Evaluation of DNA Minicircles for Delivery of Adenine Base Editors Using Activatable Reporter Imaging Systems

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Abstract

Base editing is a powerful genome editing tool with the potential to treat thousands of genetic diseases caused by single-base changes in DNA called point mutations. An adenine base editor (ABE) specifically converts adenine (A) to guanine (G) and can theoretically correct almost half of all disease-causing point mutations. Currently, there is a need for a safe and efficient method of delivering ABEs into tissues. In Chapter 2, we developed DNA minicircles as a novel non-viral delivery method for ABEs. To assess delivery efficiency, we also developed activatable reporter imaging systems for the visualization of ABE activity using fluorescence or bioluminescence imaging. Using our imaging reporters, we show for the first time that minicircles are superior to plasmids for the delivery of ABE into a variety of cancer cell types. Chapter 3 summarizes the main findings and discusses limitations and future work.

Keywords

Adenine base editors, minicircles, plasmids, fluorescence imaging, bioluminescence imaging
Summary for Lay Audience

The human genome is composed of over three billion DNA letters known as bases, with the four possible bases being: adenine (A), cytosine (C), guanine (G), and thymine (T). An error in which a single base is changed to another base is known as a point mutation, and is the cause for thousands of genetic diseases including sickle cell disease, Duchenne muscular dystrophy, and many types of cancer. Base editing is a recently developed genome editing technology that enables precise targeting and modification of DNA bases. Base editors consist of an RNA component that targets a specific location in the DNA and a protein component that makes the base change. Adenine base editors (ABEs) specifically change an A to a G and can theoretically correct almost half of all disease-causing point mutations.

One of the major challenges with base editing is delivery: we need a way to efficiently get the base editors into cells. Minicircles are small circular pieces of DNA that can be used to express a gene of interest in cells. Minicircles are more efficient and safer at delivering genes than plasmids, which are circular pieces of DNA but also contain bacterial components. In this study, we developed and tested minicircles for the delivery of ABEs for the first time. Additionally, to track the DNA edits being made, we developed imaging tools that are turned on when a base editor is working in a cell. These tools allowed us to evaluate the efficiency of minicircle delivery using two imaging techniques called fluorescence imaging and bioluminescence imaging – the latter of which might be useful one day for imaging base editor activity inside the body. We found that minicircles are better than plasmids at delivering ABE DNA into multiple cancer cell types. Our results demonstrate that minicircles are a promising method for efficient and safe delivery of base editors into cells. Future work will test these minicircles for base editing in animal models of cancer. With these tools, we will be able to develop better base editors and delivery methods for the treatment of cancer and other genetic diseases.
Co-Authorship Statement

This thesis contains work that is in preparation for submission and has been presented at multiple conferences. Study conceptualization and design was performed by me, Melissa Evans, John Ronald, and Timothy Scholl. Experimental work was performed by me, Melissa Evans, Joshua Krautner, and Rajan Leung. Melissa Evans and I are currently writing the draft of the manuscript.
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# Table of Contents

Abstract ........................................................................................................................................... ii
Summary for Lay Audience .................................................................................................................. iii
Co-Authorship Statement .................................................................................................................... iv
Acknowledgments ............................................................................................................................... v
Table of Contents ............................................................................................................................. viii
List of Tables ....................................................................................................................................... ix
List of Figures ....................................................................................................................................... x
Chapter 1 ............................................................................................................................................ 1
  1 Introduction ..................................................................................................................................... 1
    1.1 Genetic Disorders ....................................................................................................................... 1
      1.1.1 Point Mutations .................................................................................................................... 3
      1.1.2 Common Diseases Associated with Point Mutations ......................................................... 3
      1.1.3 Point Mutations in Cancers ................................................................................................. 4
    1.2 Genome Editing .......................................................................................................................... 5
      1.1.1 CRISPR Technology ............................................................................................................ 9
      1.1.2 Base Editors ....................................................................................................................... 11
    1.3 Challenges of Base Editing Technologies .................................................................................. 14
      1.3.1 Editing Efficiency and Scope ............................................................................................. 14
      1.3.2 Safety and Off-target Editing .............................................................................................. 16
      1.3.3 Delivery ............................................................................................................................ 17
    1.4 Analysis of BE Activity ............................................................................................................. 19
      1.4.1 Reporter Gene Imaging ........................................................................................................ 21
      1.4.2 Fluorescence Imaging ......................................................................................................... 22
      1.4.3 Bioluminescence Imaging ................................................................................................... 23
1.4.4 Reporter Gene Strategies for Imaging Base Editing ........................................23

1.5 Objectives and Hypotheses .................................................................................25

1.6 References ...........................................................................................................26

Chapter 2 ..................................................................................................................34

2 Evaluation of DNA Minicircles for Delivery of Adenine Base Editors Using Activatable Reporter Imaging Systems .................................................................34

2.1 Introduction ..........................................................................................................35

2.2 Materials and Methods .........................................................................................37

2.3 Results ..................................................................................................................44

2.4 Discussion ............................................................................................................57

2.5 References ...........................................................................................................63

Chapter 3 ..................................................................................................................69

3 Conclusions and Future Work ..............................................................................69

3.1 Summary ..............................................................................................................69

3.2 Limitations ..........................................................................................................71

3.3 Future Work .........................................................................................................73

3.4 References ..........................................................................................................76

Appendix ...................................................................................................................79

Curriculum Vitae .....................................................................................................81
List of Tables

Table 2.1: Primers used for Q5 Site-Directed Mutagenesis PCR reactions .......................... 39

Table 2.2: Protospacer sequences for sgRNAs ........................................................................ 39

Table 2.3: Primers used for PCR amplification of target genomic sites ............................... 42
List of Figures

Figure 1.1: Distribution of human genetic variants associated with disease. ................................. 2

Figure 1.2: DNA repair pathways following a double-stranded break........................................... 6

Figure 1.3: Comparison of genome-editing technologies that induce double-stranded DNA breaks. .................................................................................................................................................. 8

Figure 1.4: Base editors for the targeted conversion of DNA bases................................................... 12

Figure 1.5: Editing windows for ABE7.10 and ABE8. ................................................................. 15

Figure 1.6: Generation of minicircle DNA....................................................................................... 19

Figure 1.7: Methods for analyzing BE activity in cells and animal models................................. 21

Figure 1.8: GFP “Gene On” (GO) reporters for visualization of base editing activity. ........... 24

Figure 2.1: In vitro validation of GFPGO reporter for ABE activity. ............................................. 45

Figure 2.2: Comparison of PP and MC-based delivery and editing of ABE using GFPGO reporter. ........................................................................................................................................... 47

Figure 2.3: Characterization of [GFP-Akaluc]GO reporter for fluorescence and bioluminescence imaging (BLI) of ABE activity. ........................................................................................................... 49

Figure 2.4: Characterization of tdT-AkalucGO reporter for BLI of ABE activity....................... 51

Figure 2.5: In vivo jetPEI delivery of plasmid DNA to tissues via intratumoral (IT, n = 3), intraperitoneal (IP, n = 2), and intravenous (IV, n = 2) routes. ................................................................. 54

Figure 2.6: In vivo electroporation of plasmid or MC DNA to subcutaneous tumors......... 56
Chapter 1

1 Introduction

This thesis focuses on the development of new genome-editing tools called base editors for the treatment of genetic diseases caused by single nucleotide changes in DNA. We designed and validated a new non-viral delivery method for base editors called minicircles as a potentially safer and more efficient alternative to currently used delivery vectors. In addition, we built activatable imaging reporter gene systems that enable visualization of minicircle-derived base editor activity using fluorescence and bioluminescence imaging. This introductory chapter provides an overview of genetic disorders associated with point mutations, the potential to treat these diseases using base editors, the various base editor delivery methods currently used, and the value of reporter gene imaging for understanding and developing improved base editors and delivery vectors.

1.1 Genetic Disorders

The human genome is known as the blueprint of life, encoding instructions for cells to perform their functions and maintain a living organism. Genetic information is stored as a sequence of four possible nucleotide bases: adenine (A), cytosine (C), guanine (G), and thymine (T). A pairs with T and C pairs with G to form the characteristic DNA double helix.

Knowing the exact sequence of the human genome enables genes and non-coding DNA to be studied in detail to elucidate their roles. The Human Genome Project, lasting from 1990 to 2003, is one of the most important milestones in the field of biology. The project sequenced 92% of the human genome, mapping the locations of approximately 30,000 protein-coding genes. In 2022, the Telomere-to-Telomere (T2T) Consortium released for the first time the complete sequence of the human reference genome, comprised of 3.055 billion base pairs. These advancements have accelerated the study of human genetics and uncovered key information related to human development and what makes us different from other species.
Sequencing of the human genome has also greatly advanced our understanding of genetic disorders, diseases caused by mutations in the genetic code. There are over 6000 known genetic disorders in humans\(^3\), with approximately 8% of the population being affected by one or more genetic disorders\(^4\). Genetic disorders can be caused by a wide variety of changes in DNA (Figure 1.1)\(^5,6\). These include insertions and deletions, where any number of nucleotides are added or deleted from a DNA sequence. Indels refer specifically to insertions and deletions less than 10,000 base pairs in length\(^7\). Diseases may also arise from copy number gain and copy number loss, in which there is an abnormal number of copies of a particular gene. On a larger scale, a duplication occurs when a section of an entire chromosome is repeated. However, as seen in Figure 1.1, the largest class of disease-associated mutations are in fact point mutations, where a single nucleotide is changed.

![Figure 1.1: Distribution of human genetic variants associated with disease. Data was retrieved from the ClinVar database, an online archive of reports of genetic variation and its relationship to human health. Reproduced with permission from Springer Nature (2018)\(^8\).](image-url)
1.1.1 Point Mutations

Because genetic instructions are written in the sequence of bases, a single change can have drastic consequences. Point mutations in protein-coding genes can have three possible outcomes: 1) a missense mutation results in a different amino acid being encoded at a particular position in the resulting protein, 2) a nonsense mutation results in coding of a premature stop codon (TGA, TAG, or TAA) and production of truncated and usually non-functional protein, and 3) a silent mutation does not have an effect on the amino acid sequence. Missense and nonsense mutations often cause disease by altering the function and levels of protein products. On the other hand, point mutations in non-coding regions of the genome may impact gene expression through regulatory elements, such as promoters, enhancers, silencers and insulators, as well as splice sites\(^9\). These mutations usually result in overexpression or underexpression of one or multiple proteins, which are implicated in many cancers and will be discussed in section 1.1.3\(^10\).

1.1.2 Common Diseases Associated with Point Mutations

One of the most common classes of diseases associated with point mutations are blood disorders, including sickle cell disease (SCD), β-thalassemia, hereditary spherocytosis, Fanconi anemia, and Hemophilia A and B\(^11\). Of these diseases, SCD is the most common, with over 250,000 new patients each year and predominately affecting people of African ancestry\(^12,13\). It is caused by a G\(_{AG}\) to G\(_{TG}\) mutation and a change from glutamic acid to valine at the sixth amino acid position in the β-globin gene. This results in the production of abnormal hemoglobin molecules, which stick together and cause red blood cells to acquire a rigid, sickle shape as opposed to their normal, donut-like shape. SCD is characterized by anemia, hemolysis (premature destruction of red blood cells), occlusion of blood vessels, heightened inflammation, increased risk of stroke, and in severe cases, multi-organ failure resulting in death\(^14\). Current treatments typically include blood transfusions, bone marrow transplantations, and pain medications, but no gene therapy has yet been approved for SCD\(^14\).

Duchenne muscular dystrophy (DMD) is a muscle disease affecting approximately 1 in 5000 male births worldwide\(^15\). Because it is a X-linked recessive disease, it occurs
primarily in males. In contrast to SCD which is caused by a single point mutation, DMD can be caused by over 500 point mutations in the \textit{DMD} gene that encodes the dystrophin protein, whose main function is to stabilize and protect muscle fibres\textsuperscript{16}. These mutations typically consist of nonsense mutations and splice-site mutations that disrupt the protein reading frame and result in the production of non-functional, truncated dystrophin protein\textsuperscript{17}. DMD patients experience progressive muscle weakness, loss of mobility, difficulty breathing, and eventual death with an average life expectancy of only 26 years\textsuperscript{18}.

Point mutations are also known to be associated with several liver diseases. Tyrosinemia type 1 (HT1) is caused by a loss-of-function mutation in the gene coding for fumarylacetoacetate hydrolase (FAH), an enzyme involved in the production of the amino acid tyrosine\textsuperscript{19}. This may occur by a G to C mutation in the \textit{FAH} gene resulting in exon skipping and loss of functional FAH protein\textsuperscript{20}. As a result, toxic metabolites such as fumarylacetoacetate accumulate in hepatocytes and renal proximal tubules, leading to liver damage and renal tubular dysfunction. Phenylketonuria (PKU) is another liver disease caused by mutations in the \textit{PAH} gene, encoding the enzyme phenylalanine hydroxylase (PAH), which metabolizes the amino acid phenylalanine. A rise in blood phenylalanine level can have neurotoxic effects which can lead to impaired cognitive development, seizures, autism, and motor deficits\textsuperscript{21}. Approximately 62\% of pathogenic variants of \textit{PAH} are due to missense mutations, while 5\% are due to nonsense mutations\textsuperscript{22}. Lastly, alpha-1 antitrypsin deficiency (AATD) is a disease that affects both the liver and lungs, and is primarily due to a G to A missense mutation in the serine protein inhibitor-A1 (\textit{SERPINA1}) gene encoding alpha-1 antitrypsin\textsuperscript{23}. Alpha-1 antitrypsin is an enzyme that neutralizes neutrophil elastase, a component of the innate immune system that fights infection. With low levels of alpha-1 antitrypsin, neutrophil elastase begins to attack normal tissues, particularly the liver and lungs. Currently, the only resolutive therapy for these genetic liver diseases is liver transplantation, but this is limited by organ availability and post-operative complications\textsuperscript{24}.

\subsection*{1.1.3 \quad Point Mutations in Cancers}

While congenital disorders such as those described above are due to germline mutations (inherited during conception and present in every cell in the body), the majority of cancers
originate from a combination of somatic mutations acquired during one’s lifetime. These mutations include point mutations, small insertions and deletions, chromosomal changes, and epigenetic changes. In common solid cancers such as breast, brain, colon and pancreatic cancers, nearly 95% of mutations are point mutations, with 90.7% being missense mutations, 7.6% being nonsense mutations, and 1.7% in splice sites or untranslated regions immediately adjacent to start and stop codons. These mutations can be divided into two types: 1) passenger mutations, which do not actually influence a cell’s cancer-like properties, and 2) driver mutations, which cause tumorigenesis by increasing cell proliferation and invasiveness. In a study of more than 7500 tumours across 29 cancer types, it was found that a typical tumour contains 1-10 of these driver mutations. With the rapid development of methods for genome-scale analysis of tumor samples, it has been of particular interest to identify these driver mutations as potential targets for cancer gene therapy.

Examples of point mutations driving tumorigenesis are those found in the promoter for telomerase reverse transcriptase (TERT), a telomerase that preserves the ends of chromosomes. Somatic mutations in this promoter have been found to be present in over 80% of primary glioblastomas. Two hotspot positions are located at 124 and 146 bases upstream of the ATG start site, with G to A mutations at both locations. These mutations generate new binding sites for transcription factors that can increase TERT promoter activity, and thus lead to increased TERT expression which enables tumour cells to proliferate and evade cellular senescence.

