon response)\textsuperscript{40} coding for L7Ae were co-transfected into HeLa cells. The expression of L7Ae was confirmed by capillary-based immunodetection.
Casp-8 molecules through proximity-induced oligomerization. We next aimed to control cell fate (i.e., selective cell killing) by actuating the RNA nanostructured device in cell.

RNA scaffold-based cell-fate control by assembling proteins. We next aimed to control cell fate (i.e., selective cell killing) by detecting and assembling an apoptosis regulatory protein, Caspase-8 (Casp-8), through specific RNP interactions on designed RNA scaffolds formed in target cells. By naturally occurring systems, Casp-8 contains the death effector domain (DED), which is responsible for the assembly and activation of Casp-8 molecules through proximity-induced oligomerization.

We replaced DED with L7Ae, expecting that the fused L7Ae should bring multiple Casp-8 catalytic domains together on the RNA scaffold to induce caspase activation without requirement of the DED-mediated Casp-8 chain assembly. We therefore fused L7Ae with DED-defective Casp-8 (dCasp-8) and examined whether the RNP interaction-mediated accumulation of L7Ae-dCasp-8 on the RNA scaffold induces cell death (Fig. 1d).

To control the assembly of L7Ae-dCasp-8 on the RNA scaffold, we newly designed a set of RNA scaffolds that contained 1, 3, 6, 9 or 14 K-turn motifs (1Kt-33-Tri, 3Kt-33-Tri, 6Kt-33-Tri, 9Kt-33-Tri or 14Kt-33-Tri, respectively), which would collect a different number of L7Ae-dCasp-8 molecules on the RNA scaffold at nanometre scale (Supplementary Fig. 22).

The specific RNP interactions between 1-3-, 6-, 9- or 14-Kt-33-Tri and L7Ae were confirmed in vitro by EMSA and AFM (Fig. 4a and Supplementary Figs 23 and 24). In contrast, interactions between defective RNA scaffolds with reverse-complement K-turn motifs (rcKt; they do not interact with L7Ae; Supplementary Figs 23 and 25) and L7Ae were not observed. We found that the structures of the RNA scaffolds were clearly changed by L7Ae addition (Supplementary Fig. 24). Furthermore, we directly observed that the number of L7Ae proteins assembled on the scaffolds increased as the number of K-turn motifs on the scaffolds increased (Fig. 4a). We confirmed L7Ae-dCasp-8 expression in cells by capillary-based immunodetection (Supplementary Fig. 26a). We also performed a translocation repression assay and confirmed that the fusion of dCasp-8 to L7Ae maintained L7Ae-K-turn interactions in cells (Supplementary Fig. 26b, c). To investigate whether the designed RNA scaffolds could induce cell death through the accumulation of L7Ae-dCasp-8, each RNA device and L7Ae-dCasp-8-coding modRNA were co-transfected into HeLa cells and the cells were analysed by phase microscopy or flow cytometry after 24 h of transfection. Notably, we observed that 14Kt-33-Tri with L7Ae-dCasp-8 effectively induced cell death (Fig. 4b, top right picture and Fig. 4c, left), whereas the defective RNA scaffold (14Kt-33-Tri) with L7Ae-dCasp-8 did not (Fig. 4b, bottom right picture and Fig. 4c, right). This result indicated that L7Ae-dCasp-8 alone does not induce cell death effectively, but the accumulation of L7Ae-dCasp-8 on the RNA devices facilitates cell-death induction. In addition, we observed that the dead cell populations increased as the number of Kt on the RNA scaffolds increased (9Kt and 14Kt), whereas the number of rcKt on the scaffolds did not affect cell death (Fig. 4c, d). These results suggest that we can tune cell-death signals by changing the number of dCasp-8 assembled on the designed RNA scaffold.

