Development of a Genetically Validated Cell-based Reporter Assay for ADAR1 Editing Activity

Valerie Sapp, Jim Bilakovics, Ava Burr, Fernando Martins, Pavel Shashkin, Veronique Baron, Henry Zhu, Junguk Park
BPS Bioscience, San Diego, CA 92121

Abstract 2065

ABSTRACT

Introduction

Adenosine Deaminase Acting on RNA (ADAR) enzymes perform adenosine to inosine base editing in RNA, particularly targeting adenosines located within a specific double-stranded stem-loop motif.

The ADAR1 gene encodes both a universally expressed isoform (p110) and an interferon-inducible isoform (p150) that plays a role in innate immunity by mediating interferon signaling. In the context of healthy, uninfected cells, ADAR1 performs A-to-I editing on endogenous double-stranded RNA (dsRNA) to prevent it from activating downstream dsRNA sensors RIG-I and MDA5, which in turn activate a pro-inflammatory response.

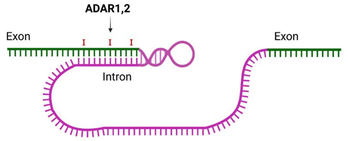

Figure 1. Example of a double-stranded mRNA loop targeted by ADAR editors.

Loss of function mutations in ADAR1 result in aberrant activation of the dsRNA sensors and are involved in autoimmune disorders. ADAR1 dysfunction also impacts cancer cell growth, proliferation, and response to immunotherapy. Thus, ADAR1 expression is increased in several types of cancer and ADAR1 knock-out seems to improve the response to certain immunotherapies such PD-1/PD-L1 blockade and to circumvent tumor immunotherapy resistance mechanisms, making ADAR1 an attractive target for therapeutic development.

Rationale

We have developed and validated a high-throughput, cell-based assay for measuring ADAR1 activity. Several ADAR reporter constructs were designed and evaluated for their relative response to ADAR1 overexpression. They all feature an ADAR1 hairpin target with a stop codon (UAG) which is susceptible to ADAR1-mediated editing to a tryptophan (UUG), located upstream of a Firefly luciferase gene.

Results

We show that transient expression of ADAR1 led to a proportional increase in the activity of the downstream Firefly luciferase, indicating dose-dependent reporter editing by ADAR1. Selected reporters were used in conjunction with ADAR2 overexpression to identify an ADAR1-biased reporter. Thus, a reporter with low luciferase activity in response to ADAR2 expression was chosen to establish stable HEK293 cell lines (which express low levels of ADAR1) with and without constitutively expressed ADAR1 protein.

ADAR1-overexpressing HEK293 cells displayed high luciferase activity, which was decreased in response to siRNA-mediated knockdown of ADAR1 expression. Finally, these validated ADAR1 activity reporter cell lines were further engineered to constitutively express Renilla luciferase under the control of a CMV promoter, serving as an internal control to determine the toxicity of ADAR1 inhibitors. This facilitates the multiplexing of the ADAR1 inhibitor assay and toxicity determination, a salient point given the known toxicity of existing ADAR1 inhibitors (Anderson *et al.* 2007, *Adis Drug Evaluation*, 67: 1633; Cotrell *et al.* 2021, *Cancer Res. Commun.* 2021, 1: 56).

CELL LINES & ASSAY PRINCIPLE

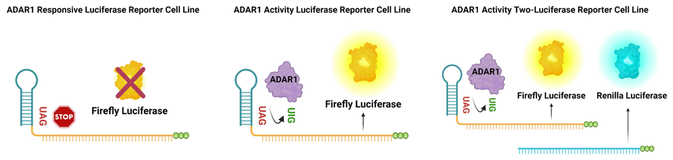

Figure 2. Illustration of the three ADAR1 Reporter cell lines.

The ADAR1 reporter construct is comprised of an ADAR1 hairpin target containing a stop codon (UAG) upstream of the sequence encoding Firefly luciferase. In the absence of ADAR1, luciferase is not transcribed and the cells show no luciferase activity. In the presence of ADAR1 activity, adenine is converted into inosine, with the resulting codon corresponding to amino acid tryptophan (UUG) and enabling transcription and expression of luciferase. ADAR1 activity, therefore, directly correlates with luciferase activity.

HEK293 cells were chosen because of their relatively low expression of endogenous ADAR1.