1.2 Genome Editing

Since the 1970s, scientists have attempted to manipulate DNA by using enzymes to cut and paste genetic material from one organism to another. This technology, called recombinant DNA, was achieved in 1972 and represents a major milestone in the field of genetic engineering. This was followed by the rapid development of genome editing, the incorporation of changes at specified genomic targets. Targeted genomic changes using nuclease typically begin with the introduction of a double-stranded break at the desired locus, which can then be repaired by the cell through one of two pathways: homology-directed repair (HDR) and nonhomologous end-joining (NHEJ) (Figure 1.2). HDR is a
precise repair mechanism that uses a homologous DNA template to replace the DNA surrounding the cleavage site. This process can be leveraged to insert an exogenous DNA sequence containing homologous arms into a desired genomic target, for example a wildtype gene to correct a mutated gene\textsuperscript{33}. The second repair pathway, NHEJ, directly joins broken ends of DNA without a DNA template, which usually results in random insertions and deletions (indels) at the cleavage site and is a desired outcome when the goal is gene disruption\textsuperscript{34}.

**Figure 1.2: DNA repair pathways following a double-stranded break.** Homology-directed repair (HDR) utilizes a donor template containing homologous arms to precisely replace DNA at the cleavage site, resulting in gene correction or insertion. Non-homologous end joining (NHEJ) is an error-prone process in which broken ends of DNA are directly joined together, resulting in small insertions and deletions.
A number of technologies exist for highly targeted editing of the genome via the introduction of double-stranded breaks (Figure 1.3). One of the first techniques is using zinc finger nucleases (ZFNs)\textsuperscript{35}. Zinc fingers were first identified as DNA-binding domains in transcription factor IIIA (TFIIIA) from *Xenopus laevis* (South African clawed frog)\textsuperscript{36}. Since then, numerous other zinc fingers have been discovered, varying widely in structure and function. The DNA-binding capability of zinc fingers was leveraged to create ZFNs, which are artificial restriction enzymes for custom site-specific DNA cleavage. ZFNs have two components: a DNA-binding domain and a DNA-cleavage domain. The DNA-binding domain consists of an array of 3 zinc fingers, each specifically binding to a set of 3-4 bases, which can be combined to recognize a longer target sequence. The DNA-cleavage domain is the FokI nuclease domain isolated from the bacterium *Flavobacterium okeanokoites*, which induces a double-stranded break in the DNA. Although ZFNs have shown considerable promise in their ability to introduce site-specific double-stranded breaks, some drawbacks are that they are time-consuming to engineer, they have low flexibility in target site selection, and sometimes show high off-target DNA editing\textsuperscript{37}. 
Zinc finger nucleases (ZNFs) consist of zinc finger DNA-binding domains and the FokI nuclease domain as the DNA-cleavage domain. Transcription activator-like effector nucleases (TALENs) also contain a FokI nuclease but instead utilize TAL effectors for more flexible DNA binding. Clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) consists of a single-guide RNA (sgRNA) which binds a genomic target upstream of a “NGG” protospacer-associated motif (PAM), and a Cas9 nuclease for DNA cleavage.

The limitations with ZFNs led to the development of a new class of genome editing tools called transcription activator-like effector nucleases (TALENs) in 2011. Like ZFNs, TALENS are composed of a DNA-binding domain and a FokI endonuclease. DNA-binding domains are derived from transcription activator-like effector (TALE) proteins secreted by
Xanthomonas bacteria when they infect plants. In TALENs, DNA-binding occurs via TAL effectors which contain a customizable sequence of 33-35 amino acids that each recognize a single base in the target DNA. Thus, TALENs have substantially greater flexibility in target site selection compared to ZFNs, in addition to more efficient on-target editing and lower off-target editing. However, one of the primary hurdles is the need to engineer a unique nuclease for every new genomic target, which can be costly and time-consuming\textsuperscript{35,39}.

1.2.1 CRISPR Technology

The development of clustered regularly interspaced short palindromic repeats (CRISPR) technology in 2012 was a key breakthrough in the field of genome editing, allowing for substantially improved simplicity and efficiency compared to previous technologies. These landmark studies resulted in the Nobel prize in Chemistry being awarded to Drs. Emmanuelle Charpentier and Jennifer Doudna in 2020\textsuperscript{40}. CRISPR is based on a naturally occurring bacterial adaptive immune system. Bacteria protect themselves against viruses by inserting fragments of virus DNA into their genome at a locus known as the CRISPR array, which is then saved as memory for fighting future infections\textsuperscript{41}. The viral DNA fragments in the CRISPR array are transcribed into RNA and can bind to re-invading virus DNA through complementary base pairing, which then induces DNA cleavage through a nuclease called CRISPR-associated protein (Cas). Taking advantage of this ability to target and cut DNA, scientists have engineered the naturally existing CRISPR system into a versatile genome editing technology\textsuperscript{42}. The most commonly used system is CRISPR/Cas9, utilizing the Cas9 nuclease from the \textit{Streptococcus pyogenes} bacteria. In this system, a 20-base pair single-guide RNA (sgRNA) is designed to be complementary to the site of interest in the DNA, which must be followed by the protospacer adjacent motif (PAM) sequence NGG, where N can be any nucleotide. The Cas9/sgRNA complex first locates the PAM, then Cas9 unwinds the DNA to check if the sgRNA matches the DNA. If a match is found, Cas9 creates a double-stranded DNA break.

The rapid expansion of CRISPR technology in basic science research and clinical trials largely stems from its simplicity and ease of design, as new genomic sites can be targeted by manipulating a short sequence of RNA rather than re-engineering a new nuclease as
was the case for ZNFs and TALENs. Despite only being a few years after its development, the first clinical trial utilizing CRISPR was in 2016, when scientists removed immune cells from lung cancer patients, disabled the gene for the immune checkpoint protein programmed cell death protein 1 (PD-1) to enhance anti-tumor response, and injected the edited cells back into the patients\textsuperscript{43}. In 2018, the first clinical trial editing somatic cells \textit{in vivo} aimed to treat a genetic retinal disease by delivering CRISPR/Cas9 directly into the retina of patients to correct abnormal splicing in a protein required for phototransduction\textsuperscript{44}. While these studies demonstrate the feasibility and potential of CRISPR for gene therapy in somatic cells, germline editing (editing in eggs, sperm, or embryos) is far more controversial due to the risk that unwanted side effects could be permanent and passed down to future generations. At the end of 2018, Dr. Jiankui He announced that he had used CRISPR to edit the C-C chemokine receptor type 5 (CCR5) gene in human embryos with the intention of conferring HIV resistance, resulting in the world’s first gene-edited babies\textsuperscript{45}. This highly controversial study has subsequently fueled debate over the ethics of CRISPR, and highlighted the need to fully understand the potential risks of gene editing technologies.

Perhaps the greatest concern with CRISPR editing is the introduction of double-stranded breaks into the DNA, which can be used for on-target treatment but can also have unintended consequences such as random insertions and deletions, as well as large chromosome rearrangements\textsuperscript{46}. For example, in a study that aimed to edit human pluripotent stem cells with high efficiency, the authors observed that double-stranded breaks induced by Cas9 were toxic and killed most stem cells\textsuperscript{47}. It has also been found that double-stranded breaks induce a cellular stress response mediated by the tumor-suppressor protein p53, and that cells with inactivating mutations in the gene encoding p53 are relatively enriched following CRISPR/Cas9\textsuperscript{47–49}. Given that 50\% of all cancers have mutations in p53, it is possible that CRISPR editing may predispose patients to cancer development. Another challenge with CRISPR is off-target effects, where double-stranded breaks are introduced in other parts of the genome which could potentially create mutations that increase cancer risk. These challenges have prompted scientists to develop and explore other genome editing technologies that may enable safer gene therapy.
1.2.2 Base Editors

In 2016, Dr. David Liu and colleagues reported that they had modified the CRISPR system to develop a new genome editing technology they called base editors (BEs), which are capable of inducing targeted single-base changes in DNA without double-stranded breaks\textsuperscript{50}. BEs consist of three main components: 1) a sgRNA that can be programmed to target a precise location in the genome, 2) a Cas9 “nickase” (Cas9n) that creates a single-stranded nick in the DNA rather than a double-stranded break, and 3) a nucleobase deaminase that converts one base to another (Figure 1.4). As mentioned, the major advantage of base editors over traditional CRISPR technology is the lack of double-stranded break production, which overcomes the associated safety concerns. In addition, because base editing does not rely on HDR to modify a gene, it can be used to edit both dividing and non-dividing cells, greatly expanding its applications.
Figure 1.4: **Base editors for the targeted conversion of DNA bases.** Cytosine base editors convert a C•G base pair to T•A, and adenine base editors convert an A•T base pair to G•C.

There are two major classes of BEs: cytosine base editors (CBEs) and adenine base editors (ABEs). CBEs convert a C•G base pair to T•A and were first developed by fusing the cytidine deaminase enzyme APOBEC1 from the rat *Rattus norvegicus* to catalytically dead Cas9, which contains Asp10Ala and His840Ala mutations that prevent the enzyme from creating double-stranded breaks\(^5\). Upon binding of the sgRNA to the target DNA
sequence, APOBEC1 deaminates C to uracil (U), creating a base pair mismatch which is then repaired by the cell’s mismatch repair pathway. Further changes were made to this initial CBE to enhance the efficiency of C•G to T•A editing. First, the authors added a uracil glycosylase inhibitor protein to the CBE, blocking removal of the U•G intermediate by the base excision repair enzyme uracil DNA glycosylase. Second, the catalytically dead Cas9 was replaced by Cas9 nickase (Cas9n) by restoring the His840Ala mutation. Cas9n creates a nick in the DNA backbone of the unedited, G-containing strand, stimulating the cell to use the strand containing the newly created U as the template for mismatch repair. After these modifications, the third generation CBE, BE3, was able to achieve editing of 15-75% of total cellular DNA across four human and murine cell lines, demonstrating high efficiency of editing in vitro.

The second class of BEs, ABEs, induce A•T to G•C base pair conversions and were developed by Dr. David Liu’s group and reported in 2017. ABEs deaminate an A to inosine (I), which is read by DNA polymerase as G. As there are no natural adenine deaminases that act on DNA, the authors created their own enzyme through seven rounds of directed evolution of the adenine deaminase TadA from the bacteria Escherichia coli. This yielded the final ABE7.10 with an average editing efficiency of ~50% across 17 genomic loci in human cells.

The development of CBEs and ABEs opened doors to the precise correction of pathogenic point mutations with considerably higher safety compared to previous genome editing technologies. Of all point mutations associated with disease, approximately 47% of them can theoretically be corrected with an ABE, and 14% with a CBE. Since their development, BEs have been rapidly expanded to new disease targets and animal models of genetic disease, including SCD, DMD, tyrosinemia, inherited hearing loss, and blindness, and cancer. In July 2022, the first base editing clinical trial began, in which patients with familial hypercholesterolemia, who are at high risk for cardiovascular disease, received an ABE that changes a base in the proprotein convertase subtilisin/kexin type 9 (PCSK9) gene, leading to mis-splicing and non-functional protein which ultimately results in decreased blood cholesterol level. The rapid progression of base editing from
the bench to bedside demonstrates the high promise of this technology for treatment, and potentially cure, of numerous genetic disorders.

1.3 Challenges of Base Editing Technologies

Despite the great potential seen in base editors and the advantages they boast over previous genome editing tools, there remain some challenges to be overcome. One of the primary limitations is editing efficiency, which can be very low at certain target sites or in particular cell types. For example, in 2018, Ryu et al. attempted to treat a mouse model of DMD via intramuscular injection of ABES delivered using adeno-associated viruses (AAV), but only achieved a 3.3% target editing rate in sequenced muscle cells\(^{54}\). In 2020, Wu et al. delivered ABES via lipid nanoparticles in a mouse model of tyrosinemia, with only a 0.4% editing rate in the liver\(^ {62}\). With the rapid expansion in BE applications, the gap between in vitro and in vivo editing is clear, and there is a need for improved BE systems that enable high efficiency editing while also minimizing off-target effects. As ABES have broader applicability than CBEs in regard to the number of disease-causing point mutations they can correct, the remainder of this thesis will focus on ABES.

1.3.1 Editing Efficiency and Scope

Following the development of the foundational ABE7.10, several studies have aimed to improve the editing efficiency as well as the scope of editing through engineering of new ABE variants. In 2018, Gaudelli et al. enhanced editing by optimizing localization of the ABE to the nucleus through the addition of SV40 nuclear-localization signals (NLS) at both the N- and C-termini of the ABE\(^ {63}\). Additionally, they codon-optimized this new variant, introducing synonymous codon changes in the gene sequence to improve protein translation efficiency in mammalian cells. These changes yielded ABEmax, which showed 5.2-7.1-fold improvements in editing over ABE7.10 in HEK293T cells.

For some applications, it is also of interest to expand the editing window of the ABE to broaden its therapeutic scope. The original ABE7.10 has an editing window of A5-A7, meaning any A at positions 5-7 of the 20-base pair sgRNA-binding region (i.e., protospacer) could be edited (Figure 1.5). However, this small editing window makes it difficult to edit some disease-associated mutations given the requirement of the NGG PAM
sequence downstream of the protospacer. To expand the editing window, Gaudelli et al. further evolved ABE7.10 and tested over 40 variants, which they called ABE8s\textsuperscript{64}. Compared to ABE7.10, these variants showed 1.5x higher editing within the original window (A5-A7), and 3.2x higher editing at A3-A4 and A8-A10. This wider window allows for an additional ~3000 disease-associated point mutations to be potentially corrected by an ABE.

**Figure 1.5: Editing windows for ABE7.10 and ABE8.** The numbers below the DNA indicate the position of the base within the 20-base pair protospacer. The height of the curve indicates the relative base editor activity at each position. Reproduced with permission from Springer Nature (2020)\textsuperscript{64}.

Another method to broaden therapeutic scope is to engineer new ABEs with different PAM requirements other than the most commonly used NGG. This can be done by linking the TadA deaminase domain to Cas homologs from other bacterial species or engineered Cas proteins which bind to different PAM sequences. In 2020, Richter et al. generated ABEs with enhanced activity and compatibility with a variety of Cas homologs, such as SaCas9 (PAM: NNGRRT, where R is G/A), LbCas12a (PAM: TTTV, where V is G/C/A), and SpCas9-NG (PAM: NG)\textsuperscript{65}. To do this, they first evolved the TadA from ABE7.10 to have higher deamination kinetics, yielding ABE8e which has a remarkably 590-fold higher rate of deamination. This is critical because the Cas domain may let go of the DNA before the
edit is made, so a faster rate of deamination increases the efficiency of editing. When TadA-8e was paired with other Cas homologs, the authors achieved up to 59-fold higher editing compared to the ABE7.10 equivalent. These advancements demonstrate the power of protein engineering for the development of novel ABEs with improved editing efficiency and targeting flexibility, which is expected to be a growing area of research as new difficult genomic targets are investigated.