To further investigate whether cell death was induced by Casp-8-mediated apoptosis through specific RNP interactions, we constructed inactive variants of Casp-8 by replacing the catalytically active cysteine residue of Casp-8 with serine (Casp-8-CS) and transfected modRNAs encoding various protein variants (L7Ae, dCasp-8, L7Ae-dCasp-8, L7Ae-dCasp-8-CS, Casp-8, L7Ae-Casp-8 and L7Ae-Casp-8-CS) together with RNA scaffolds (14Kt-33-Tri or 14rcKt-33-Tri) (Fig. 4e and Supplementary Fig. 27). We confirmed that specific interactions between L7Ae-(d)Casp-8 and 14Kt-33-Tri are necessary for the induction of effective cell death, because L7Ae or (d)Casp-8 alone did not induce cell death even in the presence of 14Kt-33-Tri (Fig. 4e and Supplementary Fig. 27). Notably, cell death was not induced by full-length Casp-8 (or L7Ae-Casp-8) alone, whereas effective cell death was observed in 14Kt-33-Tri with L7Ae-Casp-8, indicating that the expression level of Casp-8 from modRNA is not enough to activate Casp-8 under our experimental condition, but 14Kt-33-Tri could increase the local concentration of L7Ae-Casp-8 to initiate cell death. This result suggests that RNA scaffolds may function as a signal amplifier capable of inducing cell death. In addition, we observed that 14Kt-33-Tri with L7Ae-(d)Casp-8-CS abolished the cell-death induction, indicating that Casp-8 activity is essential for triggering apoptosis mediated by the RNP interaction. We also measured caspase activity using a fluorogenic substrate (IETD-AMC). The highest caspase activity was observed in cells transfected with 14Kt-33-Tri and L7Ae-dCasp-8-coding modRNA, and the addition of a caspase inhibitor (IETD-FMK) abolished the observed activity (Fig. 4f). Together, these results confirm that we can selectively control cell-death signalling by regulating the oligomerization of pro-apoptosis regulatory proteins on synthetic RNA scaffolds formed in cells.

Next, we designed an alternative RNA scaffold to detect a different protein signal and control cell fate. Lin28A protein is a DNA-binding protein that regulates the biogenesis of several miRNAs (e.g., let-7d miRNA) and has important roles in cancer, tissue regeneration and the self-renewal of stem cells.
To develop a Lin28A-responsive cell-killing system with RNP nanostructures, we constructed Lin28A-fused dCas9-8 (Lin28A-dCas-8) and a Lin28A-binding RNA scaffold, 9pre-let-7d-33, by replacing the K-turn motif of 9Kt-33-Tri with pre-let-7d (RNA sequences derived from let-7d miRNA precursors), which interacts with Lin28A (Fig. 5 and Supplementary Fig. 28). We expected that the RNP interaction between Lin28A-dCas-8 and 9pre-let7d-33 would induce cell death in a similar manner to L7Ae-dCas-8 and 9Kt-33-Tri (Fig. 5a, left). Conversely, in cells that express Lin28A, the effect of cell death should be modulated by competitive binding between Lin28A and Lin28A-dCas-8 to 9let7d-33, which should reduce the level of Casp-8 activation on the RNA scaffold (Fig. 5a, right). Transfection of 9pre-let7d-33 with Lin28A-dCas-8-coding modRNA to HeLa cells showed effective cell death comparable to the effect of 9Kt-33-Tri with L7Ae-dCas-8-coding modRNA, whereas Lin28A-dCas-8 or 9pre-let7d-33 alone did not induce cell death, confirming that the RNP interaction is essential for triggering cell death. In addition, we observed that co-transfection of Lin28A-coding modRNA together with 9pre-let7d-33 and Lin28A-dCas-8-coding modRNA reduced the number of dead cells (Fig. 5b), suggesting that Lin28A competes with Lin28A-dCas-8 to bind 9pre-let7d-33. We also confirmed the expression of Lin28A and Lin28A-dCas-8 in cells by capillary-based immunodetection (Supplementary Fig. 29).

