Retraction

CHEMISTRY, APPLIED BIOLOGICAL SCIENCES

The undersigned authors wish to note the following: “Anomalous experimental results observed by multiple members of the Pierce lab during follow-on studies raised concerns of possible research misconduct. An investigation committee of faculty at the California Institute of Technology indicated in its final report on this matter that the preponderance of the evidence and the reasons detailed in the report established that the first author falsified and misrepresented data published in this paper. An investigation at the United States Office of Research Integrity is ongoing. The undersigned authors hereby retract this paper and sincerely apologize for the inconvenience caused to other investigators.”

Robert M. Dirks
Christine T. Ueda
Niles A. Pierce

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Selective cell death mediated by small conditional RNAs

Suvir Venkataraman, Robert M. Dirks, Christine T. Ueda, and Niles A. Pierce

*Department of Bioengineering; †Department of Chemistry; and ‡Department of Applied and Computational Mathematics, California Institute of Technology, Pasadena, CA 91125

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Cancer cells are characterized by genetic mutations that deregulate cell proliferation and suppress cell death. To arrest the uncontrolled replication of malignant cells, conventional chemotherapies systemically disrupt cell division, causing diverse and often severe side effects as a result of collateral damage to normal cells. Seeking to address this shortcoming, we pursue therapeutic regulation that is conditional, activating selectively in cancer cells. This functionality is achieved using small conditional RNAs that interact and change conformation to mechanically transduce between detection of a cancer mutation and activation of a therapeutic pathway. Here, we describe small conditional RNAs that undergo hybridization chain reactions (HCR) to induce cell death via an innate immune response if and only if a cognate mRNA cancer marker is detected within a cell. The sequences of the small conditional RNAs can be designed to accept different mRNA markers as inputs to HCR transduction, providing a programmable framework for selective killing of diverse cancer cells. In cultured human cancer cells (glioblastoma, prostate carcinoma, Ewing’s sarcoma), HCR transduction mediates cell death with striking efficacy and selectivity, yielding a 20- to 100-fold reduction in population for cells containing a cognate marker, and no measurable reduction otherwise. Our results indicate that programmable mechanical transduction with small conditional RNAs represents a fundamental principle for exploring therapeutic conditional regulation in living cells.

Protein kinase R (PKR) plays a critical role in the antiviral immune response within mammalian cells. Activation by double-stranded RNA (dsRNA) leads to inhibition of protein synthesis and induction of apoptosis (3). In vitro studies suggest that PKR is activated when two PKR molecules dimerize and autophosphorylate in complex with RNA duplexes longer than ≈30 bp (7, 8). The activation efficiency increases with duplex length up to ≈85 bp; longer duplexes can activate multiple PKR dimers simultaneously (7). These properties suggest the therapeutic strategy of selectively killing cancer cells by triggering the formation of long dsRNA in cells containing mRNA cancer markers (9, 10).

Here, we describe hybridization chain reactions (HCR) (11) in which small conditional RNAs mediate a conditional immune response by mechanically transducing between detection of a cognate mRNA cancer marker and formation of long dsRNA. In normal cells lacking the marker, the small conditional RNAs remain inactive. In cancer cells containing the cognate marker, detection of the marker initiates a chain reaction of hybridization events in which small conditional RNAs sequentially nucleate and open to assemble into a long nicked dsRNA “polymer” (Fig. 1B). The resulting PKR-mediated apoptosis eliminates the cancer cell harboring the cognate marker.

Chromosomal translocations are the most common somatic mutation class among known oncogenes (12). Translocations, deletions, or insertions that yield fusion transcripts are well suited for selective detection by small conditional RNAs because the mRNA sequence in the vicinity of the splice point differs from the wild-type sequence by up to 50%. For this reason, we focus our initial efforts on selectively killing cancer cells containing markers that are mutant fusion transcripts. We design and test three HCR transducers to recognize three mRNA cancer markers each found in one of three human cancer cell lines: (M1) the Δegfr fusion resulting from deletion of exons 2–7 in the epidermal growth factor receptor gene, commonly found in glioblastomas, including the glioblastoma cell line U87MG-ΔEGFR (13), and also reported in breast, ovarian, prostate, and lung carcinomas (14), (M2) the tpchpr fusion resulting from translocation t(6;16) (p21;q22) found in the prostate carcinoma cell line LNCaP (15), and (M3) the eves/bfl fusion resulting from translocation t(11;22) (q24;q12) found in the Ewing’s sarcoma cell line TC71 (16, 17) and present in 85% of Ewing’s family tumors (18). Our studies demonstrate the efficacy, selectivity, and programmability of HCR transducers in mediating the killing of cultured human cancer cells containing cognate mRNA cancer markers.