1.3.2 Safety and Off-Target Editing

Although BEs offer the major advantage of avoiding double-stranded breaks in the DNA, they are not without safety concerns. One potential risk of base editing is that if multiple As or Cs are within the editing window, unwanted base conversions could result. These are called bystander mutations, and it is of high importance to study if they could lead to any negative effects on normal protein production or other aspects of cell function for each genomic target. Thus, in some applications it may be desirable to choose a BE with a narrower base editing window to reduce the chance of bystander mutations.

Another concern for base editing is the potential for off-target effects, in which base edits are made at sites other than the intended target site. There are two main categories of off-target editing: sgRNA-dependent and sgRNA-independent off-target editing. sgRNA-dependent off-target editing occurs due to the sgRNA binding at genomic sites with a high degree of similarity with that of the target sequence. A study showed that ABE can tolerate sites with 1-2 base mismatches within the sgRNA, and in eight sgRNAs tested, an average of eight off-target sites were found per sgRNA. Although levels of off-target editing using BEs are substantially reduced compared to conventional Cas9 nucleases, it has prompted the development of BEs using high-fidelity Cas9 variants to enhance the specificity of interactions between BEs and DNA. Another way to reduce sgRNA-dependent off-target editing is by delivering BEs using methods that allow for shorter-lived base editing activity, which will be discussed in further detail in section 1.3.3. In silico tools such as Cas-OFFinder and COSMID also exist for the computational prediction of off-target sites, which is useful for finding a sgRNA with low off-target activity for a particular target of interest.
The second category of off-target effects, sgRNA-independent off-target editing, occurs as a result of the deaminase domain rather than sgRNA sequence similarity. One study investigating these effects in mouse embryos found that embryos injected with CBE had a 20-fold higher rate of point mutations compared to control embryos and embryos injected with ABE, with the majority of these mutations being C to T mutations\textsuperscript{72}. This demonstrates that sgRNA-independent off-target editing is likely due to the cytosine deaminase domain binding to DNA independently of the sgRNA. To overcome this, some groups have engineered new CBE variants with mutations in the deaminase domain that decrease its kinetics of deamination, resulting in several fold decreases in sgRNA-independent off-target editing\textsuperscript{73,74}. On the other hand, similar off-target effects have been very low with ABEs, so most engineering efforts have focused on enhancing their kinetics and on-target effects instead. Thus, engineering of BEs requires finding the right balance while optimizing on-target effects and minimizing off-target effects.

### 1.3.3 Delivery

One of the most difficult challenges of in vivo base editing is delivering the BE into tissues of interest with high efficiency. It is also crucial to maintain a balance between high expression of BEs and avoiding accumulation of off-target editing that may result from prolonged BE expression. Numerous delivery methods for BEs have been tested in animal models, with each having their own advantages and limitations.

Viral-based delivery is a popular method for in vivo base editing. Compared to other delivery systems, viruses are highly proficient at transferring genes into cells, and while many different viral vectors exist for delivery of genome editing tools, the most common for BEs are adeno-associated viruses (AAVs). AAVs insert their cargo into the nuclei of cells but do not directly integrate their cargo into the genome. Therefore, transgenes are not replicated during cell division, allowing for transient BE expression in cells and thus low levels of off-target editing\textsuperscript{75}. Additionally, there are multiple AAV serotypes with different tissue tropisms, meaning different types of AAVs can be used to target different organs, such as the heart, lung, pancreas, and skeletal muscle\textsuperscript{76}. However, using AAVs to deliver BEs is challenging because the size of BEs (~5.2 kb for BEs containing Cas9) exceeds the packaging limit of AAVs (~4.7 kb). To overcome this limit, BEs have been
split into two halves using a split intein system that splices together N- and C-terminal peptides, with the sgRNA typically encoded on one or both halves of the AAV constructs. However, this decreases their editing efficiency because of the need for the two halves to rejoin once delivered\textsuperscript{77}. Single AAV vectors are also being developed for the delivery of smaller BEs using compact Cas9 enzymes, but these can be limited by PAM specificity\textsuperscript{78,79}.

Non-viral delivery methods have also been extensively explored for \textit{in vivo} base editing. A promising choice is mRNA delivery of the BE combined with synthesized sgRNA, which is advantageous because mRNA is not limited by transgene size. Like AAVs, mRNA is also beneficial for its transient expression and low off-target editing levels\textsuperscript{80}. mRNA can be injected directly into the circulation, or it can be encapsulated in lipid nanoparticles which protect the mRNA from degradation. However, BEs delivered by lipid nanoparticles end up mostly in the liver, which can make it difficult to target other organs\textsuperscript{24}.

Another common non-viral delivery method, particularly for \textit{in vitro} base editing, is using plasmids encoding the BE and sgRNA as DNA. Plasmids are relatively easy to produce in large quantities, but they contain bacterial backbones which are undesired for several reasons. First, they increase the size of the vector, limiting their ability to transfect cells with high efficiency. Bacterial DNA contain unmethylated CpG motifs, which can induce an immune response in a mammalian host and subsequently result in elimination of the vector and transfected cells\textsuperscript{81}. Transfer of antibiotic resistance genes from the bacterial backbone to patients is also another serious safety concern\textsuperscript{82}. Finally, bacterial backbone DNA has been shown to result in transcriptional silencing of the encoded transgene\textsuperscript{83}.

DNA minicircles (MCs) may be an appealing alternative to plasmids; they are small circular DNA vectors containing the transgene(s) of interest and lacking a bacterial backbone. MCs can be produced from “parental plasmids” (PPs) containing attB and attP recombination sites on opposite sides of the transgene\textsuperscript{84}. When the PPs are grown in a special host bacterial strain, addition of arabinose induces expression of an integrase that recombines the attB and attP sequences, resulting in MCs that contain only the cassette encoding the transgenes and regulatory sequences of interest (Figure 1.6). Because of their smaller size, MCs enter cells and nuclei more readily resulting in higher transgene
expression, which may be beneficial in cell types that are difficult to transfect. However, MCs have not yet been explored for the delivery of BEs.

**Figure 1.6: Generation of minicircle DNA.** Minicircles are created from parental plasmids containing the transgene of interest, attB and attP recombination sites, and a bacterial backbone. Parental plasmids are grown in an engineered E. coli strain (ZYCY10P3S2T) which can be induced by arabinose to express 1) a ΦC31 integrase that recombines the attB and attP sites, and 2) a Sce-I endonuclease that digests the bacterial backbone at a region containing 32 copies of SceI cut sites, leaving only minicircles which express the transgene. The minicircles can then be isolated at high yield from these engineered bacteria using standard plasmid isolation kits.

### 1.4 Analysis of BE Activity

With the rapid expansion of BE tools and delivery vehicles, there is a need to evaluate these new tools in cells and animal models for each disease target (Figure 1.7). For *in vitro* studies, the most common method is extracting DNA from cells following a period of editing and subjecting this to deep DNA sequencing, which yields a percentage change in
a nucleotide at a particular position in the genome. For in vivo BE analysis, it may be possible to characterize the function or expression level of a protein whose gene has been edited. For example, functional analysis has also been used to evaluate base editing in a mouse model of retinal degeneration, where mice received a green light stimulus and photopic electroretinography was used to measure electrical response from edited photoreceptors. However, most BE analysis in animal models is performed ex vivo after the animal has been sacrificed and tissues extracted. These methods include DNA sequencing and tissue immunohistochemistry, which may provide information about restored expression of a certain protein, for example dystrophin for DMD and FAH for tyrosinemia. Although these techniques yield valuable insights on editing efficiency and restoration of functional protein, they cannot provide information about editing on a whole-body scale, which is important for knowing where BE delivery vehicles travel once inside the body and the location(s) of successful BE activity. For the evaluation of new BE variants or delivery vectors, it would be advantageous to map the spatial distribution of BE activity so that we can answer questions such as: which tissues/cells are edited? What are the kinetics of editing? How long are the BEs are active for once delivered? These key questions are difficult to answer using the one-dimensional and often tissue destructive techniques currently available.
Figure 1.7: Methods for analyzing BE activity in cells and animal models. For *in vitro* analysis, the most common method is deep sequencing of genomic targets. Some imaging strategies have also been developed for the visualization of BE activity *in vitro*. For animal models, methods may exist to characterize protein levels *in vivo* via functional assays, but typically tissues are extracted following euthanasia and subjected to sequencing or histology to evaluate protein expression. *In vivo* imaging (theoretical example shown on the middle-right) allows for non-invasive, longitudinal analysis of BE activity.

1.4.1 Reporter Gene Imaging

Reporter gene imaging may be a powerful tool for the non-invasive visualization of the timing and spatial distribution of BE activity across an entire animal or person. An imaging
reporter gene expresses a protein whose presence or effects can be detectable with imaging, allowing spatial mapping of gene expression and biological processes such as signal transduction pathways, protein-protein interactions, and trafficking of proteins or cells in living subjects. Cells can be genetically engineered to express a reporter gene, which may be constitutive (always expressed) or activatable (expressed under certain conditions). Multiple modalities exist for reporter gene imaging such as fluorescence imaging (FLI) and bioluminescence imaging (BLI), as well as clinically-used modalities like magnetic resonance imaging (MRI), positron emission tomography (PET), and photoacoustic imaging (PAI). This thesis will focus on FLI and BLI, as they are relatively easy to use and cost-effective methods for the development and refinement of new reporter gene technologies. Additionally, they are highly sensitive and specific at visualizing cells at the single-cell level in cell culture and tissues and at the whole-body level for small animals.

1.4.2 Fluorescence Imaging

FLI uses a fluorescent reporter protein that is excitable by light of a certain wavelength and emits the absorbed energy as light of a longer wavelength. One of the most widely used FLI reporter proteins is green fluorescent protein (GFP), first isolated from the jellyfish *Aequorea aequorea* in 1962. These discoveries were awarded the Nobel Prize in Chemistry to Drs. Roger Tsien, Martin Chalfie, and Osamu Shimomura in 2008. Many synthetic variants of GFP have since been created, such as the commonly used enhanced GFP (eGFP), which has improved brightness and stability. Development of FLI proteins of other colours such as blue, yellow, orange, and red has also enabled the study of multiple proteins within a cell or living subject, which can be easily done by switching the excitation and emission filters. One of the major advantages of FLI reporters is that they do not require any cofactors or substrates other than oxygen, making them feasible for a wide variety of applications. However, for *in vivo* imaging, FLI suffers from low sensitivity due to background tissue fluorescence, known as autofluorescence, as well as light scattering from tissues.
1.4.3 Bioluminescence Imaging

BLI is another optical imaging technique that is broadly used in preclinical research. It relies on the expression of a luciferase enzyme, which reacts with a substrate to produce light that is then detected by a cooled charge-coupled device (CCD) camera\(^9\). The most widely used luciferase is firefly luciferase (FLuc) from *Photinus pyralis*, which oxidizes its substrate d-luciferin in the presence of oxygen, ATP, and Mg\(^{2+}\) to produce yellow light with an emission peak at 578 nm\(^9\). Unlike FLI which suffers from autofluorescence, there is no intrinsic bioluminescent signal in living subjects, allowing for high sensitivity and specificity. BLI is also advantageous for its relative simplicity and affordability, and the ability to perform simultaneous whole-body imaging of multiple (usually up to 5) small animals such as mice\(^9\). However, BLI is also limited by light scattering and absorption in tissue, making it difficult to detect sparse cell populations at greater tissue depths\(^9\). This is due to the presence of hemoglobin, which acts as a tissue chromophore and impairs transmission of wavelengths below 600 nm.

The challenges of deep tissue BLI has prompted the development of improved BLI reporters and substrates with greater sensitivity\(^1\). Recent efforts have focused on shifting bioluminescence into red wavelengths and the near-infrared region (700-900 nm), where there is reduced tissue absorption and scattering. In 2016, Kuchimaru et al. synthesized a novel luciferin analog called AkaLumine-hydrochloride (AkaLumine-HCl), which emits light peaking at 677 nm when paired with FLuc\(^1\). Iwano et al. further optimized this system by mutating FLuc to produce brighter emissions with AkaLumine-HCl, leading to the creation of the novel luciferase Akaluc\(^1\). In this study, *in vivo* imaging with Akaluc/AkaLumine-HCl produced 1000 times more intense light signal than FLuc/d-luciferin and remarkably enabled detection of single cells in the lungs of mice. Akaluc has since been applied for the sensitive tracking of breast cancer metastasis, glioma expansion, CAR-T cell therapy, and mesenchymal stromal cells in animal models\(^1\)-\(^6\).

1.4.4 Reporter Gene Strategies for Imaging Base Editing

In the past few years, a number of imaging reporters have been developed for the visualization of base-edited cells *in vitro*. In 2019, Martin et al. created three GFP reporters,
each containing a different missense mutation in the GFP sequence which disrupts protein folding and ablates fluorescence\textsuperscript{107}. These mutations can be corrected via C-to-T editing by CBE, allowing for restoration of GFP expression. Using this reporter, the authors were able to quantify CBE activity and compare the editing efficiencies of CBEs containing different deaminases.

In 2020, Katti et al. developed “Gene On” (GO) reporter systems to track BE activity in living cells using a variety of FLI and BLI reporter genes (Figure 1.8)\textsuperscript{108}. For the CBE system, they created GFP, mScarlet, and FLuc reporters containing start codons mutated from ATG to ACG, preventing translation of the mRNA transcript. Upon C-to-T editing at this site, the ATG start codon is restored and reporter gene expression is activated. For evaluating ABEs, a premature stop codon (TAG) was placed at the beginning of the GFP sequence, causing early termination of protein translation. A-to-G editing converts the stop codon into TGG, which codes for tryptophan and thus allows full protein expression. Using these GO systems, Katti et al. visualized and quantitatively compared the activity of various BE enzymes \textit{in vitro}.

\textbf{Figure 1.8: GFP “Gene On” (GO) reporters for visualization of base editing activity.}

These reporters, developed by Katti et al. in 2020, consist of mutated forms of GFP that prevent proper protein production until corrected by cytosine base editor (CBE) or adenine base editor (ABE). The reporters also contain a nuclear localization signal (NLS) that directs transport of GFP to the nucleus for easier visualization of edited cells.
Compared to the three GFP reporters developed by Martin et al., the GO reporters are more flexible as they can be easily adapted to other reporter genes by simply mutating start codons or inserting premature stop codons. However, there is currently no GO reporter for BLI of ABE activity. Furthermore, no in vivo imaging of base editing using these GO systems has been reported to date, and thus the sensitivity and specificity of these reporters in vivo have not been characterized.

1.5 Objectives and Hypotheses

The objectives of this thesis are to:

1. Test MCs as a novel delivery vector for ABE and compare them to plasmid delivery. We hypothesize that MC delivery will lead to enhanced base editing compared to plasmids.