To further investigate whether the system could selectively control cell fate by detecting endogenous expression levels of Lin28A, we constructed a stable HeLa-Lin28A cell line that expresses Lin28A from the genome (Supplementary Fig. 29). We compared Lin28A expression levels in HeLa-Lin28A and human induced pluripotent stem cells (hiPSCs) (Fig. 5c). We observed that cells treated with 9pre-let7d-33 with Lin28A-dCas-8 did not induce cell death, whereas cells treated with 9Kt-33-Tri with L7Ae-dCas-8 increased the number of dead cells. These results indicate that cell-death signals initiated by 9pre-let7d-33 with Lin28A-dCas-8 can be attenuated when Lin28A is detected in the cell. In addition, we transfected these RNA nanodevices in hiPSCs and found that Lin28A-dCas-8 with 9pre-let7d-33 did not induce cell death, whereas L7Ae-dCas-8 with 9Kt-33-Tri did in a manner similar to that in HeLa-Lin28A cells (Fig. 5d and Supplementary Fig. 31). Based on these results, we concluded that RNA-scaffold mediated cell-death control is adaptable to not only the L7Ae-K-turn interaction, but also the Lin28A-pre-let-7d interaction.

Fig. 5 RNA scaffold-mediated cell-death control by Lin28A detection. a Schematic representation of a Lin28A-responsive RNA nanostructured device composed of 9pre-let7d-33 and Lin28A-dCas-8. b Lin28A-responsive control of cell fate using 9pre-let7d-33 and Lin28A-dCas-8 in HeLa cells. L7Ae-responsive 9Kt-33-Tri was used as a control. Cell death is induced in the presence of both 9pre-let7d-33 and Lin28A-dCas-8. The induction of cell death is partially repressed by adding Lin28A-coding modRNA as a competitor. c, d Selective cell-fate control by detecting Lin28A in HeLa-Lin28A cells (c) and hiPSCs (d). Dead cells were stained with Pacific Blue Annexin V and analysed using flow cytometry. The data are presented as the mean ± SD (n = 3). NS not significant, *P < 0.05, **P < 0.01 (Welch’s t-test)
Actuation of protein-driven RNA devices by miRNA signals. Finally, in order to detect intracellular RNA signals and actuate protein-responsive RNA nanodevices, we constructed a system that responds to endogenous miRNAs and regulates Casp-8 protein signals. We designed synthetic mRNA\(^4\) that controls L7Ae-dCasp-8 expression in response to endogenous miRNAs expressed in target cells (miR-L7Ae-dCasp-8 switch). We linked this switch to an RNA device, 14Kt-33-Tri (Fig. 6a). To selectively control cell-death signals through miRNA detection, we applied our system to HeLa cells and hiPSCs, which express active miR-21-5p (miR-21) and miR-302a-5p (miR-302), respectively. Accordingly, we designed miR-21- and miR-302-responsive L7Ae-dCasp-8 mRNA switches. Transfection of miR-21 (miR-302)-L7Ae-dCasp-8 switch with the defective RNA scaffold (14rcKt-33-Tri) did not induce cell death in either cell type (Fig. 6b, c), indicating that endogenous miR-302 and miR-21 can control cell-death signals activated by the formation of RNP nanostructures. We also observed that the addition of a miR-302 or miR-21 mimic with the corresponding switches repressed the death of HeLa cells and hiPSCs, respectively (Fig. 6b, c). Together, these results suggest that we can control RNA scaffold-mediated cell death by detecting endogenous miRNAs and modulating protein signals in target cells.

Discussion

In this study, we demonstrated the construction of protein-triggered, functional RNA nanostructured devices in vitro and within mammalian cells. Both the structures and the functions of the devices can be controlled via specific RNP interactions, which produce various output signals (i.e., ON/OFF switching of...
aptamer formation, intracellular protein-responsive actuation and cell-fate control by detecting intracellular protein or miRNA signals). Protein-responsive RNA-nanostructured devices may regulate various biological processes, because the spatiotemporal regulation of naturally occurring RNP complexes has a crucial role in the control of gene expressions, signal transductions and cell-fate conversions.