Author contributions: S.V., R.M.D., and N.A.P. designed research; S.V., R.M.D., and C.T.U. performed research; S.V. and N.A.P. analyzed data; and S.V. and N.A.P. wrote the paper.

Conflict of interest statement: We declare competing financial interests in the form of a US patent and pending patent applications in the United States and EU.

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†To whom correspondence should be addressed. E-mail: niles@caltech.edu.

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**Results**

**HCR Transduction Mechanism.** Each HCR transducer (11) consists of two species of small conditional RNA (hairpins A and B in Fig. 1B) that are designed to coexist metastably in the absence of a cognate mRNA cancer marker (M) that serves as the input to the transducer. Each HCR hairpin consists of an input domain with an exposed toehold and an output domain with a toehold sequestered in the hairpin loop. Within a cancer cell, hybridization of the marker to the input domain of A (labeled “a-b” in Fig. 1B) opens the hairpin to expose its output domain (“c*-b*-”). This hairpin conformational change occurs via toehold-mediated branch migration (19), in which M first nucleates with A via base-pairing to toehold (“a”), and subsequently opens the hairpin via branch migration. Hybridization of the output domain of A to the input domain of B (“b-c”) opens hairpin B to expose an output domain (“b*-a*-”) identical in sequence to M. Regeneration of the marker sequence provides the basis for a chain reaction of alternating A and B polymerization steps leading to formation of a nicked dsRNA “polymer.” This dsRNA is the output of HCR transduction, intended to activate PKR and induce apoptosis. It is not clear a priori whether nicks in the dsRNA will interfere with PKR activation. If they do not, amplified transduction can be achieved if polymers grow to be longer than ≈85 bp (7), enabling simultaneous activation of multiple PKR dimers in response to detection of a single mRNA molecule containing the marker. If the marker is absent, the hairpins are metastable (i.e., kinetically impeded from polymerizing) due to the sequestration of the output-domain toeholds in the hairpin loops.

**Engineering Small Conditional RNAs for HCR Transduction.** In dimensioning the components of the HCR hairpins, it is critical to achieve two key properties: hairpin metastability in the absence of the marker, and hairpin polymerization in the presence of the marker. Hairpin polymerization is promoted by increasing toehold/loop size (leading to the formation of additional base-pairs during each polymerization step); hairpin metastability is promoted by decreasing toehold/loop size (improving steric sequestration of the toehold in the hairpin loop). We previously dimensioned DNA hairpins (6-nt toeholds/loops, 18-bp duplex stem) that satisfy these competing requirements and execute HCR transduction in test tube studies (11, 20). Here, we engineered RNA hairpins with smaller 4-nt toeholds/loops and a shorter 14-bp duplex stem that are suitable for performing HCR transduction. Critically, the dsRNA stem in each hairpin is too short to activate PKR (7), a key requirement for achieving conditional rather than systemic immune response.

For therapeutic applications, the sequence of the marker is constrained to be a subsequence of the mutant mRNA (for the present studies, a subsequence containing the splice point of the fusion). This constraint dramatically restricts the design space for the hairpin sequences because only the loop of hairpin A and the toehold of hairpin B (domains “c-” and “c,” respectively, in Fig. 1B) are independent of the marker sequence. Design of an HCR transducer therefore corresponds to simultaneous selection of the marker sequence M and hairpin sequences A and B. Here, we designed small conditional RNAs for three HCR transducers (HCR1, HCR2, and HCR3) that accept the corresponding mRNA cancer markers (M1, M2, and M3) as their inputs. Native polyacrylamide gel electrophoresis confirms strong polymerization in the presence of the cognate marker (Fig. 2A, lane 3), but not in the absence of markers (lane 4), in the presence of wild-type markers (lanes 5 and 6), or in the presence of noncognate cancer markers (lanes 7 and 8). The longest polymers have hundreds of base-pairs, suggesting the potential for amplified transduction. These results confirm the programmability of the HCR transduction mechanism and demonstrate that this kinetically controlled mechanism can be encoded in RNA sequences despite the severe sequence constraints imposed by the cognate marker sequences.