2. Develop an activatable BLI reporter for the visualization of ABE activity. To do this, we build off the GO systems designed by Katti et al. to create a new activatable Akaluc reporter, called Akaluc\textsuperscript{GO}. We hypothesize that Akaluc\textsuperscript{GO} can enable highly sensitive and specific imaging of base-edited cells both in vitro and in vivo.
1.6 References


Chapter 2

2 Evaluation of DNA Minicircles for Delivery of Adenine Base Editors Using Activatable Reporter Imaging Systems

ABSTRACT:

Background: There are over 30,000 point mutations associated with genetic disease including sickle cell disease, muscular dystrophy, and numerous types of cancers. CRISPR base editors (BEs) are recently developed genome editing tools for the targeted conversion of a single base. Adenine base editors (ABEs) specifically convert an adenine (A) to a guanine (G) nucleotide and can correct ~48% of disease-causing point mutations. Currently, in vivo base editing is limited by poor delivery into tissues of interest. Our objective was to evaluate DNA minicircles (MCs) as a novel non-viral delivery vector for ABE delivery and to compare them to plasmid delivery. To enable non-invasive visualization of adenine base editing, we also develop activatable imaging reporter systems for the tracking of ABE activity using fluorescence and bioluminescence imaging (BLI).

Methods: We cloned plasmids expressing ABE (PP-ABE) or Cas9n as a control (PP-Cas9n). From these plasmids, minicircles (MC-ABE and MC-Cas9n) were produced. We then built activatable “Gene On” (GO) systems for two imaging reporter genes: green fluorescent protein (GFP) for in vitro fluorescence imaging and Akaluc for in vitro and in vivo BLI. These reporter genes were mutated to contain a premature TAG stop codon which will stop proper protein production, and should only produce imaging protein and imaging signal when TAG (stop codon) is edited to TGG (tryptophan codon) by an ABE. HEK293T or HeLa cells were transduced via lentivirus to express GFP\textsuperscript{GO} or Akaluc\textsuperscript{GO}, respectively. To measure reporter gene activation, cells were transfected with PP-Cas9n, MC-Cas9n, PP-ABE, or MC-ABE. After 48 h, GFP\textsuperscript{GO} cells were analyzed by flow cytometry and Akaluc\textsuperscript{GO} cells were analyzed by BLI.

Results: For the GFP\textsuperscript{GO} system, both PP-ABE and MC-ABE transfection resulted in significantly higher % GFP-expressing cells compared to Cas9n controls (p<0.0001). Additionally, MC-ABE led to higher GFP expression compared to PP-ABE (p<0.01).
Similarly, the Akaluc GO system showed significantly higher BLI signal from PP-ABE and MC-ABE transfection compared to Cas9n controls (p<0.0001), with MC-ABE resulting in higher signal than PP-ABE (p<0.0001).

**Discussion:** Here we show for the first time that minicircle delivery of ABE leads to higher levels of *in vitro* adenine base editing compared to plasmids, which is likely due to their smaller size. We have developed highly sensitive and specific activatable imaging reporter systems for the visualization of ABE activity. Future work will test these systems *in vivo* in preclinical cancer models with BLI. These systems can be used to evaluate new ABEs and delivery systems being developed for the treatment of genetic diseases.

### 2.1 Introduction

Point mutations are single base pair changes in DNA and are associated with thousands of genetic diseases including sickle cell disease, Duchenne muscular dystrophy, β-thalassemia, and numerous types of cancers\(^1\)–\(^3\). Point mutations are the largest class of human pathogenic mutations, comprising approximately 58% of human genetic variants associated with disease\(^4\)–\(^5\). Technologies capable of editing the genome through highly targeted modification of DNA sequences have long been sought, and may potentially offer a cure for genetic diseases caused by point mutations. These technologies can also be used to introduce point mutations to knock-out genes of interest via various mechanisms such as introduction of a premature stop codon. The development of the clustered regularly interspaced short palindromic repeat (CRISPR) system in 2012 revolutionized the field of genome editing because of its simplicity, ease-of-design, and cost-effectiveness\(^6\)–\(^7\). CRISPR generates double-stranded breaks in DNA which can be used to specifically modify a DNA sequence using a donor template, but can also lead to unintended insertions, deletions, and chromosome rearrangements\(^8\).

In 2016, Dr. David Liu and colleagues developed a new CRISPR-based genome editing technology called base editing which enables conversion of single nucleotide bases without creating double-stranded breaks, thus overcoming some of the associated safety concerns
of genome editing tools that rely on double-stranded breaks. Base editors (BEs) consist of three key components: 1) a single guide RNA (sgRNA) for programmable targeting of a genomic locus, 2) a mutated version of Cas9, called a Cas9 nickase (Cas9n), that creates a single-stranded nick in the DNA rather than a double-stranded break, and 3) a nucleobase deaminase that catalyzes the base conversion. Cytosine base editors (CBEs) induce cytosine-to-thymidine (C → T) conversions, while adenine base editors (ABEs) induce adenine-to-guanine (A → G) conversions. Since their development, BEs have been applied to correct point mutations associated with numerous diseases including sickle cell disease, Duchenne muscular dystrophy, tyrosinemia, inherited hearing loss and blindness, and cancer.

Although these studies have demonstrated the versatility and safety of base editing, one of the major outstanding challenges is efficient and safe delivery of BEs to tissues of interest. Non-viral delivery methods are of interest due to their versatility and improved safety profile over viral vectors such as adeno-associated viruses. However, most non-viral delivery vectors suffer from low efficiency of gene transfer when compared to viral vectors. Minicircles (MCs) are small circular DNA vectors produced from “parental plasmids” (PPs) and lack a bacterial backbone consisting primarily of an antibiotic resistance gene and origin of replication. The small size of MCs allows for higher transgene expression compared to plasmids, and their lack of bacterial components avoid potential immune responses or transfer of antibiotic genes to mammalian hosts. Thus, DNA minicircles may be a promising non-viral delivery vector for efficient and safe delivery of BEs.

With the development of new BEs and delivery methods, it is also critical to reliably evaluate the efficiency of these technologies in cells and animal models. Current methods include next-generation sequencing (NGS) of DNA isolated from cells or tissues and immunohistochemistry of tissues for analysis of protein expression. However, these methods provide bulk information about base editing activity and do not provide spatial or kinetic information at the single cell or whole-animal scale. In 2020, Katti et al. developed the “Gene On” (GO) reporter systems to track base editing activity in vitro. They created mutated versions of GFP, that they called GFPGO, that can be corrected and activated in the
presence of CBEs or ABEs and appropriate sgRNAs, allowing for quantification of the efficiencies of various BEs. However, in vivo imaging of BE activity with GFP is not ideal for highly sensitive detection of BE events due to high tissue autofluorescence and light scattering\textsuperscript{28,29}. Akaluc is a bioluminescence imaging (BLI) reporter gene that oxidizes its substrate, Akalumine-hydrochloride (Akalumine-HCl) to produce light peaking at 650 nm\textsuperscript{30}. Akaluc BLI has been shown to be highly effective at visualizing engineered cells, even single cells, in deep tissues of mice\textsuperscript{31,32}.

The focus of this study is on ABEs as they can correct a larger number of pathogenic mutations compared to CBEs\textsuperscript{4,5}. We developed and evaluated MCs as a novel non-viral delivery vector for ABE-based editing. Specifically, we compared the editing efficiency of MC-based ABE editing to that of their parental plasmid (PP) counterparts in cancer cells stably expressing a GFP\textsuperscript{GO} reporter or a novel Akaluc\textsuperscript{GO} reporter. We also demonstrated non-invasive imaging of ABE activity in a preclinical cancer model using Akaluc\textsuperscript{GO}.

### 2.2 Materials and Methods

**GO Reporter System Lentiviral Constructs**

A lentiviral transfer plasmid encoding GFP\textsuperscript{GO} (pRRL-GFPAdGO2-PGK-Neo) was a gift from Lukas Dow (Addgene plasmid #136899)\textsuperscript{27}. A [GFP-Akaluc]\textsuperscript{GO} lentiviral plasmid was generated by amplifying a T2A-Akaluc fragment from LV-pEF1\textalpha-tdT-Akaluc\textsuperscript{32} and inserting it after GFP\textsuperscript{GO} in pRRL-GFPAdGO2-PGK-Neo using In-Fusion HD Cloning (Takara Bio, CA, USA). A tdT-Akaluc\textsuperscript{GO} lentiviral plasmid with constitutive tdT and ABE-activatable Akaluc was generated by inserting an ATG to TAG mutation at the 265\textsuperscript{th} amino acid position in Akaluc within the LV-pEF1\textalpha-tdT-Akaluc plasmid using Q5 Site-Directed Mutagenesis Kit (New England BioLabs, MA, USA). The optimal position to insert the premature stop codon into Akaluc was determined using Benchling’s CRISPR guide design feature (benchling.com/crispr), where the sgRNA with the highest specificity and efficiency score was selected to allow an A → G conversion within the editing window for ABE\textsuperscript{8e} (positions 4-8). NEBaseChanger (nebasechanger.neb.com) was used to design primers for all Q5 Site-Directed Mutagenesis PCR reactions (Table 2.1).
Cell Culture and Engineering with GO Reporter Systems

HEK293T cells, HeLa cells, MDA-MB-231 cells, and OVCAR8 cells were obtained from American Type Culture Collection (ATCC, VA, USA). Cells were cultured in DMEM (HEK293T, HeLa, MDA-MB-231) or RPMI (OVCAR8) supplemented with 10% fetal bovine serum and 5% (v/v) antibiotic-antimycotic (ThermoFisher Scientific, MA, USA) at 37°C and 5% CO₂. All cells were routinely verified as free of mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza, NY, USA).

Third generation lentiviral packaging and envelope-expression plasmids pMDLg/pRRE, pRSV-Rev, and pMD2.G were gifts from Didier Trono (Addgene plasmids #12251, #12253, and #12259, respectively). To create lentivirus expressing GO reporters, HEK293T cells were seeded at ~80% confluence in a 10 cm dish. Twenty-four hours later, cells were transfected with 4 μg pMDLg, 1 μg pRSV-Rev, 1 μg pMD2.G, and 6 μg of one of the lentiviral transfer plasmids encoding a GO reporter system using Lipofectamine 3000 Transfection Reagent (ThermoFisher). Viral media was collected after 24 and 48 hours, centrifuged and filtered with a 0.45 μm filter, and stored at -80°C. Cells were engineered by plating them at ~70% confluence in a 6-well plate and incubating them the next day with a 1:4 to 1:10 dilution of GO reporter lentivirus as previously described, with 8 μg/mL polybrene. After 24 hours, medium was replaced with complete medium and after 48 hours cells were washed and plated in new dishes. Cells engineered to express GFP_GO or [GFP-Akaluc]GO were selected with 400 μg/mL Geneticin for 7-10 days. Cells engineered to express tdT-Akaluc_GO were sorted for constitutive tdT expression using a FACS Aria III fluorescence-activated cell sorter (BD Biosciences, CA, USA). Flow cytometry was performed on a FACSCanto and analyzed using FlowJo software (BD Biosciences).

Base Editor Plasmids and MCs

A plasmid encoding a highly efficient ABE (ABE8e(TadA-8e V106W)) was a gift from David Liu (Addgene plasmid #138495). A plasmid containing a sgRNA specific for GFP_GO with constitutive tdTomato (tdT) (sgRNA-tdT) was a gift from Lukas Dow (Addgene plasmid #136911). Protospacer sequences for all sgRNAs are listed in Table
2.2. As a Cas9n control plasmid for 2-plasmid ABE/sgRNA delivery experiments, we used a LV-Cas9nHifi-P2A-Puro plasmid which was previously constructed by Genscript (NJ, USA) using a high-fidelity Cas9n gene insert and a pLenti-Cas9-P2A-Puro backbone (Addgene plasmids #136902 and #110837, respectively)\(^{27,35}\). To generate an “All-in-one” PP expressing both ABE8e and the GFP\(^{GO}\) sgRNA, the vector PP-pSurvivin-SEAP\(^{23}\) was used. The pSurvivin-SEAP expression cassette was replaced with both the pCMV-ABE fragment from ABE8e(TadA-8e V106W) and the pU6-sgRNA fragment from sgRNA-tdT using In-Fusion HD Cloning. This vector is referred to as PP-ABE\(^{GFP}\). A negative control plasmid PP-Cas9n\(^{GFP}\) was generated by deleting the TadA adenine deaminase domain and 32-amino acid linker\(^{34}\) from PP-ABE\(^{GFP}\) using Q5 Site-Directed Mutagenesis. To generate all-in-one PP-ABE\(^{Akaluc}\) and PP-Cas9n\(^{Akaluc}\) constructs to target tdT-Akaluc\(^{GO}\), the sgRNA protospacer sequences were substituted using Q5 Site-Directed Mutagenesis.

Table 2.1: Primers used for Q5 Site-Directed Mutagenesis PCR reactions.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
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<tbody>
<tr>
<td>tdT-Akaluc(^{GO})</td>
<td>GGTCGTGCTCTAGTACCGCTTCG</td>
<td>CGAAAGCCGCAGATCAAG</td>
</tr>
<tr>
<td>PP-Cas9n(^{GFP})</td>
<td>GACAAGAAGTACAGCATC</td>
<td>GACTTTCCGCTTCTTCTTTG</td>
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<tr>
<td>PP-ABE(^{Akaluc})</td>
<td>CGCTTCGAGGGTTTAAGAGCTAT GCTGGAAC</td>
<td>GTACTAGAGCGGTTGTCTCTTGA CTTTCCAC</td>
</tr>
<tr>
<td>PP-Cas9n(^{Akaluc})</td>
<td>CGCTTCGAGGGTTTAAGAGCTAT GCTGGAAC</td>
<td>GTACTAGAGCGGTTGTCTCTTGA CTTTCCAC</td>
</tr>
</tbody>
</table>

Table 2.2: Protospacer sequences for sgRNAs.

<table>
<thead>
<tr>
<th>Target</th>
<th>Protospacer (5’-3’)</th>
<th>PAM</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP(^{GO})</td>
<td>GTTTAGAGTGAGCCATGTA</td>
<td>TGG</td>
</tr>
<tr>
<td>[GFP-Akaluc](^{GO})</td>
<td>GTTTAGAGTGAGCCATGTA</td>
<td>TGG</td>
</tr>
<tr>
<td>tdT-Akaluc(^{GO})</td>
<td>GCTCTAGTACCGCTCGAGG</td>
<td>AGG</td>
</tr>
</tbody>
</table>

Minicircles (MCs) expressing ABE (MC-ABE\(^{GFP}\) and MC-ABE\(^{Akaluc}\)) and Cas9n (MC-Cas9n\(^{GFP}\) and MC-Cas9n\(^{Akaluc}\)) were generated from the respective PP constructs using the protocol outlined in Kay et al.\(^{36}\) and the MC-Easy Minicircle DNA Production Kit (System Biosciences, CA, USA). Briefly, PPs were transformed into ZYCY10P3S2T E. coli, and colonies were selected and cultured in terrific broth (TB) overnight at 30°C. Activity of the
ΦC31 integrase was then induced via addition of an equal volume of lysogeny broth (LB) containing 0.001% (v/v) L-arabinose and 4 mL 1N NaOH, and incubation at 30°C for 3 hours followed by 37°C for 1 hour. Both MCs and PPs were purified from E. coli using the Endotoxin-free Maxi Kit (QIAGEN, ON, Canada). The size and purity of PPs and MCs were verified by digestion with EcoRV and agarose gel electrophoresis.