Our system provides a tool for controlling the localization and accumulation of signal proteins on RNA scaffolds that were designed on a nanometre scale. In fact, we found that the oligomerization of L7Ae-dCasp-8 proteins (i.e., more than nine dCasp8 proteins) on a designed RNA scaffold is important for the induction of effective cell death. In naturally occurring systems, the assembly and activation of Casp-8 are induced by the formation of death-inducing signalling complex (DISC), which is a key step in the death receptor-mediated activation of apoptotic pathways. Dickens et al.46 reported that DISC contains ninefold more Casp-8 than Fas-associated protein with death domain (FADD) and they proposed a DED chain assembly model for DISC formation. They demonstrated that the recruitment of many Casp-8 molecules to a single FADD molecule was required for DISC formation and subsequent apoptotic cell death. The DISC model seems to agree well with our observation that the assembly of more than nine L7Ae-dCasp-8 proteins on a single RNA scaffold facilitated effective Casp-8 activation and cell-death induction (Fig. 4). To further examine the effect of RNA structural configuration on cell-killing activity, we designed an alternative scaffold that formed ‘zigzag-shaped’ nanostructures (14Kt-27-28-Zig) by RNP interaction (Supplementary Fig. 32). The cell-killing activity of 14Kt-27-28-Zig was comparable to that of 14Kt-33-Tri (Supplementary Fig. 33), suggesting that the assembly and oligomerization of dCasp-8 proteins on the RNA scaffolds are important for cell-death induction.

The molecular design of RNA nanodevices can be applied to various RNA motifs that bind to target proteins, including naturally occurring motifs and in vitro selected aptamers. Indeed, we designed and constructed alternative RNA nanodevices that control their structures by responding to U1A protein by employing U1A-binding motifs47 (Supplementary Fig. 34). We observed that U1A protein interacted with the RNA device to induce RNA conformational change (Supplementary Fig. 35). To actuate RNA nanodevices in response to a target protein within mammalian cells, RNA-binding proteins of interest (e.g., Lin28A) could be fused to functional proteins (e.g., dCasp-8) and the protein-binding RNA motifs can be incorporated into the RNA nanodevices. We designed a Lin28A-responsive, cell-fate control system using the 9pre-let7d-33 nanodevice (Fig. 5). We found that Lin28A expressed in HeLa-Lin28A cells or hiPSCs inhibited Lin28A-dCasp-8-mediated cell killing activity, probably due to the competitive binding between Lin28A and Lin28A-dCasp-8 to 9pre-let7d-33. These data suggest that the RNA scaffold-mediated cell death system could be modular and controllable by detecting various protein signals.

To detect endogenous miRNAs and control protein signals on the RNA scaffold, we combined our protein-responsive RNA nanostructured devices with miRNA-responsive miRNAs (miR-switches)45. The combination of 14Kt-33-Tri and miR-L7Ae-dCasp-8 switch selectively induced cell death in HeLa cells or hiPSCs by responding to the endogenous miRNA signatures (miR-21 or miR-302) and by regulating Casp-8-mediated apoptosis signals on the RNA scaffold. Transfection of full-length Casp-8- or L7Ae-Casp-8-encoding mRNA did not induce cell death (Fig. 4e), indicating that simple use of miRNA switches that express Casp-8 cannot control the cell phenotype efficiently. In contrast, RNA scaffold 14Kt-33-Tri amplified cell-death signals produced from miR-L7Ae-dCasp-8 switch and increased the number of dead cells in the absence of the target miRNAs. We propose that the combination of miRNA switches and synthetic RNA scaffolds can facilitate the control of miRNA-responsive cell-signalling pathways for the following reasons: (1) the simple use of miRNA switches that encode cell-death inducers may cause leaky expression of the proteins even in the presence of miRNA, which could blur the threshold between different cell types, because the expression balance between miR-switches and miRNA is critical for controlling cell death; (2) synthetic RNA scaffolds that assemble and oligomerize target proteins at nanometer scale can amplify the effect of protein signals by increasing the local concentration of the proteins on the scaffold; signal amplification processes using RNA scaffolds facilitate to control cell fate; and (3) RNA scaffolds can tune the signals by changing the assembly number of the apoptosis regulatory proteins on the scaffold (Fig. 4a–d).