- The ADAR1 Responsive Luciferase Reporter Cell Line (BPS Bioscience #82238) was generated following transduction and stable integration of the reporter construct. These cells must be transfected to express ADAR1 for luciferase to be induced.
- The ADAR1 Activity Luciferase Reporter Cell Line (BPS Bioscience #82239) was generated by transduction of ADAR1 into the ADAR1 Responsive Luciferase Reporter Cell Line followed by antibiotic selection and cloning.
- The ADAR1 Activity Two-Luciferase Reporter Cell Line (BPS Bioscience #82240) was generated from ADAR1 Activity Luciferase Reporter Cell Line to constitutively express Renilla luciferase. Renilla luciferase activity serves as a proxy for cell viability, allowing the user to assess compound toxicity in parallel with its effect on ADAR1 activity.

REPORTER OPTIMIZATION

HEK293 cell pools were generated for each of three reporter designs and were assayed using transient transfection of an ADAR1-encoding plasmid to identify the construct producing the most sensitive, robust response. Given the substantial overlap in RNA target sequences for ADAR1 and ADAR2, we also assessed the effect of ADAR2 overexpression. Figure 3A shows that two of the three ADAR reporter hairpins triggered luciferase activity upon ADAR1 overexpression. Reporter #2 showed no response upon ADAR2 expression and demonstrated better specificity towards ADAR1 compared to Reporter #1 (Figure 3B). Therefore, Reporter #2 was used to generate a stable cell line.

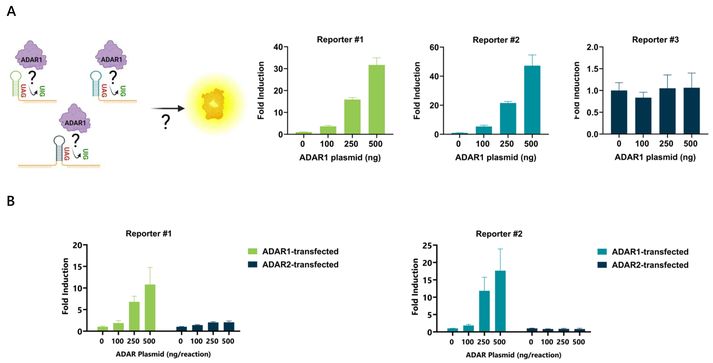

Figure 3. ADAR Reporter #2 was selected for best response and ADAR1 selectivity. HEK293 cells transduced with three distinct ADAR reporter constructs were grown with selection antibiotic for enrichment of stably transduced cell populations (cell pools). Each cells pool was plated at 30,000 cells/well in a 96-well plate. The next day, cells were transfected with increasing amounts of an ADAR1 or an ADAR2-encoding plasmid and incubated for 24 hours. Luciferase activity was measured using ONE-Step™ Luciferase Assay System (BPS Bioscience #60690). Results are expressed as fold induction of luminescence signal compared to mock-transfected control.

ADAR1 RESPONSIVE REPORTER CELL LINE

A stable, clonal cell line was generated from the HEK293 cell pool expressing hairpin Reporter #2. Luciferase activity resulting from transient transfection of ADAR2 or ADAR1 isoforms p150 or p110 was compared. Results indicated preferential response to ADAR1 p150 activity compared to p110, with minimal luciferase activity detected upon ADAR2 overexpression. ADAR1 Responsive Reporter Cell Line maintained robust luciferase activity over time when cells at low passage were compared to cells cultured for 21 passages.

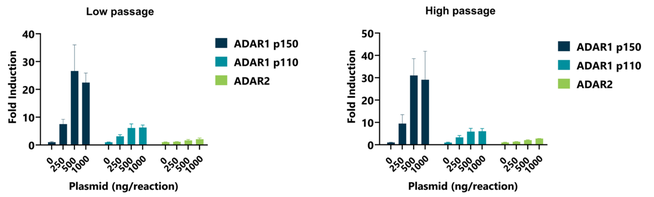

Figure 4. The stable ADAR1 Responsive Reporter Cell Line responds to ADAR1 but not to ADAR2 expression. *Stable ADAR1 Responsive Reporter HEK293 cells at low passage or cultured for 21 passages were plated at 30,000 cells/well in a 96-well plate. The day after, cells were transfected with increasing amounts of either ADAR1 (p150 or p110) or ADAR2-encoding plasmids and incubated for 24 hours. Luciferase activity was measured using ONE-Step™ Luciferase Assay System. Results are expressed as fold induction of luminescence signal compared to mock-transfected controls.*

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ADAR1 ACTIVITY LUCIFERASE REPORTER CELL LINE

To provide a cellular model in which ADAR1-directed drug candidates such as small molecules can be studied, a stable, clonal ADAR1 overexpressing HEK293 cell line was generated by lentivirus-mediated transduction of ADAR1 p150 isoform into the ADAR1 Responsive Reporter HEK293 Cell Line. siRNA-mediated knockdown of ADAR1 resulted in a dose-dependent decrease in luciferase activity, confirming that luciferase activity in these cells results from ADAR1 overexpression (Figure 5B). ADAR1 knockdown did not induce cytotoxicity (Figure 5C). Therefore, cell death did not account for the observed decrease in luciferase activity. Comparing 96-well and 384-well formats showed that stable ADAR1 overexpression results in a reliable, robust signal when compared to parental controls (Figure 6).