To test whether HCR transduction mediates selective PKR activation in a test tube, we employed Western blots to assay for phosphorylation at residue Thr-451 (Fig. 2B). Strong PKR activation is observed if and only if a cognate cancer marker is present (lane 3). This result suggests that dsRNA polymers containing nicks are suitable for mediating PKR activation.

**Efficacy and Selectivity of Small Conditional RNAs in Mediating Cell Death.** To test efficacy and selectivity in killing cultured human cancer cells, we transfected each of three HCR transducers into each of three human cancer cell lines. HCR1 targets marker M1 in cell line U87MG-ΔEGFR. HCR2 targets marker M2 in cell line LNCaP. HCR3 targets marker M3 in cell line TC71. The images and cell counts of Fig. 3A and B reveal high efficacy and selectivity in killing cultured human cancer cells. Each HCR transducer causes a 20- to 100-fold reduction in population for the cancer cell line containing the cognate marker, while causing no measurable reduction in the population of the two cancer cell lines that lack the cognate marker.

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**Fig. 1.** Conditional therapeutic regulation via programmable mechanical transduction with small conditional RNAs. (A) Concept: The drug remains inactive in normal cells, minimizing side effects. The drug activates allosterically in cancer cells by mechanically transducing between detection of a cognate cancer marker and activation of an independent therapeutic pathway. (B) HCR mechanism: In normal cells, small conditional RNAs (hairpins A and B) coexist metastably. In cancer cells containing a cognate mRNA cancer marker (M; here, a mutant fusion transcript with the splice point denoted by a black circle), small conditional RNAs undergo a chain reaction to mechanically transduce between detection of the marker and formation of a long nicked dsRNA “polymer” that activates PKR, inducing an innate immune response that leads to cell death. Arrowheads denote 3’ ends. Asterisks denote complementarity.

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As a further test of selectivity, we performed additional studies on the related cell lines: U87MG-ΔEGFR (13) and U87MG-wtEGFR (21). U87MG-ΔEGFR cells overexpress the Δegfr transcript containing fusion marker M1. U87MG-wtEGFR cells overexpress the wild-type egfr transcript which lacks the marker M1 (the two segments that form the fusion in the mutant transcript are not contiguous in the wild-type transcript). Hence, transducers that target marker M1 would be expected to selectively kill U87MG-ΔEGFR cells without affecting U87MG-wtEGFR cell populations. We transfected each of three transducers into U87MG-ΔEGFR and U87MG-wtEGFR cells (Fig. 3 C and D). HCR1 and HCR5 target M1 using two different sets of hairpin sequences, both achieving 20-fold reductions in U87MG-ΔEGFR cell populations with no measurable reduction in U87MG-wtEGFR cell populations. HCR4 targets a marker (M4) present in both transcripts and both cell lines, leading to 20-fold population reductions for both cell types, and confirming that U87MG-wtEGFR cells are amenable to hairpin-mediated cell death. Taken together, these results demonstrate the high selectivity of transducers HCR1 and HCR5, both of which avoid killing U87MG-wtEGFR cells that overexpress the wild-type transcript from which their cognate marker (M1) is formed via a deletion mutation.

Efficiency in Killing Cells That Survive Initial Treatment. Noting that a small fraction of cells survive treatment with a cognate HCR transducer (between 1% and 5% in Fig. 3), we wished to establish whether these surviving cells were resistant to HCR-mediated cell death. To examine this question, we transfected HCR transducers into cells grown up from the surviving populations. The cell counts of Fig. 4 reveal that HCR transducers mediate cell death with undiminished efficacy in these regrown populations (20- to 100-fold population reductions). These results indicate that resistance to HCR-mediated cell death is not responsible for the survival of a small fraction of cells following transfection with cognate HCR transducers.