To develop functional nanodevices in mammalian cells for future therapeutic applications, the introduction and formation of nanostructures inside target cells should be safe and efficient. Thus, we thought that the formation of RNP nanostructures in cells by RNA-only delivery is ideal for the following reasons: (1) the gene delivery of synthetic RNA rather than DNA is more likely to avoid potential genomic damage caused by random genomic integration of devices40; (2) modRNA can produce RNA-binding proteins without an immune response40, (3) the function of RNA devices can be transiently actuated inside cells, reducing the risk of potential side effects; and (4) the self-folding of RNA strands in vitro before transfection may facilitate the efficient detection of intracellular proteins via RNP interactions in the cytoplasm and the subsequent control of cell functions, because neither transcription nor RNA export from the nucleus are required in the case of RNA delivery; (5) RNA delivery approaches may have diverse medical applications, such as RNA vaccines48, cell reprogramming49, cell purification45 and cell-fate control40.

The construction of protein-responsive nucleic acid nanodevices in vitro has been reported. Examples of such nanodevices include nanomechanical DNA origami that detects proteins at the single-molecule level8, DNA nanorobots that recognize the cell surface and control cell signalling by utilizing the interaction between DNA aptamers and protein10, and protein-triggered DNA nanomachines on gold nanoparticles that control the assembly and cleavage of DNA components50. However, the detection of proteins by DNA nanodevices has been limited to in vitro or cell surfaces. Protein-assembled RNA nanostructures have also been constructed in vitro31–34, although these RNA nanostructures had rather static scaffolds and their conformations and functions were not controlled by the protein signal. Our RNA nanostructured devices can be actuated via their conformational changes both in vitro and inside mammalian cells in response to extracellular and intracellular protein signals, respectively. In addition, our RNA devices are signal transducers that are capable of converting the input protein signal into the work of RNA (e.g., the formation of active aptamers) or protein (e.g., apoptosis induction) by employing a dynamic RNA scaffold. We believe that RNA and RNP nanodevices can be employed as a material to construct molecular robots that regulate their structures and produce desired functions by sensing various types of extracellular and intracellular information.

Methods

Plasmid construction. DNA templates of 14Kt-33-Tri-long and 14Kt-33-Tri-short and 14Kt-27-28-Zig-long and 14Kt-27-28-Zig-short with BamHI and Xhel sites were purchased from Life Technology. Preparation of 9pre-let7d-33-long plasmid was delegated to FASMAC. The DNA templates were inserted into the BamHI–Xhel sites of pcDNA4/TTO to give the desired plasmids. We used
Cy3- and Cy5-labelling of RNA. Cy3- and Cy5-labelled RNAs were prepared by ligation using T4 RNA ligase (Ambion), Pcp-Cy3 (Jena Bioscience) and Pcp-Cy5 (Jena Bioscience). Cy3- and Cy5-labelling was performed with 150 pmol RNA using 10 U T4 RNA ligase, 3 mmol Pcp-Cy3 or Pcp-Cy5 and 10% (v/v) dimethyl sulfoxide in 10 µl at 16 °C for 36–48 h. The Cy3- and Cy5-labelled RNAs were purified using an RNAeasy MinElute Cleanup Kit (Qiagen). After the recovery of RNA by denaturing polyacrylamide gel electrophoresis, the RNA concentration was measured using NanoDrop (Thermo Scientific). The RNA was treated with Antarctic Phosphatase (New England Biolabs) at 37 °C for 30 min. The RNA samples were denatured by heating at 80 °C for 5 min and then cooled at room temperature for 10–30 min. The excitation wavelength was 512 nm and the emission and excitation bandwidths were 10 nm. Fluorescence measurements were carried out with Infinite M1000 Plate Reader (TECAN).

Electrophoretic mobility shift assay. Mixtures of L-RNA and S-RNA (each 20–50 nM) in 20 mM phosphate buffer, pH 7.0, 1.5 mM MgCl2 and 150 mM KCl were incubated at 80 °C for 3 min and then cooled at room temperature for 10 min. After the addition of L7Ae (50–1000 nM), the mixtures were incubated at room temperature for 10–30 min. The mixtures were mixed with 10 × loading buffer (0.25% bromophenol blue, 30% glycerol) and were subjected to electrophoresis through a native polyacrylamide gel with 0.5 × Tris/Borate/EDTA buffer at room temperature. After electrophoresis, the gels were stained for 10 min with SYBR Green I and II (Lonza). Images of the gels were collected using a Gel Doc EZ Imager (BIO-RAD) or a Typhoon FLA 7000 laser scanner (GE Healthcare).