**Evaluation of 2-Plasmid ABE/sgRNA Delivery using GFP<sup>GO</sup>**

To validate the GFP<sup>GO</sup> reporter system previously developed by Katti et al.<sup>27</sup>, HEK293T cells engineered to express GFP<sup>GO</sup> (referred to as 293T-GFP<sup>GO</sup>) were seeded at 7x10⁴ cells/well in 500 µL media in a 24-well plate. The next day, cells were transfected with sgRNA-tdT plasmid (167 ng) and either ABE8e(TadA-8e V106W) plasmid or LV-Cas9nHiFi-P2A-Puro plasmid (333 ng), after complexation with 1 µL jetPEI transfection agent (Polyplus Transfection, PA, USA). After 48 hours, tdT and GFP expression were visualized using an EVOS FL Auto Imaging System (ThermoFisher Scientific) and quantified using flow cytometry.

To visualize the kinetics of transfection and base editing, 293T-GFP<sup>GO</sup> cells transfected with sgRNA-tdT and ABE8e(TadA-8e V106W) plasmids were imaged using the CytoSMART Lux3 FL incubator microscope (CytoSMART Technologies BV, AZ Eindhoven, Netherlands). Images were taken immediately following transfection and every 10 minutes for up to 70 hours. Red and green object counts were obtained from the CytoSMART software.

**Comparison of All-in-one PP and MCs using GFP<sup>GO</sup>**

To compare the efficiency of ABE delivery using PPs and MCs, 293T-GFP<sup>GO</sup> cells were seeded at 7x10⁴ cells/well in 500 µL media in a 24-well plate. The next day, cells were transfected with 500 ng PP-Cas9n<sup>GFP</sup>, MC-Cas9n<sup>GFP</sup>, PP-ABE<sup>GFP</sup>, or MC-ABE<sup>GFP</sup> after complexation with 1 µL jetPEI transfection agent. After 48 hours, GFP expression were visualized using an EVOS FL Auto Imaging System and quantified using flow cytometry.

**Characterization of Akaluc<sup>GO</sup> Systems**
First, the [GFP-Akaluc]GO reporter system was tested by seeding engineered and naïve HeLa cells at $7 \times 10^4$ cells/well in 500 µL media in a 24-well plate. The next day, HeLa-[GFP-Akaluc]GO cells were transfected with 500 ng PP-Cas9nGFP or PP-ABE GFP after complexation with 1 µL jetPEI transfection agent. After 48 hours, GFP expression was visualized using an EVOS FL Auto Imaging System. Akaluc BLI signal was visualized by adding 5 µL 5 mM Akalumine-HCl in 0.9% saline to each well and images were acquired within 5 min on auto exposure using an IVIS Lumina XRMS In Vivo Imaging System (PerkinElmer, MA, USA). Regions of interest (ROIs) were manually drawn around wells using LivingImage software to measure bioluminescent average radiance (p/s/cm²/sr).

The second AkalucGO reporter design, tdT-AkalucGO, was tested next. HeLa cells, MDA-MB-231 cells, and OVCAR8 cells were engineered to express tdT-AkalucGO (referred to as HeLa-AkalucGO, OVCAR8-AkalucGO, and 231-AkalucGO, respectively). Cells were seeded at $7 \times 10^4$ cells/well in 500 µL media in a 24-well plate. The next day, cells were transfected with 500 ng MC-Cas9nAkaluc, PP-ABE Akaluc, or MC-ABE Akaluc after complexation with 1 µL jetPEI transfection agent (for HeLa cells). MDA-MB-231 and OVCAR8 cells were transfected using 1.5 µL Lipofectamine 3000 and 1 µL P3000 transfection agent. After 48 hours, Akaluc BLI signal was quantified using the same procedure as described above.

**Genomic DNA Analysis**

To further analyze ABE editing, genomic DNA was isolated from cells 48 hours after transfection using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, CA, USA). The GFPGO and AkalucGO target sites were amplified by PCR using primers that bind approximately 200 base pairs upstream and downstream of the target A (Table 2.3). The PCR products were then purified using the QIAquick PCR Purification Kit (Qiagen, CA, USA), and analyzed by Sanger sequencing. Base Editing Analysis Tool (BEAT, [http://www.hanlab.cc/beat](http://www.hanlab.cc/beat))37 was used to quantify base editing from Sanger sequencing data.
Table 2.3: Primers used for PCR amplification of target genomic sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>Forward Primer (5’-3’)</th>
<th>Reverse Primer (5’-3’)</th>
</tr>
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<tbody>
<tr>
<td>GFP&lt;sup&gt;GO&lt;/sup&gt;</td>
<td>CCCAGATATGCGCCAACCCT</td>
<td>GTAGGTCAGGGGTGGTCACG</td>
</tr>
<tr>
<td>tdt-Akaluc&lt;sup&gt;GO&lt;/sup&gt;</td>
<td>CGCCCTGATCATGAACAGT</td>
<td>TGGTAGGCGGAAGCGTTTG</td>
</tr>
</tbody>
</table>

**In Vivo Characterization of tdt-Akaluc<sup>GO</sup> in HeLa Tumors**

Animals were cared for in accordance with the standards of the Canadian Council on Animal Care, and under an approved protocol of the University of Western Ontario’s Council on Animal Care (2020-025). Six to eight-week-old female nu/nu athymic nude mice were obtained from Charles River Laboratories (Willington, MA, USA). For the subcutaneous tumor model, 4x10⁶ HeLa-Akaluc<sup>GO</sup> cells in 50 µL PBS mixed with 50 µL Matrigel were injected into the hind flank. Tumor volume was assessed weekly using a caliper and was calculated using the following formula: tumor volume = 0.5*(length × width²).

When subcutaneous tumors reached ~100mm³, mice received intratumoral injections of MC-ABE<sub>Akaluc</sub> complexed with *in vivo* jetPEI (Polyplus Transfection). Complexes were prepared by diluting 50 µg MC and 8 µL jetPEI each in 25 µL of 5% glucose to achieve an N/P ratio of 8 (N/P ratio is the number of nitrogen residues in *in vivo*-jetPEI per nucleic acid phosphate). The dilutions were then mixed and incubated for 20 min at room temperature. The complexes (50 µl) were injected intratumorally in multiple quadrants of the tumor over a period of ~60 seconds. BLI was performed on days -1, 1, 2, 4, and 7 following MC-ABE<sub>Akaluc</sub> delivery by injecting mice intraperitoneally with 100 µL 5 mM Akalumine-HCl and acquiring images over ~20 min on auto exposure using an IVIS Lumina XRMS In Vivo Imaging System. ROIs were manually drawn around tumor borders using LivingImage software to measure bioluminescent average radiance (p/s/cm²/sr).

**In Vivo Transfection Analysis**

To assess the transfection efficiency of *in vivo* jetPEI, intratumoral, intraperitoneal, and intravenous delivery routes were tested in female nude mice with a plasmid constitutively
expressing Akaluc (pEF1α-tdT-Akaluc). For intratumoral delivery, HeLa naïve tumors ~100mm³ in size were intratumorally injected with 25 µg pEF1α-tdT-Akaluc and 4 µL jetPEI (N/P = 8), complexed as described above. For intraperitoneal delivery, 100 µg pEF1α-tdT-Akaluc and 16 µL jetPEI (N/P = 8) were each diluted in 200 µL of 5% glucose, and 200 µL of the complex was injected into the lower left and right peritoneum each for a total of 400 µL. For intravenous delivery, 40 µg pEF1α-tdT-Akaluc and 6.4 µL jetPEI (N/P = 8) were each diluted in 100 µL of 5% glucose, and 200 µL of the complex was injected into the tail vein. Akaluc expression was measured by BLI for up to 48 hours following injection. ROIs were manually drawn around tumor borders (for subcutaneous delivery), the peritoneum (for intraperitoneal delivery), and lungs (for intravenous delivery) using LivingImage software to measure bioluminescent average radiance (p/s/cm²/sr).

In Vivo Electroporation

Six to eight-week-old female NOD scid-gamma (NSG) mice were obtained from Jackson Laboratories (Farmington, CT, USA). Mice received subcutaneous injections of 3x10⁶ OVCAR8 naïve cells (left flank) and 3x10⁶ OVCAR8-AkalucGO cells (right flank) in 50 µL PBS mixed with 50 µL Matrigel. Once tumors reached ~100mm³, electroporation was performed as previously described38. Mice received intratumoral injections of 25 µg DNA in sterile water (pEF1α-tdT-Akaluc plasmid into OVCAR8 naïve tumors and MC-ABE_Akaluc into OVCAR8-AkalucGO tumors). Electroporation was then performed immediately by delivering two series of four 5-ms square-wave pulses of 300 V in perpendicular directions at a frequency of 1 Hz. Pulses were delivered with a NEPA21 Electroporator (Sonidel Limited, Dublin, Ireland) and CUY650P5 tweezer electrodes (Bulldog Bio, NH, USA). The electrodes were covered with Aquasonic CLEAR ultrasound gel (Parker Laboratories, NJ, USA) to improve contact of the electrodes with the skin and control tissue impedance.

Statistics

Statistical tests were performed using Graphpad Prism software (Version 8.1.2 for Mac OS X, GraphPad Software Inc., CA, USA, www.graphpad.com). Unpaired two-tailed t test
was performed to compare GFP\textsuperscript{GO} and Akaluc\textsuperscript{GO} activation between two groups. Ordinary one-way ANOVA and Tukey’s multiple comparison post hoc test were used to compare GFP\textsuperscript{GO} and Akaluc\textsuperscript{GO} activation from PP and MC delivery of ABE or Cas9n. Repeated measures one-way ANOVA and Tukey’s multiple comparison post hoc test was used to compare Akaluc signal across time points for the electroporation experiment. A \( p \)-value less than 0.05 was considered statistically significant.

2.3 Results

Evaluation of 2-Plasmid ABE/sgRNA Delivery using GFP\textsuperscript{GO}

We first validated the ABE GFP\textsuperscript{GO} reporter system previously developed by Katti et al.\textsuperscript{27}. This reporter consists of GFP containing a premature TAG stop codon, which prevents full translation of protein (Figure 2.1A). Base editing via ABE corrects the TAG to TGG (tryptophan), allowing for full GFP expression. HEK293T (human embryonic kidney) cells were engineered to express GFP\textsuperscript{GO} (referred to as 293T-GFP\textsuperscript{GO}), then transfected with plasmid encoding sgRNA and tdT (sgRNA-tdT) and a separate plasmid encoding either ABE (which consists of Cas9\textsubscript{n} and the fused adenosine deaminase) or Cas9\textsubscript{n} as a negative control (Figure 2.1B). After 48 h, tdT expression was observed from both groups, indicating successful transfection of sgRNA-encoding plasmid (Figure 2.1C). Strong GFP expression was observed in cells transfected with ABE, while cells transfected with Cas9\textsubscript{n} showed minimal GFP expression. Analysis using flow cytometry showed that a significantly higher percentage of tdT+ cells were also GFP+ when transfected with ABE compared to Cas9\textsubscript{n} (Figure 2.1D; \( p < 0.001 \)). Sanger sequencing of the GFP\textsuperscript{GO} target site in genomic DNA extracted from cells confirmed the conversion of A to G nucleotides after transfection with ABE but not Cas9\textsubscript{n} (Figure 2.1E). It was also interesting to note a bystander A to G edit at the second A within the editing window. Live-cell analysis of cells transfected with sgRNA-tdT and ABE plasmids using a CytoSMART Lux3 FL incubator microscope showed tdT fluorescence beginning \( \sim 9 \) h after transfection and GFP fluorescence beginning \( \sim 12 \) h after transfection (Figure 2.1F). Red and green object counts obtained from CytoSMART software increased over time and reached a plateau by 70 h.
Figure 2.1: In vitro validation of GFP\textsuperscript{GO} reporter for ABE activity. (A) Schematic of the GFP\textsuperscript{GO} reporter. Insertion of a premature stop codon (TAG) into the reporter prevents expression of full-length functional GFP. ABE with a sgRNA targeting the stop codon performs an A to G conversion which restores GFP expression. The nuclear localization signal (NLS) enables targeting of GFP to the nucleus for easier visualization of GFP+ cells. (B) 2-plasmid system for delivery of sgRNA along with tdTomato (sgRNA-tdT), and ABE or Cas9n as a negative control. (C) Fluorescence microscopy of cells 48 h after transfection with sgRNA and ABE or Cas9n. (D) Flow cytometry analysis of cells 48 h after transfection (n = 3). Data are presented as mean ± SD (**p < 0.001). (E) Sanger
sequencing of the GFP<sup>GO</sup> target site in genomic DNA extracted from cells 48 h after transfection. (F) Live-cell analysis of tdT and GFP expression over time following transfection with sgRNA-tdT plasmid and ABE plasmid.

Comparison of All-in-one PP and MCs using GFP<sup>GO</sup>

We next constructed all-in-one parental plasmids (PPs) to simplify delivery of sgRNA and ABE or Cas9n (Figure 2.2A). Propagation of these PPs in ZYCY10P3S2T *E. coli* and addition of arabinose induces the ΦC31 integrase which recombines the attB and attP sites in the PP backbone. This leads to the production of bacterial component-free MCs encoding sgRNA and either ABE or Cas9n. Digestion of PPs and MCs with EcoRV confirmed the production of MCs of the expected size with minimal PP contamination (Figure 2.2B).

We next used the GFP<sup>GO</sup> reporter to compare the efficiency of ABE delivery using the PPs and MCs. 293T-GFP<sup>GO</sup> cells were transfected with 500 ng PP-Cas9n<sup>GFP</sup>, MC-Cas9n<sup>GFP</sup>, PP-ABE<sup>GFP</sup>, or MC-ABE<sup>GFP</sup>. After 48 h, GFP expression was visible only in cells transfected with PP-ABE<sup>GFP</sup> or MC-ABE<sup>GFP</sup>, but not the Cas9n controls (Figure 2.2C). Flow cytometry analysis revealed that both PP-ABE<sup>GFP</sup> and MC-ABE<sup>GFP</sup> transfection resulted in significantly higher % GFP+ cells compared to Cas9n controls (p < 0.0001). Additionally, MC-ABE<sup>GFP</sup> led to higher GFP expression compared to PP-ABE<sup>GFP</sup> (p < 0.01).
Figure 2.2: Comparison of PP and MC-based delivery and editing of ABE using GFP<sup>GO</sup> reporter. (A) All-in-one delivery system combines sgRNA and ABE or Cas9n delivery into a single DNA vector. Parental plasmids (PPs) contain attB and attP recombination sites, which are joined together after arabinose induction of the ΦC31 integrase, leading to production of minicircles (MCs) free of bacterial DNA components. (B) Agarose gel electrophoresis following EcoRV restriction digest confirming production of PPs and MCs expressing Cas9n or ABE. (C) Fluorescence microscopy of 293T-GFP<sup>GO</sup> cells 48 h after transfection with PP-Cas9n<sup>GFP</sup>, MC-Cas9n<sup>GFP</sup>, PP-ABE<sup>GFP</sup>, or MC-ABE<sup>GFP</sup>. (D) Flow cytometry analysis of cells 48 h after transfection (n = 3). Data are presented as mean ± SD (**p < 0.01, ****p < 0.0001).