Validation of HCR Transduction Mechanism in Cells. To test whether cell death is mediated by the intended HCR mechanical transduction pathway, as opposed to an unintended pathway involving HCR hairpins, we further examined the function of transducer HCR1 in U87MG-ΔEGFR cells containing cancer marker M1. The HCR transduction cascade features alternating polymerization of A1 and B1 hairpins, and is hence only possible if both hairpin species are present. In contrast to cotransfection of A1 and B1, transfection of either A1 or B1 alone has no measurable effect on cell population (Fig. 5 B and C). This result suggests that interactions between A1 and B1 are crucial in mediating cell death.

To test whether A1 and B1 polymerize within the cell, we reprogrammed the hairpin sequences to disrupt (and then restore) different features of the polymerization mechanism, and measured the efficacy of the modified hairpins in mediating cell death. First, we disrupted hairpin nucleation by designing modified hairpin A5, with a mutated loop sequence that is noncomplementary to the toehold of B1. To restore polymerization, we also synthesized hairpin B5, containing a mutated toehold sequence complementary to the loop of A5. Native polyacrylamide gel electrophoresis demonstrates the expected properties: A5 and B1 do not polymerize in the presence of M1 and polymerization is restored using A5 and B5 to detect M1 (Fig. 5 A, lanes 2 and 3). This loss and restoration of polymerization is mirrored in the efficacy of the hairpins: cotransfection of A5 and B1 has no measurable effect on cell population while cotransfection of A5 and B5 leads to a 20-fold reduction in cell population (Fig. 5 B and C).

Second, we disrupted hairpin opening by designing modified hairpin B4, with a mutated stem sequence that cannot undergo branch migration with the exposed output domain of A1. To restore polymerization, we also synthesized hairpin A4 containing a mutated stem sequence complementary to the stem of B4. To allow testing of these modified hairpins in the same cell line, the stem mutations were chosen so that A4 detects the alternate sequence M4, located elsewhere in the Δegfr transcript (and containing the same toehold sequence as M1). As expected, A1 and B4 do not polymerize in the presence of M1, and polymerization is restored using A4 and B4 to detect M4 (Fig. 5 C, lanes 6 and 7). Likewise, cotransfection of A1 and B4 has no measurable effect on cell population, and cotransfection of A4 and B4 leads to a 20-fold reduction in cell population (Fig. 5 B and C).

Third, we disrupted the periodicity of HCR by designing modified hairpin B6 with a mutated loop sequence that is noncomplementary to the toehold of A1. The chain reaction should proceed normally until, upon formation of the trimer M1·A1·B6, the opening of hairpin B6 fails to regenerate the marker sequence M1, preventing nucleation of an A1 hairpin and thus terminating the chain reaction. As expected, A1 and B6 do not polymerize in the presence of M1, but instead generate short products (Fig. 5 A, lane 11). Cotransfection of A1 and B6 lead to a 2.5-fold reduction in cell population, in contrast to the 20-fold reduction in cell population observed using A1 and B1 hairpins that have the capacity to form long polymers (Fig. 5 B and C). This result is consistent with the reduced efficiency of PKR activation by dsRNAs shorter than ≈85 bp (7).

In summary, disruption of the HCR transduction mechanism at either the hairpin-nucleation step or the hairpin-opening step eliminates the killing capacity of the hairpins. Restoration of complementarity relationships consistent with hairpin nucleation or hairpin opening restores the killing capacity of the hairpins. Disruption of the periodicity of HCR prohibits the formation of long polymers, greatly reducing efficacy. Taken together, the mechanism studies of Fig. 5 suggest that within cultured human cancer cells, small conditional RNAs mediate cell death by performing HCR mechanical transduction via sequential nucleation and opening of A and B hairpins to form long dsRNA polymers.
activated in those cell populations where HCR mediates cell death, we performed Western blots using an antibody that selectively binds PKR phosphorylated at residue Thr-451. Phosphorylation of Thr-451 is critical to the kinase function of PKR in phosphorylating translation initiation factor eIF2α at residue Ser-51. eIF2α phosphorylation leads to inhibition of protein synthesis and is sufficient for inducing apoptosis. We observe strong PKR activation if and only if an HCR transducer is transfected into cells containing the cognate cancer marker (Fig. 6A, compare lane 2 to lanes 3–9). Likewise, strong eIF2α phosphorylation is observed if and only if strong PKR activation is observed (Fig. 6A). These results are consistent with HCR-mediated activation of PKR in cultured human cancer cells.