Atomic force microscopy. The RNA and RNP nanostructures were observed in solution. Their samples were prepared as described for the EMSA. The AFM images were collected using Nano Live Vision (Research Institute of Biomolecules Technology (RIB)) or BIXAMP (Olympus). Small cantilevers (9 µm long, 3.5 µm wide and 130 nm thick; BL–AC10EGS, Olympus) having a spring constant of ~0.1 N m⁻¹ and a resonant frequency of ~300–600 kHz in water were used to scan the sample surface. The 320 × 240-pixel images were collected at a scan rate of 0.2 frames per second. A fresh mica surface was coated with 0.1% 3-amino propyl triethoxy silane. The samples (20–50 nM RNA nanostructure with or without 600–1000 nM protein) were prepared in RNP binding buffer and diluted five- (for RNP nanostructures) or tenfold (for RNA nanostructures) with AFM observation buffer (20 mM Tris–HCl (pH 7.6) and 10 mM MgCl2). Then, they were applied to the mica for 5 min at room temperature and were washed with buffer solution. The image sequences were analysed using ImageJ (http://imagej.nih.gov/ij/) software.

FRET experiment. Cy3- and Cy5-labelled RNAs (each 50 nM) were heated at 80 °C for 3 min and then cooled at room temperature for 10 min in 20 mM phosphate buffer (pH 7.0) containing 1.5 mM MgCl2 and 150 mM KCl. Trans-w (20%) and either protein buffer (20 mM phosphate buffer, pH 7.0, 1.5 mM MgCl2, 150 mM KCl and 40% glycerol) or L7Ae (600 nM) dissolved in protein buffer was added to the mixtures and incubated at room temperature for 10–30 min. The excitation wavelength was 512 nm and the emission and excitation bandwidths were 10 nm. Fluorescence measurements were carried out with Infinite M1000 Plate Reader (TECAN).

Fluorescence measurement of biMGA. biMGA-conjugated RNA nanostructures (100 nM) were heated at 80 °C for 3 min and then cooled at room temperature for 10 min in 20 mM phosphate buffer (pH 7.0) containing 1.5 mM MgCl2 and 150 mM KCl. MG (2 µM), Twen-20 (0.1%) and either protein buffer (20 mM phosphate buffer, pH 7.0, 1.5 mM MgCl2, 150 mM KCl and 40% glycerol) or L7Ae (600 nM) dissolved in protein buffer were added to the mixtures and incubated at room temperature for 10–30 min. The excitation wavelength was 610 nm and the emission and excitation bandwidths were 10 and 20 nm, respectively. Fluorescence measurements were carried out with Infinite M1000 Plate Reader (TECAN).

Cell cultures. HeLa cells (originally obtained from ATCC) were cultured at 37 °C in 5% CO2 in Dulbecco’s modified Eagle’s medium—High Glucose (Nacalai Tesque) containing 10% fetal bovine serum (Japan Bio Serum), non-essential amino acids (Invitrogen), sodium pyruvate (Sigma) and antibiotic antymycotic solution (Sigma). HeLa cells stably expressing RNAi-Lin28A (a kind gift from Dr. Kozo Tomita, Tokyo University) were established by transfecting pIR5-puro vector containing Flag-tagged Lin28A sequence into HeLa cells. Positive clones were obtained by selection with puromycin (0.5 µg ml⁻¹) for 2 weeks. Human iPSCs (2017b, a kind gift from Dr Masato Nakagawa, Kyoto University) were maintained in feeder-free condition⁴ with StemFit AK03 (Ajinomoto) on laminin-511 E8 (iMatrix 511, Nippi).

RNA transfection. Mixtures of L-RNA and S-RNA (each 0.1–4 µM) in 20 mM phosphate buffer, pH 7.0, 1.5 mM MgCl2 and 150 mM KCl were heated at 80 or 95 °C for 3 min and then cooled at room temperature for 10 min. The mixtures of RNA nanostructures (0.1–8 pmol) and modRNAs (10–500 ng) were used for RNA delivery using Stemfect RNA Transfection Reagent (STEMGENT) according to the manufacturer’s protocol.