In Vitro Characterization of Akaluc<sup>GO</sup> Systems

To expand the GO system to allow <em>in vivo</em> BLI of adenine base editing, we chose to incorporate Akaluc, a highly sensitive luciferase with red-shifted emission, into the GFP<sup>GO</sup> system. To do this we first cloned Akaluc downstream of GFP<sup>GO</sup>, separated by a T2A self-cleaving peptide sequence, and called this construct [GFP-Akaluc]<sup>GO</sup> (Figure 2.3A).
Because this Akaluc sequence lacks a start codon and depends on the translation start site of GFP for expression, we hypothesized that Akaluc expression would only be activated upon base editing of GFP. To test this system, we transfected HeLa (cervical cancer) cells that were engineered to express [GFP-Akaluc]G0 with 500 ng PP-Cas9nGFP or PP-ABEGFP. After 48 h, GFP expression was only visible in cells transfected with PP-ABEGFP (Figure 2.3B). However, BLI of these cells revealed an unexpectedly high level of background Akaluc expression in the Cas9n control, although PP-ABEGFP did show significantly higher signal (p < 0.001, Figure 2.3C/D). Cells transfected with PP-Cas9nGFP also showed lower background signal than non-transfected engineered cells, presumably due to some toxicity from the transfection agent. We hypothesized that this background signal was due to translation initiation of Akaluc at alternative in-frame start codons within its gene sequence, which resulted in the production of truncated but functional Akaluc protein in the absence of ABE.
Figure 2.3: Characterization of \([\text{GFP-Akaluc}]^{\text{GO}}\) reporter for fluorescence and bioluminescence imaging (BLI) of ABE activity. (A) Schematic of the \([\text{GFP-Akaluc}]^{\text{GO}}\) reporter. Adenine base editing corrects the premature stop codon and restores translation of both GFP and Akaluc. (B) Fluorescence microscopy and (C) BLI of HeLa-[\(\text{GFP-Akaluc}]^{\text{GO}}\) cells 48 h after transfection with PP-Cas9\(^n\)GFP or PP-ABE\(^GFP\). Non-transfected [\(\text{GFP-Akaluc}]^{\text{GO}}\) cells did not receive any DNA. (D) Quantification of BLI signal (n = 3). Data are presented as mean ± SD (**p < 0.001).

We next aimed to reduce this background signal by re-engineering the Akaluc\(^{GO}\) system. We chose an in-house plasmid, LV-pEF1\(\alpha\)-tdT-Akaluc, and inserted a premature stop codon into the middle of the Akaluc sequence to turn off Akaluc expression while maintaining constitutive tdT expression (Figure 2.4A). We reasoned this premature stop
codon would prevent production of any functional truncated Akaluc protein. HeLa cells engineered to express this construct (HeLa-tdT-Akaluc GO cells) were FACS-sorted for various levels of tdT expression (low, medium, high) to investigate the effect of the relative number of Akaluc GO copies per cell on the system (Figure 2.4B). Cells were transfected with 500 ng MC-Cas9 nAkaluc, PP-ABE Akaluc, or MC-ABE Akaluc. After 48 h, BLI showed minimal background Akaluc signal in all groups transfected with MC-Cas9n except the high sort group (Figure 2.4C/D). We observed significant increases in Akaluc signal in all groups when comparing PP-ABE Akaluc to MC-Cas9nAkaluc, with higher tdT levels resulting in higher BLI signal. The medium sort and high sort groups showed significant increases in Akaluc signal from MC-ABE Akaluc over PP-ABE Akaluc (p < 0.0001 and p < 0.05, respectively), while the unsorted and low sort groups did not show significant differences. We also calculated the ratio of signal from PP-ABE Akaluc or MC-ABE Akaluc over the signal from MC-Cas9n as a measure of how well the system activates above background levels for each sort group (Figure 2.4E). The medium sort group showed the highest ABE/Cas9n ratio for both PP-ABE Akaluc and MC-ABE Akaluc, and therefore we chose this group to continue with further evaluation.
Figure 2.4: Characterization of tdT-Akaluc\(_{\text{GO}}\) reporter for BLI of ABE activity. (A) Schematic of the Akaluc\(_{\text{GO}}\) reporter. Adenine base editing corrects the premature stop codon and restores translation of full functional Akaluc protein. (B) Flow cytometry analysis of tdTomato (tdT) expression following lentiviral transduction of HeLa cells with Akaluc\(_{\text{GO}}\) and sorting for low, medium, and high tdT expression. (C) BLI of HeLa-tdT-Akaluc\(_{\text{GO}}\) cells 48 h after transfection with MC-Cas9\(_n\)^{Akaluc}, PP-ABE\(^{Akaluc}\), or MC-ABE\(^{Akaluc}\). (D) Quantification of BLI signal from HeLa-tdT-Akaluc\(_{\text{GO}}\) cells (n = 3). (E) Activation levels as determined by the signal from cells transfected with ABE divided by that of Cas9n (n = 3). (F) BLI of Akaluc\(_{\text{GO}}\) activation in OVCAR8 and MDA-MB-231 cells (medium tdT expression). (G) Quantification of BLI signal from OVCAR8 and MDA-MB-231 Akaluc\(_{\text{GO}}\) cells (n = 3). (H) Percent editing of the target site in HeLa, OVCAR8 and MDA-MB-231 Akaluc\(_{\text{GO}}\) cells (medium tdT expression) as quantified by BEAT analysis of Sanger sequencing (n = 3). Data are presented as mean ± SD (“ns” non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

To test the generalizability of the tdT-Akaluc\(_{\text{GO}}\) reporter to other cell lines, we also engineered OVCAR8 (ovarian cancer) and MDA-MB-231 (breast cancer) cells to express tdT-Akaluc\(_{\text{GO}}\). Cells were sorted for a medium level of tdT expression using the same fluorescence intensity gate used to sort the HeLa cells. Forty-eight h after transfection, cells showed minimal background Akaluc signal in the MC-Cas9n\(^{Akaluc}\) groups (Figure 2.4F/G). MC-ABE\(^{Akaluc}\) transfection resulted in significantly higher Akaluc signal compared to PP-ABE\(^{Akaluc}\) for both OVCAR8 and MDA-MB-231 cells (p < 0.01 and p < 0.05, respectively).

Since BLI does not give a measure of the percentage of cells expressing Akaluc as does the GFP\(_{\text{GO}}\) system, we performed Sanger sequencing analysis on edited cells to obtain an estimate of the percent editing efficiency. Genomic DNA was extracted from HeLa, OVCAR8, and 231-tdT-Akaluc\(_{\text{GO}}\) cells (all medium tdT sort) 48 h following transfection. The target site was PCR-amplified and subject to Sanger sequencing. Analysis was performed using Base Editing Analysis Tool (BEAT), a computer program written in Python that analyzes and quantifies base-editing events from Sanger sequencing data in a
batch manner\textsuperscript{37}. It does this by calculating average background noise for each base from trace data (e.g. G peak value under A, C, or T peaks), subtracting average noise for each base at each position, then calculating the percentage of each peak value over sum of each base at that position. The average A to G conversion for HeLa cells was 45.6±3.6% for PP-ABE\textsuperscript{Akaluc} and 60.6±7.0% for MC-ABE\textsuperscript{Akaluc} (p < 0.05, Fig. 2.4H). However, for OVCAR8 and MDA-MB-231 cells, A to G conversion was less than 3% and no significant differences were observed between editing with PP-ABE\textsuperscript{Akaluc} and MC-ABE\textsuperscript{Akaluc}.

\textit{In Vivo Characterization of tdT-Akaluc\textsuperscript{GO} in HeLa Cells}

We next aimed to demonstrate proof of concept \textit{in vivo} visualization of adenine base editing using tdT-Akaluc\textsuperscript{GO} in a preclinical tumor model. In a pilot experiment, female nude mice were implanted with subcutaneous HeLa-tdT-Akaluc\textsuperscript{GO} (medium sort) tumors (n = 4). Once tumors reached ~100mm\textsuperscript{3}, mice received intratumoral injections of 50 µg MC-ABE\textsuperscript{Akaluc} complexed with \textit{in vivo} jetPEI transfection agent. \textit{In vivo} BLI was performed on days -1, 1, 2, 4, and 7 following MC-ABE\textsuperscript{Akaluc} delivery. Unfortunately, we could not detect Akaluc signal in these tumors at any time point (data not shown). We did note some DNA precipitation when complexing the 50 µg MC-ABE\textsuperscript{Akaluc}, which may have been due to the large amount of DNA in a small volume, and so we performed a booster injection of 25 µg MC-ABE\textsuperscript{Akaluc} in the same volume (which did not result in any visible precipitation) on day 8. However, this injection also did not result in any Akaluc signal. We hypothesized that inefficient delivery of MC-ABE\textsuperscript{Akaluc} to tumor cells was a hindering our ability to visualize activation of Akaluc\textsuperscript{GO}, and so we next aimed to analyze the efficiency of \textit{in vivo} jetPEI transfection of tissues.

\textit{In Vivo Transfection Analysis}

To determine the best route of DNA delivery to tissues using \textit{in vivo} jetPEI transfection agent, we tested intratumoral (n = 3), intraperitoneal (n = 2), and intravenous (n = 2) delivery routes with a plasmid constitutively expressing Akaluc (pEF1\textalpha-tdT-Akaluc). All \textit{in vivo} jetPEI transfections were performed with an N/P ratio of 8 using recommended conditions as described in the manufacturer’s protocol. For intratumoral delivery, subcutaneous HeLa naïve tumors ~100mm\textsuperscript{3} in size were intratumorally injected with 25
µg of pEF1α-tdT-Akaluc. A slight increase in BLI signal was observed in tumors at 24 and 48 h post transfection (Figure 2.5A/B). Intraperitoneal delivery of 100 µg pEF1α-tdT-Akaluc in healthy mice (i.e., without tumors) did not yield any detectable signal at 24 and 48 h post transfection. For intravenous delivery, 40 µg pEF1α-tdT-Akaluc was injected into the tail vein in healthy mice. At 24 and 48 h post injection, strong Akaluc signal was visible in the lungs.

Figure 2.5: In vivo jetPEI delivery of plasmid DNA to tissues via intratumoral (IT, n = 3), intraperitoneal (IP, n = 2), and intravenous (IV, n = 2) routes. A plasmid constitutively expressing Akaluc (pEF1α-tdT-Akaluc) was used to track transgene delivery. (A) Representative BLI images of mice following Akaluc plasmid delivery. (B) Quantification of BLI signal. Data are presented as mean ± SD.

In Vivo Electroporation for Intratumoral DNA Delivery

As an alternative to in vivo jetPEI transfection, we also explored in vivo electroporation for DNA delivery into tumors. This method delivers electric pulses to create temporary pores in cell membranes, facilitating the transfer of foreign DNA into cells. For this experiment we switched to subcutaneous OVCAR8 tumors in more immunocompromised NSG mice,
as we previously experienced difficulty establishing HeLa tumors in some of our nude mice. Female NSG mice (n = 5) were implanted with OVCAR8 naïve cells on the left flank and OVCAR8-Akaluc<sup>GO</sup> cells on the right flank (Figure 2.6A). Once tumors reached ~100 mm<sup>3</sup>, OVCAR8 naïve tumors were electroporated with 25 µg pEF1α-tdT-Akaluc plasmid and OVCAR8-Akaluc<sup>GO</sup> tumors were electroporated with 25 µg MC-ABE<sup>Akaluc</sup>. Akaluc signal was observed in 3 of 5 naïve tumors 24 h and 48 h after electroporation, though not significantly different from pre-injection signal (Figure 2.6B). For the OVCAR8-Akaluc<sup>GO</sup> tumors, a significant increase in Akaluc signal was observed at 24 h (p < 0.01) but not at 48 h (Figure 2.6C). It is also important to note that some background Akaluc signal was seen in these tumors pre-injection of MC-ABE<sup>Akaluc</sup>. 
Figure 2.6: In vivo electroporation of plasmid or MC DNA to subcutaneous tumors. 

(A) Schematic of mouse model and electroporation procedure. (B) BLI images and signal quantification of left flank tumors of mice 1-5 (M1-5). (C) BLI images and signal
quantification of right flank tumors of M1-5. “ns” non-significant, **p < 0.01 when compared to pre-injection signal.

2.4 Discussion

Base editing has shown great promise as a versatile genome editing tool with the potential to treat thousands of genetic disorders. However, translation of this technology into the clinic requires methods for safe and efficient delivery of base editors into tissues of interest. DNA MCs may be an appealing alternative to currently used delivery vectors including plasmids, mRNA, and AAVs due to their high transgene expression, low immunogenicity, and easy production. This is the first study demonstrating the use of MCs for the delivery of ABE to cells.

To assess the efficacy of minicircles in comparison to plasmids, we used the GFP Gene On (GO) imaging reporter system developed by Katti et al.27. We first validated their system by using two separate plasmids to deliver the ABE and sgRNA-tdT into HEK293T cells engineered to stably express the GFPGO reporter. We observed successful editing with approximately 42% of tdT+ cells also expressing GFP. This is comparable to the ~60% reported by Katti et al., though some notable differences are that they tested MDA-MB-231 cells engineered to stably express ABE and sgRNA via lentivirus transduction, as well as a different version of ABE. In this experiment, we also detected ~3% GFP+ cells in the Cas9n control, which we believe was due to spillover of strong tdT signal into the green channel during flow cytometry, even with the presence of single colour compensation controls. This was confirmed by performing flow cytometry on non-transfected 293T-GFPGO cells, where we observed minimal (<1%) background GFP expression. In our time-course analysis of cells transfected with sgRNA-tdT and ABE plasmids, we observed tdT fluorescence beginning ~9 h after transfection and GFP fluorescence beginning ~12 h after transfection, indicating the time course between delivery of ABE vectors, expression of ABE and activation of GFP fluorescence. Interestingly, our analysis showed the green object count surpassing the red object count at 32 h. This may be due to proliferation of edited GFP+ cells while sgRNA-tdT plasmid is diluted with cell division.
We next designed and produced all-in-one PPs and MCs encoding both the ABE and sgRNA. Using the GFP\textsuperscript{GO} reporter, we observed a significant improvement in editing using MCs over PPs. These results agree with previous studies demonstrating higher transgene expression using MCs compared to plasmids both \textit{in vitro} and \textit{in vivo}\textsuperscript{23,24,39,40}. The improved transgene expression is likely due to the smaller size of our MCs (~61.5% the size of PPs). It has been previously shown that there is an inverse relationship between the size of the DNA vector and levels of transgene expression\textsuperscript{41,42}. \textit{In vitro} transfection of DNA vectors is a multi-step process including: 1) endocytosis of the DNA complex through the cell membrane, 2) diffusion of DNA into the cytoplasm, and 3) entry of DNA into the nucleus. Larger DNA vectors have higher diffusion coefficients due to increased molecular weight, limiting their transport into the nucleus and thus resulting in lower transgene expression\textsuperscript{43}. Additionally, because equal masses of MC and PP were transfected, cells theoretically received ~1.6 times more copies of the ABE transgene in the MC transfection compared to the PP transfection, which may also explain the increased editing with MC delivery.