If the observed cell death results from selective activation of PKR, we would expect that chemical inhibition of PKR activation would disrupt HCR mediation of eIF2α phosphorylation and cell death. 2-aminopurine (2-AP) is an adenine analog that inhibits dsRNA-mediated activation of PKR in vitro and in vivo. Analyses of protein expression and phosphorylation patterns in cultured cells suggest that 2-AP is not a general kinase inhibitor, exhibiting selective but not specific inhibition of PKR. Here, introduction of 2-AP blocks the downstream effects of HCR transduction: PKR activation and eIF2α phosphorylation return to basal levels (Fig. 6A, compare lanes 6 and 3) and no measurable reduction in cell populations is observed (Fig. 6B). These results are consistent with conditional regulation in which HCR transduction accepts a cognate cancer marker as input and activates PKR as an output, leading to selective phosphorylation of eIF2α and selective cell death. It is possible that HCR transduction selectively activates additional endogenous pathways that contribute to cell death (e.g., the 2-5A system).

Selective Apoptosis. As PKR is known to induce apoptosis in response to activation by dsRNA, we wished to establish whether the observed selective cell death occurs via apoptosis. Internucleosomal DNA cleavage during apoptosis produces DNA fragments of ~180 bp and multiples thereof, resulting in characteristic DNA laddering when assayed by agarose gel electrophoresis. Here, we observe DNA laddering in those cell populations where HCR mediates cell death (Fig. 6C, compare lane 2 with lanes 3–9). Likewise, strong eIF2α phosphorylation is observed if and only if strong PKR activation is observed (Fig. 6A). These results suggest that HCR-mediated selective cell death occurs via apoptosis.

Discussion

Small conditional RNAs that perform HCR transduction mediate cell death with striking efficacy and selectivity. In studies with four cultured human cancer cell lines, we observe a 20- to 100-fold reduction in population when a cognate mRNA marker...
is present and no measurable reduction otherwise. The efficacy of HCR transducers in mediating cell death is undiminished in populations grown from cells that survive initial treatment, suggesting that survival does not indicate resistance to HCR-mediated cell death. Disruption and restoration of HCR hairpin complementarity relationships lead to the expected disruption and restoration of killing capacity, suggesting that hairpins mediate cell death by the intended HCR mechanical transduction mechanism. Strong and selective PKR activation and eIF2α phosphorylation are observed in cell populations that undergo HCR-mediated cell death, and chemical inactivation of PKR inhibits cell death, suggesting a central role for PKR in the observed selective cell death. Selective DNA laddering suggests that HCR-mediated cell death occurs via apoptosis.

It is instructive to compare and contrast our approach with efforts to develop chemotherapies based on RNA interference (RNAi) (35, 36). Since the discovery of RNAi in Caenorhabditis elegans in 1998 (37), therapeutic RNAi research has progressed rapidly, with multiple human trials now underway (35, 36). The approach relies on exogenous small RNAs to mediate the recognition and cleavage of a target mRNA by protein enzymes (4). The significant therapeutic potential of RNAi follows from the feasibility of designing small RNAs to target diverse disease-related mRNAs of known sequence. Our approach shares this crucial property of programmability. The fundamental difference is that our small RNAs are conditional, mechanically transducing a target that activates treatment by binding to PKR (responsible for selectivity but not efficacy). Mechanical transduction converts previously inactive HCR hairpins into a long dsRNA HCR polymer that activates treatment by binding to PKR (responsible for efficacy but not selectivity). By comparison, the small RNAs that mediate RNAi do not perform mechanical transduction, do not functionally decouple diagnosis and treatment, and are not conditional. As a result, for RNAi therapeutics, the choice of target mRNA affects both selectivity and efficacy—priorities that are in conflict if the target is specific to diseased cells but does not facilitate effective treatment, or vice versa. For this reason, targeted delivery technologies provide an important means of conferring selectivity on the scope of RNAi treatment (36, 38). The challenge of delivering small RNAs for therapeutic RNAi remains the subject of extensive academic and commercial research.