Flow cytometric-based FRET assay. A total of 5 × 10⁴ cells were seeded into 12-well plates. After a 24 h culture, RNA nanodevices (8 pmol) and modRNAs (500 ng) were co-transfected into the cells using 2 µl of Stemfect RNA Transfection Reagent (STEMGENT). The medium was changed after 2 h. After 12 or 24 h of transfection, the cells were washed with phosphate-buffered saline (PBS) and incubated in 200 µl of 0.25% Trypsin-EDTA (Nacalai Tesque) at 37 °C for 3 min. After the addition of 100 µl of Dulbecco’s PBS containing 2% fetal bovine serum, the cells were analysed with a FACSArria cell sorter (BD Bioscience). A 532 nm laser and a 775/25 nm optical filter were used to detect Cy3 fluorescence. A 532 nm laser and a 670/30 nm optical filter were used to detect the Cy5 fluorescence attributed to FRET. The flow cytometric analysis was performed using a FACS Canto II (Becton Dickinson). The excitation wavelength was 488 nm and the emission and excitation bandwidths were 530 and 670 nm, respectively. The excitation wavelength was 405 nm and the emission and excitation bandwidths were 525 and 585 nm, respectively. The flow cytometric analysis was performed using a FACS Canto II (Becton Dickinson). The excitation wavelength was 405 nm and the emission and excitation bandwidths were 525 and 585 nm, respectively. The excitation wavelength was 405 nm and the emission and excitation bandwidths were 525 and 585 nm, respectively.
the mean of the ratio between the Cy3 and Cy5 fluorescence intensities. Enhancement of the Cy3/Cy5 mean was calculated from the Cy3/Cy5 mean nor-
tomized to cells transfected with 2DStk-17-Z-Z and either MS2CP- or L7Ae-coding modRNA. The obtained means were normalized to the mean of negative control cells transfected with MS2CP-coding modRNA and 2DStk-17-Z-Z for calculating relative Cy3/Cy5 mean intensities.

**Cell-death assay.** A total of 5 x 10^6 cells were seeded into 24-well plates. After a 24 h culture, the corresponding RNA nanostructures (0.1 pmol) and modRNA (10 ng) were co-transfected into the cells using 1 μl of Stemfect RNA Transfection Reagent (STEMGENT). After 2 h, the medium was changed. Cell images were taken after 24 h of transfection. The supernatants and cells were recovered and stained with Pacific Blue-labelled Annexin V (Life Technologies). Dead cells were stained with SYTOX AADvanced Dead Cell Stain, respectively; 450/50 and 660/20 nm optical filters were used to detect the fluorescence of Pacific Blue and SYTOX AADvanced Dead Cell Stain, respectively. Cells with high Pacific Blue fluorescence intensity were counted as apoptotic and dead cells.

**miRNA-responsive cell-death regulation assay.** A total of 5 x 10^4 cells (HeLa cells and hiPSCs) were seeded into 24-well plates. After a 24 h culture, the corresponding RNA nanostructures (0.1 pmol for HeLa cells and 0.2 pmol for hiPSCs) and miRNA-responsive modRNA switch (10 ng for HeLa cells and 30 ng for hiPSCs) were transfected into the cells using 1 μl of Stemfect RNA Transfection Reagent (STEMGENT). MiR-21-5p inhibitor (2 pmol) and miR-302a-5p mimic (0.5 pmol) were used for HeLa cells. MiR-21-5p mimic (2 pmol) and miR-302a-5p inhibitor (2 pmol) were used for hiPSCs. The following protocols were similar to 'Cell-death assay.'