Having demonstrated the benefit of MC delivery for editing of GFP\textsuperscript{GO}, we next aimed to develop an \textit{in vivo} compatible reporter system for non-invasive tracking of ABE activity by incorporating the Akaluc BLI reporter. For our [GFP-Akaluc]\textsuperscript{GO} design, we observed good activation of GFP upon ABE transfection but saw high background Akaluc signal in the absence of ABE. This was unexpected since both GFP and Akaluc translation should be initiated at the 5’ end of GFP, with Akaluc being cleaved via the T2A peptide. Thus, a premature stop codon in GFP should theoretically also prevent translation of Akaluc. We reasoned that the background Akaluc expression could be explained by translation initiation at other in-frame start codons downstream of the proper start codon, allowing for production of functional Akaluc protein. It has previously been shown in eukaryotes that if the first start codon is in an unfavorable sequence context (i.e., the nucleotides surrounding the start codon are not optimal), this codon may be “skipped” resulting in translation initiation at downstream start codons\textsuperscript{44}. Another mechanism that could explain alternative translation is a process known as translation re-initiation. If a start codon is followed shortly by a stop codon, the ribosome may remain attached to the mRNA and continue scanning for downstream start codons\textsuperscript{45–47}. Methods such as ribosome profiling,
which involves deep sequencing of ribosome-protected mRNA fragments, may provide further insight into the leakiness of our [GFP-Akaluc]GO reporter\textsuperscript{48,49}.

To minimize the possibility of leakiness resulting from alternative translation initiation, we built the tdT-Akaluc\textsuperscript{GO} reporter by inserting a premature stop codon into Akaluc, approximately halfway through the Akaluc gene. Sorting for various levels of tdT expression in engineered HeLa cells allowed us to evaluate the implications of engineering cells with different levels of the reporter. As shown by the BLI signal of cells transfected with the Cas9 control, background Akaluc signal was observed in cells sorted for a high level of tdT expression, while the unsorted, low sort, and medium sort cells showed minimal levels of background signal. We believe it is unlikely that the background signal is due to production of functional truncated protein, as it has been shown for FLuc (the enzyme from which Akaluc was engineered) that much of the active site is in the C terminal beyond the position of our premature stop codon\textsuperscript{50}. Rather, we postulate this leakiness to be due to translational readthrough of the premature stop codon. Stop-codon readthrough is a phenomenon that has been observed in viruses, prokaryotes, and eukaryotes\textsuperscript{51–53}, and its strength largely depends on the type of stop codon and the surrounding sequence context\textsuperscript{54,55}. Although the frequency of readthrough is typically less than 1\%\textsuperscript{56}, engineering cells with high copy numbers of the Akaluc reporter could still lead to significant leakiness. These results demonstrate that engineering cells to strongly express an activatable reporter may not always be favorable, and that balancing strong activation with background expression may be necessary.

Comparison of the different tdT sorts of HeLa-Akaluc\textsuperscript{GO} cells showed that a medium level of tdT expression provided the best level of Akaluc activation above background levels. Using these medium sort cells we also demonstrated that MC delivery of ABE significantly enhances editing compared to PPs, achieving ~60\% A to G conversion. We validated the generalizability of these results by testing the tdT-Akaluc\textsuperscript{GO} reporter in two other cell lines, OVCAR8 and MDA-MB-231. We observed significant increases in Akaluc BLI signal with MCs compared to PPs in both cell lines. However, sequencing revealed low A to G conversion (<3\%), which may be because these cell types are more difficult to transfect compared to HeLa cells\textsuperscript{57}. Differences in editing efficiency may also be explained by
mutations in DNA repair enzymes across different cancer cell lines, such as base excision repair and single-strand break repair enzymes which are necessary for A to G conversion\textsuperscript{58,59}. Although the percent editing was low in OVCAR8 and MDA-MB-231 cells, these results demonstrate that Akaluc\textsuperscript{GO} can enable sensitive detection of low levels of base editing. Thus, Akaluc\textsuperscript{GO} is a powerful tool for the monitoring of ABE activity and can be used to compare the efficiency of different ABE delivery vectors \textit{in vitro}.

In a preliminary \textit{in vivo} experiment, mice bearing subcutaneous HeLa-tdT-Akaluc\textsuperscript{GO} (medium sort) tumors received intratumoral injections of MC-ABE\textsuperscript{Akaluc} complexed with jetPEI, but no Akaluc activation could be detected. We then analyzed the efficiency of transgene delivery in this tumor model by transfecting naïve HeLa tumors with a plasmid constitutively expressing Akaluc. However, BLI at 24 and 48 h post injection revealed minimal Akaluc BLI signal in these tumors. Thus, a low level of intratumoral gene transfer using \textit{in vivo} jetPEI in this model likely explains the undetectable ABE activity in our pilot experiment. Previous attempts at intratumoral jetPEI transfection of GFP or FLuc-expressing plasmids have also shown inefficient delivery\textsuperscript{38,60}. This may be due to poor diffusion of the DNA complexes within the tumor mass and poor uptake into cells following injection. Another plausible explanation is poor blood supply to the tumor, resulting in low delivery of the substrate and oxygen, both of which are required for the luciferase reaction\textsuperscript{61,62}. In healthy mice, we then analyzed two other \textit{in vivo} jetPEI delivery routes: intraperitoneal and intravenous. Intraperitoneal delivery did not yield any Akaluc signal across the mice, which was different from previous results with an FLuc-expressing plasmid from the company that sells the \textit{in vivo} jetPEI reagent. In contrast, intravenous delivery resulted in detectable signal in the lungs, which has been previously demonstrated in studies using plasmids expressing FLuc or RLuc\textsuperscript{23,63}. Intravenous delivery likely results in transgene expression mostly in the lungs because of the large surface area and thin endothelial/epithelial junction, allowing for the DNA complexes to easily diffuse from the vasculature into neighboring cells\textsuperscript{63}. Additionally, the high perfusion of the lungs ensures availability of substrate and oxygen for the luciferase reaction. Thus, intravenous delivery of ABE DNA with \textit{in vivo} jetPEI is a promising model for base editing of normal lung tissue or tumors within lungs and will be explored in future studies.
We also explored *in vivo* electroporation for gene delivery to subcutaneous tumors. Electroporation allows for targeted transfection of both superficial and deep tissues (e.g., using catheter-based electroporation devices)\(^6^4\) and has been tested in the clinic for gene therapy of metastatic melanoma\(^6^5,6^6\). To assess the efficiency of this method, we electroporated a plasmid constitutively expressing Akaluc into OVCAR8 naïve tumors. Akaluc signal was detected in 3 of the 5 tumors following electroporation, demonstrating successful uptake of the plasmid into cells. The lack of Akaluc signal in the other 2 mice may have been due to the size and shape of the tumors, as they were smaller and flatter which made it more difficult to maintain adequate contact between the tumor and electrodes. A previous study demonstrated electroporation of an FLuc-expressing plasmid into subcutaneous murine colorectal cancer tumors, where the signal obtained was \(~10\)-fold higher than observed in our study\(^3^8\). Thus, while we were able to achieve plasmid delivery into tumors, we will need to further optimize our electroporation parameters (e.g., voltage and pulse length) to improve DNA uptake. For MC-ABE electroporation into OVCAR8-Akaluc\(^{GO}\) tumors, significantly increased BLI signal was seen at 24 h post electroporation. However, background signal in these tumors pre-injection made it difficult to quantify the actual level of base editing, which we will next evaluate by sequencing the genomic DNA of these tumors.

While we have shown that MCs enable higher levels of base editing compared to plasmids *in vitro*, a potential limitation of MCs is that they may result in more persistent transgene expression, which may not be favorable for delivery of base editors. For example, Munye et al. compared MC and plasmid transfection of FLuc in the lungs through oropharyngeal instillation (a method to introduce fluids into the lungs via the pharynx)\(^2^4\). At 2 weeks after transfection, FLuc lung signal was persistent in mice that received MCs but was undetectable in most mice that received plasmids. While persistent transgene expression can enable higher levels of base editing, it also increases the likelihood of undesired off-target editing. Jang et al. compared sgRNA-dependent off-target effects of ABE *in vitro* following plasmid, mRNA, and ribonucleoprotein (RNP) delivery in HEK293T cells, and found that plasmid delivery exhibited the highest off-target editing for all four target sites tested\(^6^7\). This is likely due to the inherent stability of DNA resulting in prolonged ABE expression, while mRNA and RNPs are rapidly degraded in cells\(^6^8\). However, in an *in vivo*...
study by Song et al., tail vein injection of plasmid encoding ABE to correct a mouse model of tyrosinemia did not result in any detectable off-target editing in the liver\textsuperscript{14}. Thus, high off-target editing observed \textit{in vitro} may not necessarily translate \textit{in vivo}, and further studies are needed to analyze the off-target editing caused by MC delivery of ABE in comparison to other delivery methods. Moreover, using MCs for editing highly proliferative cancer cells would limit the persistence of ABE expression as the episomal MCs would be diluted over time. We have seen that MC transgene expression in tumors \textit{in vivo} is transient in nature compared to somatic non-dividing tissues like the lungs\textsuperscript{23,69,70}.

There are also some limitations to our Akaluc\textsuperscript{GO} reporter system which necessitate further exploration and optimization. First, the background signal seen in the OVCAR8 tumors could be further reduced, either by sorting the cells for a lower level of tdT, or by optimizing the location of the premature stop codon within the Akaluc gene. Second, BLI itself is limited by its depth penetration due to the absorption and scattering of light by tissues\textsuperscript{71}. While Akaluc has been engineered to emit more red-shifted light to reduce tissue absorption, sensitive imaging of deep tissues and in larger animal models remains a challenge. As seen in our \textit{in vivo} transfection experiments, BLI may also be limited by the availability of substrate and oxygen, making it difficult to accurately assess Akaluc expression in solid tumors. To overcome some of these limitations, it would be of interest to test the GO system with other sensitive and clinically relevant imaging reporters, such as organic anion transporting polypeptide 1B3 (OATP1B3) for magnetic resonance imaging\textsuperscript{72} and sodium iodide symporter (NIS) for positron emission tomography\textsuperscript{73}.

In conclusion, this study was the first to demonstrate MCs as a promising vector for efficient delivery of ABES \textit{in vitro} across multiple cancer cell types. Additionally, we developed Akaluc\textsuperscript{GO}, a BLI reporter for visualization of adenine base editing. We showed that Akaluc\textsuperscript{GO} can detect \textit{in vitro} ABE activity in cells with high sensitivity and specificity, and can also enable visualization of \textit{in vivo} ABE activity. Future work will further optimize the Akaluc\textsuperscript{GO} reporter to reduce background Akaluc signal \textit{in vivo}, followed by testing in other tumor models such as lung and liver to demonstrate tracking of organ-specific adenine base editing. We also aim to explore new genomic disease targets, new GO
reporters, and other ABE delivery methods to aid expansion of the toolbox of genome editing for the treatment of genetic diseases.

2.5 References


Chapter 3

3 Conclusions and Future Work

This chapter summarizes the objectives, experimental findings, and limitations of this thesis, as well as directions for future work.

3.1 Summary

Genome editing is a rapidly evolving technology with the potential to treat and cure genetic disorders by modifying DNA at the single nucleotide level. Base editing has shown tremendous promise for the correction of point mutations without the need to induce double-stranded breaks into DNA, thereby overcoming the concerns of unwanted insertions, deletions, and chromosome rearrangements associated with previous genome editing tools\(^3,4\). Together, ABEs and CBEs can theoretically correct ~61% of all disease-causing point mutations, including sickle cell disease, Dystrophin muscular dystrophy, inherited blindness, and some forms of cancer\(^5,6\). With the rapid expansion of applications, there is a need to test the efficiency and safety of new BEs and delivery vectors in preclinical models. DNA MCs are non-viral delivery vectors with enhanced transfection efficiency compared to plasmids due to their smaller size, as well as an improved safety profile because they lack a bacterial backbone\(^7,9\). Evaluating MCs also raises the requirement for a method to assess the activity of BEs \textit{in vivo}. Current evaluation tools predominately consist of DNA sequencing and tissue immunohistochemistry, which are \textit{ex vivo} methods and therefore cannot accurately measure BE activity in a spatiotemporal manner. Reporter gene imaging may overcome these limitations by enabling longitudinal non-invasive visualization of BE activity in cultured cells and animal models.

The main objectives of this thesis were to:

1. Test MCs as a novel delivery vector for ABE and compare them to plasmid delivery. We hypothesized that MC delivery will lead to enhanced base editing compared to plasmids.
2. Develop an activatable BLI reporter for the visualization of ABE activity by building off the previous “Gene On” (GO) systems designed by Katti et al. to create
a new activatable Akaluc reporter, called Akaluc\textsuperscript{GO}. We hypothesized that Akaluc\textsuperscript{GO} can enable highly sensitive and specific imaging of base-edited cells both in vitro and in vivo.

In Chapter 2, we validated the GFP\textsuperscript{GO} reporter previously developed by Katti et al.\textsuperscript{10} using a 2-plasmid ABE/sgRNA delivery system and observed robust induction of GFP expression in HEK293T cells stably expressing GFP\textsuperscript{GO}. We next built the first all-in-one MCs for the delivery of both ABE and sgRNA in a single compact DNA vector. Using the GFP\textsuperscript{GO} reporter, we showed significantly enhanced adenine base editing with MCs compared to their PP counterparts. To create an in vivo-compatible GO reporter, we added the Akaluc BLI reporter gene to the end of GFP\textsuperscript{GO} which was separated by a T2A self-cleavage peptide linker. However, this [GFP-Akaluc]\textsuperscript{GO} reporter showed unexpectedly high background Akaluc BLI signal, which we hypothesized was due to alternative translation initiation resulting in truncated but functional forms of Akaluc protein.

To circumvent this issue, we built a second BLI reporter called tdT-Akaluc\textsuperscript{GO}, which contains a premature stop codon mutation halfway through the Akaluc gene. HeLa cells were engineered to stably express this reporter, then sorted for various levels of tdT expression (low, medium, high). Cells with medium tdT expression showed minimal background Akaluc BLI signal and strong Akaluc activation upon transfection with PPs and MCs encoding ABE. Furthermore, MCs induced significantly increased Akaluc signal compared to PPs. The enhanced editing of tdT-Akaluc\textsuperscript{GO} with MCs was also demonstrated in two other cell lines, OVCAR8 and MDA-MB-231. These results indicate that MCs are a promising non-viral delivery vector for ABEs, and that tdT-Akaluc\textsuperscript{GO} is a useful tool for assessing ABE activity in different cancer cell lines.

In a pilot in vivo study, we injected MC-ABE\textsuperscript{Akaluc} directly into HeLa-tdT-Akaluc\textsuperscript{GO} subcutaneous tumors in mice following complexation with in vivo jetPEI transfection agent. However, no Akaluc BLI signal was observed in any tumors, which may be due to low efficiency of gene transfer to tumor cells and poor delivery of substrate and oxygen to the tumor\textsuperscript{11,12}. Further in vivo transfection analysis with a plasmid constitutively expressing Akaluc revealed that intratumoral DNA delivery with jetPEI was not efficient, whereas
intravenous delivery resulted in strong Akaluc BLI signal in the lungs 24 and 48 h post injection. These findings show that in vivo delivery of transgenes remains a major challenge for genome editing. Future work will further explore base editing in a mouse model of lung cancer as well as other base editor delivery methods.

In another preliminary in vivo study, we tested electroporation as a method of delivering DNA to subcutaneous tumors. We were able to detect transfer of an Akaluc-expressing plasmid into OVCAR8 tumor cells, as well as visualize ABE activity following MC delivery into OVCAR8-Akaluc<sup>GO</sup> tumors. These findings demonstrated that electroporation is a promising method for DNA delivery, but future work is needed to further improve the efficiency of gene transfer in subcutaneous tumors and other tumor models.