**Fig. 5.** Validation of the HCR transduction mechanism in cells. To determine whether small conditional RNAs mediate cell death via the intended HCR transduction mechanism (Fig. 1B), we disrupted and restored different features of the mechanism to measure the effect on efficacy. (A) Native polyacrylamide gel assay for loss and recovery of conditional polymerization. Lane 13: dsRNA ladder. (B, C) Micrographs and cell counts 20 h posttransfection. (Scale bar: 100 μm). Normalized means over six sets of experiments; error bars depict standard deviations. Positive control: Hairpins A1 and B1 target marker M1. Single hairpin study: absence of either hairpin prevents polymerization. Hairpin-nucleation study: modified hairpin A5 disrupts nucleation of B1; modified hairpin B5 restores nucleation. Hairpin-opening study: modified hairpin B4 disrupts branch migration; modified hairpin A4 restores branch migration (detecting marker M4 located elsewhere in the Δegfr fusion transcript). HCR periodicity study: modified hairpin B6 prevents regeneration of marker sequence M1, terminating polymerization after two steps.

**Fig. 6.** Conditional immune response. (A) Western blots for selective PKR activation and eIF2α phosphorylation (compare lane 2 to lanes 3–9). β-actin serves as a loading control. (B) HCR-mediated cell death is blocked by chemical inhibition of PKR with 2-AP. Normalized means over six sets of experiments; error bars depict sample standard deviations. (C) Selective apoptosis. DNA laddering is observed if and only if HCR transducers are transfected into cells containing cognate cancer markers (compare lane 2 to lanes 3–9). Lane 1: DNA ladder.
efforts (35, 36). It is a matter of considerable practical significance that progress in delivering small (nonconditional) RNAs for therapeutic RNAi will significantly benefit future translational efforts with small conditional RNAs.

In this initial study of HCR transduction in living cells, we focused our efforts on detecting cancer markers that are mutant fusion transcripts resulting from deletions or translocations. In the coming era of patient and tumor genotyping (39), it is a strength of our approach that HCR transducers can be programmmed to recognize a mutant fusion splice point that is specific to a particular patient or tumor. It remains to be seen whether HCR transduction is suitable for detecting more subtle mutations, including point mutations.

During the last decade, researchers in the field of nucleic acid nanotechnology have exploited the programmable chemistry of nucleic acid base-pairing to engineer devices and systems that execute diverse functions including catalysis, amplification, logic, and locomotion (5, 6). Here, we exploit design principles drawn from this experience to engineer small conditional RNAs that interact and change conformation within cultured human cells, mechanically transducing between detection of an mRNA cancer marker and activation of an endogenous cell death pathway. In doing so, we have engineered a conditional regulatory link that operates autonomously within a complex cellular regulatory network that is not fully characterized. Using the kinetically-controlled self-assembly mechanism of HCR, molecular interactions occur via a base-matched-branch migration (19) and the sequential ordering of interactions is controlled by conditional sequestration and desequstration of toeholds in the loops of metastable hairpins (11). It is significant that these fundamental mechanisms for controlling molecular interactions, which were originally invented and validated in the context of DNA molecules in a test tube, can be adapted to operate robustly using RNA molecules in cultured human cells. By diversifying molecular mechanisms and reprogramming sequences, mechanical transduction with small conditional RNAs offers a versatile framework for introducing conditional regulatory links into living cells.

Methods Summary
HCR hairpins were transfected into cells using commercially available reagents. For each transfection, the total concentration of RNA in solution was 100 nM (using equimolar amounts of A and B hairpin for solutions containing both species). Cell survival rates were measured by flow cytometry, gating for three properties: forward scatter, side scatter, and low fluorescence in the presence of an exclusion dye.

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