**Lin28A-responsive cell-death regulation assay.** A total of 5 x 10^4 cells (HeLa cells, HeLa-Lin28A cells and hiPSCs) were seeded into 24-well plates. After a 24 h culture, the corresponding RNA nanostructures (0.2 pmol for HeLa cells and HeLa-Lin28A cells, 0.4 pmol for hiPSCs) and Lin28A-dCas9-8 (40 ng for HeLa cells, HeLa-Lin28A cells and hiPSCs) or L7Ae-dCas9-8 (10 ng for HeLa cells, HeLa-Lin28A cells, 30 ng for hiPSCs) coding modRNA were co-transfected into the cells using 1 μl of Stemfect RNA Transfection Reagent (STEMGENT). Lin28A- or MS2CP-coding modRNA (10 ng for HeLa cells) was used as a competitor for the data in Fig. 6b. The following protocols were similar to 'Cell-death assay.'

**Measurement of caspase activity.** A total of 2 x 10^5 HeLa cells were seeded into six-well plates and incubated for 24 h. The corresponding RNA nanostructures (0.4 pmol) and modRNA (40 ng) were co-transfected to the cells using 4 μl of Stemfect RNA Transfection Reagent (STEMGENT). After 2 h, the medium was changed. The measurement of caspase activity was performed after 6 h of transfection. The caspase activity was measured using the LPS-HPLC assay kit (M-PER Mammalian Protein Extraction Reagent from Thermo Scientific), and the debris of the cells were removed by centrifugation. The total amount of protein in the supernatant was determined by BCA assay kit (Thermo). GAPDH, Myc-tag and Lin28A were detected by an automated capillary-based immunodetection system (Wes SimpleManner, Proteom). Aliquots (100 μl) of 100-fold diluted Anti-GAPDH (Santa Cruz, FL-355, sc-25778), 20-fold diluted Anti-Myc (Santa Cruz, sc-789) and 100-fold diluted Anti-Lin28A (R&D Systems, AF3757) antibodies as primary antibodies, respectively. HRP-conjugated Anti-rabbit (ProteinSimple, 042-206) and 100-fold diluted Anti-goat (Thermo, 81-1620) antibodies were used as secondary antibodies. The expression levels of the target proteins were quantified by Compass software (ProteinSimple).

**Statistical analysis.** The statistical significance was analysed by Welch’s t-test (KaleidaGraph 4.5.1, HULINKS). The levels of significance are denoted as *P < 0.05, **P < 0.01, ***P < 0.001, NS not significant. All data are presented as the mean ± SD.

**Data availability.** The data that support the findings of this study are available from the corresponding author upon request.

Received: 13 June 2017 Accepted: 29 June 2017 Published online: 14 September 2017

**References**


Acknowledgements
We thank Kei Endo (The University of Tokyo) and Shunnnichi Kashida (ENS) for fruitful discussions. We also thank Kanae Mitsunaga (Kyoto University) for supporting flow cytometry experiments, Peter Karagannis (Kyoto University) and Callum Parr (Kyoto University) for reading the manuscript, and Takuya Yamamoto (Kyoto University) for advice about statistical analysis. Stable HeLa-Lin28A cells were a kind gift from Kozo Tomita and Takashi Nagaike (The University of Tokyo). This work was supported by Grant-in-Aid for Young Scientist (B) (number 26810089) (to T.S.), Grant-in-Aid for Scientific Research on Innovative Areas ‘molecular robotics’ (24104002) (to H. Saito), Grant-in-Aid for Scientific Research (S) (15H05722) (to H. Saito) from The Ministry of Education, Culture, Sports, Science, and Technology, Japan, Naito Foundation (to H. Saito), Canon Foundation (to H. Saito) and Nakatani Foundation (to H. Saito).

Author contributions
T.S. and H. Saito conceived the project and designed the experiments. T.S., Y.F., H.O. and K. Hayashi performed the experiments in vitro and inside cells. Y.F., K.K.R. and S.K. performed the immunodetection of proteins. T.S. and K. Hidaka performed the AFM measurements. T.S. and H. Saito discussed the results of apoptosis. T.S. and H. Saito wrote the manuscript. All authors discussed the results and commented on the manuscript.

Additional information
Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00459-x.

Competing interests: There are potential competing interests. Kyoto University has filed a patent application broadly relevant to this work. T.S. and H. Saito are the investigators of record listed on the patent application. The remaining authors declare no competing financial interests.

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