### 3.2 Limitations

Despite the promising results in this study for MC delivery of ABE, there is room for further characterization of this delivery system. First, we did not characterize their kinetics of editing, as we only evaluated the cells 48 hours after transfection. Measuring editing at various time points will provide deeper insight into when editing begins and how long the ABEs are active for. We did perform time-course live cell imaging of 293T-GFP<sup>GO</sup> cells transfected by sgRNA-tdT and ABE plasmids, however it is difficult to accurately characterize the kinetics of editing since HEK293T cells are highly proliferative. Therefore, we cannot discern whether the increase in GFP fluorescence is due to more cells being edited or due to proliferation of edited cells. More accurate methods to assess the kinetics of editing would be using flow cytometry or sequencing, though they are more time-consuming to perform.

Second, we did not characterize the levels of off-target editing in cells transfected by MCs. Given their higher on-target editing compared to plasmids, it is important to assess whether they also result in higher off-target editing. Off-target editing has been found to be linked to the persistence of ABE expression, which is dependent on the delivery vector<sup>13</sup>. For example, in a study comparing plasmid, mRNA, and RNP delivery of ABE in vitro, the authors found the highest off-target editing with plasmids due to their longer persistence in
cells\textsuperscript{14}. Since it has been shown that MCs provide prolonged transgene expression following delivery to the lungs, the off-target editing with MCs may also be higher than with plasmids\textsuperscript{15}.

There are also some limitations to our tdT-Akaluc\textsuperscript{GO} reporter for the assessment of ABE activity. First, BLI provides a measure of light output from a sample of cells, which although is useful for comparing relative Akaluc expression between groups, does not provide a measure of the percentage of cells edited. Akaluc\textsuperscript{GO} also cannot enable visualization of single edited cells, which is possible using fluorescence microscopy with GFP\textsuperscript{GO}-expressing cells. To further analyze base editing of Akaluc\textsuperscript{GO}, we performed Sanger sequencing of the target site in genomic DNA extracted from cells 48 h after transfection. Sanger sequencing alone provides qualitative information on the proportion of each DNA nucleotide at a certain position. We used BEAT, a computer program developed to quantify base editing from Sanger sequencing data based on the peak values of each nucleotide at each position. Quantification of A to G editing using BEAT analysis yielded similar trends as seen using BLI, with MC delivery showing increased A to G conversion compared to plasmids in HeLa cells. However, for OVCAR8 and MDA-MB-231 cells, BEAT analysis yielded <3\% A to G conversion, with no significant differences between MC and plasmid delivery. These results should be interpreted with caution, as BEAT has been shown to be less reliable at quantifying <5\% editing due to the inherit sensitivity limit with Sanger sequencing\textsuperscript{16}. For more accurate quantification of low levels of editing, it would be necessary to perform next-generation deep sequencing. Despite the low levels of editing in OVCAR8 and MDA-MB-231 cells, these results demonstrate that Akaluc\textsuperscript{GO} can detect very low levels of editing with high sensitivity and specificity \textit{in vitro}.

Finally, there are also limitations with base editing technology itself for the correction of point mutations. First is that ABEs are restricted to performing A to G edits and CBEs to C to T edits. This means \~39\% of disease-causing point mutations cannot be corrected with base editing\textsuperscript{5,6}. Base editors are also restricted to genomic targets where there is a PAM site in close proximity, which is required for the binding of Cas9 to the DNA\textsuperscript{17}. Lastly, bystander editing of multiple As or Cs could occur if they are within the editing window (e.g. positions 5-7 for the original ABE and positions 4-8 for ABE8e used in this study)\textsuperscript{18}. 
Therefore, correcting a desired point mutation could result in the unwanted change of another base. To overcome some of these limitations, Dr. David Liu’s group developed a new genome editing method called prime editing\(^\text{19}\). Like base editors, prime editors consist of a Cas9n and sgRNA, but instead of a deaminase they use a reverse transcriptase. The reverse transcriptase uses the sgRNA as template to copy new sequences into the target DNA. Because of the extreme flexibility of the sgRNA sequence, prime editors can install all possible base conversions, as well as small insertions and deletions. Although they are a promising alternative to base editors, prime editors have not been as extensively optimized and currently show lower on-target editing efficiencies\(^\text{10,20}\).

### 3.3 Future Work

To further assess the safety of MCs as an ABE-delivery vector, future work will measure off-target editing rates. Potential off-target sites can be determined using \textit{in silico} tools such as Cas-OFFinder\(^\text{21}\) and COSMID\(^\text{22}\). These are bioinformatic tools that computationally predict off-target sites by scanning the target genome for potential sites based on homology to the on-target sequence, then ranking the sites based on the degree of homology\(^\text{23}\). Once the top potential off-target sites are determined, we will isolate genomic DNA from edited cells or tissues and perform deep sequencing at these sites. Significant editing at these off-target sites would indicate a need to further optimize the delivery vector or engineer new base editors with improved on-target specificity.

Future work will also further test the Akaluc\(^\text{GO}\) reporter in preclinical cancer models. Based on our \textit{in vivo} transfection analysis, transgenes can be delivered to the lungs with tail vein delivery of DNA. Thus, a potential mouse model to explore would be a metastatic cancer model in which Akaluc\(^\text{GO}\)-expressing cancer cells (e.g., HeLa or MDA-MB-231) are injected via the tail vein, which would then become trapped in the lungs\(^\text{24,25}\). Following the establishment of lung nodules, MCs encoding ABE will be complexed with \textit{in vivo} jetPEI transfection agent and delivered via the tail vein. BLI will then allow for visualization of base editing in Akaluc\(^\text{GO}\)-expressing cancer cells in the lungs. This lung model would be useful for studying ABE delivery and activity for the treatment of genetic lung diseases. For example, cystic fibrosis is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, resulting in defective ion transport across epithelial...
cell membranes and the buildup of thick, sticky mucus in the airways\textsuperscript{26}. There are over 240 disease-causing point mutations in the CFTR gene, of which 46% can theoretically be corrected by ABEs and 15% by CBEs\textsuperscript{27,28}. Base editing of the CFTR gene has been demonstrated in human intestinal and airway epithelial organoids and human airway epithelial cells\textsuperscript{28–30}.

Our GO reporter can also be used to track \textit{in vivo} editing with other delivery methods. For example, lipid nanoparticle delivery of ABE-encoding mRNA and sgRNA is well suited for targeting of the liver, as demonstrated in animal models for the treatment of tyrosinemia and familial hypercholesterolemia\textsuperscript{31–33}. Lipid nanoparticles can also be used to deliver ribonucleoproteins (RNPs), which allows for the fastest \textit{in vivo} response since the ABE does not have to be transcribed or translated\textsuperscript{14}. RNPs also have the advantage of being rapidly degraded in cells, which reduces the likelihood of off-target editing. Lastly, adeno-associated viruses (AAVs) enable delivery of DNA base editors with transient gene expression, and have the advantage of targeted tissue delivery via the selection of a particular AAV serotype\textsuperscript{34}. For example, efficient \textit{in vivo} editing of cardiomyocytes was recently demonstrated in a mouse model of hypertrophic cardiomyopathy, a genetic heart disease\textsuperscript{35}. The authors expressed the ABE gene in an AAV9 vector, a specific serotype that enables high transgene expression in the heart. The ABE gene was also expressed under the control of the cardiomyocyte-specific promoter chicken troponin T (Tnnt2). With this system, they achieved over 70% editing in cardiomyocytes, with very low editing in other organs. These studies demonstrate the rapid progress and expansion of ABE technologies, but also highlight the need to non-invasively track these new tools in animal models.

Future work will also aim to expand the GO systems to other imaging modalities to overcome the limitations associated with BLI, mainly the attenuation of light by tissue which impedes the accurate evaluation of base editing in deep tissues\textsuperscript{36}. Magnetic resonance imaging (MRI) is a clinical imaging modality that provides high spatial resolution and soft-tissue contrast. An MRI reporter gene we aim to explore is human organic anion transporting polypeptide 1B3 (OATP1B3), a transmembrane protein that mediates uptake of gadolinium-ethoxybenzyl-diethylenetriaminepentaacetic acid (Gd-EOB-DTPA), a clinically approved contrast agent\textsuperscript{37}. Our group has recently demonstrated
in vivo visualization of cell-cell communication using an activatable OATP1B3 reporter system\textsuperscript{38}. Another clinical imaging modality we aim to explore is positron emission tomography (PET), which is highly sensitive and can provide quantifiable information. We have previously complemented OATP1B3-MRI with PET imaging using the reporter gene human sodium iodide symporter (NIS), which uptakes the radiotracer [18F]tetrafluoroborate, to sensitively visualize breast cancer cells in mice\textsuperscript{39}. By incorporating these MRI and PET reporter genes, we hope to improve the sensitivity and clinical translatability of the GO reporter systems.

In conclusion, here we demonstrate for the first time that MC delivery of ABE leads to higher in vitro base editing levels compared to plasmid delivery. We have also developed Akaluc\textsuperscript{GO}, the first BLI GO reporter for the visualization of adenine base editing. This reporter can be used to complement existing ABE assessment methods for non-invasive, spatiotemporal tracking of ABE activity and kinetics, which can aid the development of new ABEs and delivery strategies for the treatment of genetic diseases.
3.4 References


Appendix

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Western University, London, ON
Supervisor: Dr. John Ronald

AWARDS AND SCHOLARSHIPS

2022 Ontario Graduate Scholarship
Competitively selected to receive funding to support the 2nd year of my Master’s research
Amount: $15,000

2022 Alfred Jay Award for Biological and Biophysical Systems Research
Awarded for the best paper in basic biophysical research at Western University
Amount: $2500

2021-2022 Western Graduate Research Scholarship
Awarded to graduate students who maintain an average of 80% or more
Amount: $5000

2021 1st Place Oral Presentation Award
Imaging Network of Ontario Symposium

2021 Strik Couprie Inch Cancer Research Course Prize
Awarded for the highest mark in MEDBIO 4467: Radiobiology and Radionuclides
Amount: $300
2021  **CIHR Canada Graduate Scholarship – Master’s Award**  
Competitively selected to receive funding to support the 1st year of my Master’s research  
Amount: $17,500

2020  **Top Poster Prize**  
5th Biennial International Cancer Research Conference

2017-2021  **NSERC Undergraduate Student Research Award**  
Competitively selected to receive funding to support 3 summers of undergraduate research  
Amount: $4500

2019  **Richard Konrad Scholarship in Science**  
Awarded for academic excellence entering 3rd year in the Medical Biophysics program  
Amount: $1500

2019  **Excellence in Leadership Award, UWO Biology Mentorship Program**  
Awarded for my innovative contributions and dedication to the mentorship of students in 1st year Biology

2017  **Western Continuing Admission Scholarship**  
Awarded for entering undergraduate studies with a graduating average of 95% or higher

2017  **Research Western Imagination Prize, Thames Valley Science and Engineering Fair**  
Awarded for the project displaying the most imaginative work, both from the perspective of the concept and its implementation

2016  **Canada-Wide Science Fair Finalist**  
Selected as one of the top 10 regional projects to compete at the national science fair

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**RESEARCH EXPERIENCE**

2021-present  **MSc Thesis**  
*Dr. John Ronald and Dr. Timothy Scholl, Western University, London, ON*  
Developed and evaluated a novel DNA minicircle system for the delivery of CRISPR base editors using activatable imaging reporter systems
2020-2021  **Undergraduate Honors Thesis**  
*Dr. John Ronald, Western University, London, ON*  
Developed a clinically relevant MRI reporter gene system to evaluate base editing activity

2020  **NSERC Undergraduate Student Research Award**  
*Dr. John Ronald, Western University, London, ON*  
Established the first mouse model of synchronous bilateral breast cancer and used dual-bioluminescence imaging to track metastatic cross-seeding

2019  **NSERC Undergraduate Student Research Award**  
*Dr. John Ronald, Western University, London, ON*  
Characterized the sensitivity and kinetics of the recently developed Akaluc and Antares2 reporters for in vivo dual-bioluminescence imaging

2018  **NSERC Undergraduate Student Research Award**  
*Dr. John Ronald, Western University, London, ON*  
Developed a synthetic reporter gene imaging system to enable tracking of the crosstalk between breast cancer cells and T-cells for immunotherapy evaluation using MRI and bioluminescence imaging

2016  **Partners in Experiential Learning (PEL) High School Co-op Program**  
*Dr. John Ronald, Western University, London, ON*  
Investigated the strengths of various gene promoters for improving reporter gene tracking of cancer cells

**PUBLICATIONS**


**CONFERENCE PRESENTATIONS**


TEACHING AND MENTORSHIP

2022-2023  **Mentor, MEDBIO 3970Z (General Biophysics Laboratory)**  
Western University, London, ON  
Mentee: Rajan Leung

2021-2022  **Mentor, MEDBIO 3970Z (General Biophysics Laboratory)**  
Western University, London, ON  
Mentees: Joshua Krautner, Ma’az Syed

2021-2023  **Graduate Teaching Assistant**  
Course: MEDBIO 3970Z (General Biophysics Laboratory)  
Department of Medical Biophysics, Western University, London, ON  
Course Coordinator: Dr. John Ronald

2018-2021  **Mentor, Biology Mentorship Program**  
Courses: B1001A (Biology for Science I) and B1002B (Biology for Science II)  
Department of Biology, Western University, London, ON  
Course Coordinator: Dr. Niki Sharan

LEADERSHIP AND VOLUNTEERING

2021-present  **Chair, Medical Biophysics Student Research Symposium**  
*Western University, London, ON*  
- Led the organization of the annual department symposium for undergraduate students to share their research through oral and poster presentations  
- Arranged meetings, oversaw and delegated tasks, communicated with department representatives

2021-present  **Board Member, Thames Valley Science and Engineering Fair**  
*Youth Science London, London, ON*  
- Participated in the organization of the science fair for youths in the London region  
- Created promotional materials, mentored students, and served on the judging committee

2021-2022  **Co-President, Biophysics Network of Students (BONeS)**  
*Western University, London, ON*  
- Attended monthly meetings with Medical Biophysics Faculty members to discuss issues and student concerns within the department  
- Chaired the subcommittee which planned educational online social events for biophysics students
2020-2021  **Co-President, Biology Mentorship Program**  
*Western University, London, ON*
- Coordinated a team of biology mentors to provide learning support and study resources to 2300 first year Biology students, including weekly workshops, exam review sessions, and an official Facebook group
- Worked with first year Biology professors to communicate students’ unmet needs and ideas for improvement in the course

2020-2021  **A.C.E. Learning Co-Founder**  
*London, ON*
- Developed a non-profit organization dedicated to helping students in Grades 1-8 with online learning and skill-building in response to changes brought by COVID-19
- Recruited and matched volunteer tutors, designed a website, and collaborated with community non-profit organizations to implement literacy and art programs

2018-2020  **Emergency Department Volunteer**  
*University Hospital, London, ON*
- Actively involved in supporting the emergency team in providing patient-centered care
- Ensured smooth patient flow, answered patient and visitor inquires, and provided comfort measures to patients and families