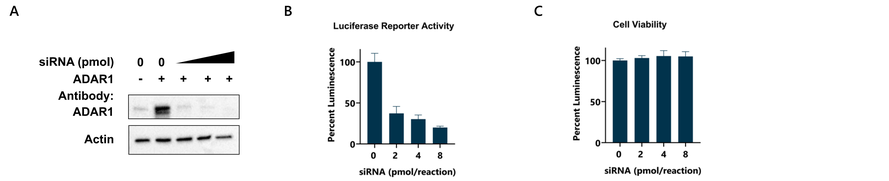

*Figure 5. Luciferase activity in ADAR1 Activity Luciferase Reporter HEK293 Cell Line results from ADAR1 expression. Cells were transduced with an ADAR1-targeting siRNA (Horizon #M-008630-01-0005). After 72 hours, three analyses were performed in parallel. **(A) Western Blotting:** Cells were lysed and ADAR1 protein levels were analyzed by Western Blot using rabbit anti-ADAR1 (Abcam #ab126745) and anti-Actin (Cell Signaling Technology #4970). Untransduced parental cells were included as ADAR1 (-) control. **(B) Determination of luciferase activity.** Luciferase activity was measured using ONE-Step™ Luciferase Assay System. **(C) Determination of cell viability.** The effect of ADAR1 knockdown on cell viability was assessed using CellTiter-Glo® Luminescent Cell Viability Assay (Promega #G7570). In (B) and (C), results are expressed as percent of luminescence signal compared to mock-transfected control.*

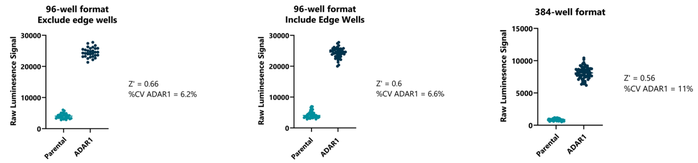

Figure 6. ADAR1-dependent luciferase activity in ADAR1-overexpressing reporter cells shows low variability. *Cells were plated at densities of 40,000 cells/well (96-well) or 5,000 cells/well (384-well) the day before measuring luciferase activity using the ONE-Step™ Luciferase Assay System. Results are shown as raw luminescence signal. Z' statistic and %Coefficient of Variance were calculated.*

ADAR1 ACTIVITY TWO-LUCIFERASE REPORTER CELL LINE

Finally, we generated a cellular ADAR1 model allowing for simultaneous assessment of ADAR1 activity and compound-induced cytotoxicity. The ADAR1 Activity Reporter Luciferase HEK293 Cell Line was engineered to achieve stable, constitutive expression of Renilla luciferase. In this assay system, both Firefly and Renilla luciferase activities correlate with cell density (Figure 7).

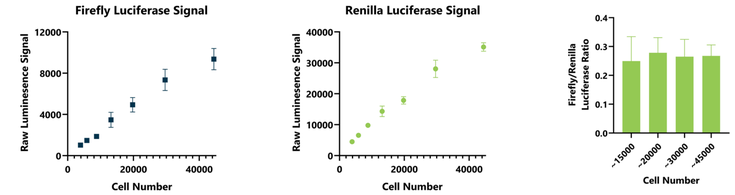

Figure 7. Firefly luciferase and Renilla luciferase signals correlate with cell numbers in ADAR1 Activity TWO-Step Luciferase Reporter Cell Line. *Cells were plated at increasing densities in a 96-well plate. Firefly and Renilla luciferase activities were measured using the TWO-Step Luciferase Assay System (BPS Bioscience #60683).*

CONCLUSION

In conclusion, our sensitive & robust cell-based assays provide well-validated resources to study ADAR1 in a cellular context. Three cell lines were generated to support different aspects of ADAR1 research:

- ADAR1 Responsive Luciferase Reporter HEK293 Cell Line is designed to compare the effect of ADAR1 modifications and mutations on ADAR1 activity, for example when studying structure/function relationships, or when designing ADAR1 variants for RNA editing.
- ADAR1 Activity Luciferase Reporter HEK293 Cell Line stably expresses ADAR1 and is suitable to assess the efficacy of ADAR1 modulators such as small molecule inhibitors. This assay is amenable to high-throughput screening.
- ADAR1 Activity Two-Luciferase HEK293 Cell Line allows the multiplexing of efficacy measurements in parallel with toxicity determination of ADAR1-directed compounds such as small molecule inhibitors.

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