In the Business of Base Editors: Evolution from Bench to Bedside

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Abstract

With the advent of recombinant DNA technology in the 1970s, the idea of using gene therapies to treat human genetic diseases captured the interest and imagination of scientists around the world. Years later, enabled largely by the development of CRISPR-based genome editing tools, the field has exploded, with academic labs, startup biotechnology companies, and large pharmaceutical corporations working in concert to develop life-changing therapeutics. In this essay we highlight base editing technologies and their development from bench to bedside. Base editing, first reported in 2016, is capable of installing C•G to T•A and A•T to G•C point mutations, while largely circumventing some of the pitfalls of traditional CRISPR/Cas9 gene editing. Despite their youth, these technologies have been widely used by both academic labs and therapeutics-based companies. Here, we provide an overview of the mechanics of base editing and its use in clinical trials.

1. Introduction

Precision medicine has long been a major focus of biological application-based research, and the development of CRISPR-derived genome editing tools has propelled progress in this area forward in recent years. In particular, base editors (BEs) have demonstrated their worth as especially powerful tools for the development of genome editing therapies. BE technologies were derived from CRISPR/Cas9 systems but avoid the use of double-strand breaks (DSBs) that traditional genome editing systems use. Bypassing the use of DSBs largely prevents the introduction of stochastic genome editing byproducts (such as indels). However, the tradeoff for this enhancement in genome editing precision is that BEs can only perform certain types of single base pair edits (transition mutations – purine to purine or pyrimidine to pyrimidine mutations), rather than the insertion, deletion, or replacement of any stretch of DNA desired. Fortunately, the ability to install transition point mutations with high precision and efficiency can be leveraged for a variety of therapeutical applications (not only the correction of monogenic disease-causing point mutations), making BEs fitting tools for the clinic.

In this essay, we describe the initial development of BEs, and discuss their limitations and the subsequent improvements made to the original BE constructs. We focus on modifications made to improve the efficiency, precision, and specificity of BEs, particularly in the context of therapeutics. We then provide an overview of the four current BE clinical trials, focusing on the general genome editing strategies employed by each trial. We finish with a brief commentary on future BE clinical trials in the immediate pipeline, additional emerging next-generation genome editing tools, and ethical considerations to consider as genome editing therapeutics become more prevalent.

2. Base Editing Technologies

a. Cytosine Base Editors (CBEs)

Currently, two classes of BEs exist: cytosine base editors (CBEs) and adenine base editors (ABEs). In the first example of targeted point mutation introduction via a non-DSB mechanism, the original CBE (named BE1) was created by fusing a catalytically inactive, or 'dead' Cas9 (dCas9) enzyme with the naturally occurring cytidine deaminase enzyme APOBEC1 (rAPOBEC1 sourced from *Rattus norvegicus*) [1]. The dCas9 protein complexes with a pre-programmed guide RNA (gRNA) and subsequently locates and binds to a specific DNA sequence (the protospacer) through the formation of an R-loop, driven by base-pairing between the protospacer and the first 20 nucleotides of the gRNA (the spacer, **Figure 1**) [2]. For the gRNA to bind to the protospacer, the protospacer must also be immediately adjacent to a protospacer adjacent motif (PAM) sequence (**Figure 1**). In the case of the widely used *Streptococcus pyogenes* Cas9 (spCas9), the PAM sequence is 5'-NGG-3', which has been calculated to occur approximately once every ~42 bases throughout the human genome [3].

Figure 1: Overview of cytosine base editor (CBE) and adenine base editor (ABE) principal components. ***Top left***, CBE architecture shown with principal components: Cas9 nickase (Cas9n) in grey (outline of crystal structure obtained from PDB: 6VPC), CBE deaminase APOBEC3A in red (outline of crystal structure obtained from PDB: 5SWW), and uracil glycosylase inhibitor (UGI) in purple (outline of crystal structure obtained from PDB: 1UGI). The deaminase and UGI components are tethered to nCas9 via short amino acid linkers (grey). Overlayed on top of the principal components is a general schematic of the mechanism of action; the guide RNA (gRNA, brown) will bind to the DNA protospacer (sequence of 20 nucleotides proximally located to the 3-nucleotide protospacer adjacent motif (PAM, violet) sequence), in the process exposing a single-stranded DNA "bubble" open for cytosine deamination. Deamination produces a U•G intermediate, which is processed by the cell to produce an overall C•G to T•A conversion (shown in base conversion inset). Concurrently, nCas9 will nick the unedited DNA strand (blue triangle) to increase editing efficiency. Similarly, the addition of the UGI component increases editing efficiency. ***Top right***, ABE architecture, simplified mechanism schematic, and overall base conversion are shown. Key differences of the ABE architecture are: ABE deaminase TadA-8e, similarly in red, (outline of crystal structure obtained from PDB: 6VPC) replaces CBE

deaminase and the lack of a UGI component, as ABE utilizes an inosine intermediate, compared to the CBE architecture. ***Bottom***, a non-comprehensive sampling of notable variations on key CBE and ABE principal components are shown. Collectively, these substitute components serve an array of purposes including increased on-target editing, decreased off-target editing, and relaxed PAM requirements for broadened utility. Development of new and enhanced BE principal components is a populated field of study with new results being published rapidly.

Following formation of the Cas9:gRNA:DNA ternary complex, a subset of one DNA strand is now single-stranded and accessible to rAPOBEC1 for deamination chemistry (**Figure 1**). Cytidines that are within this "editing window" are deaminated by rAPOBEC1, which produces a C•G to U•G conversion. The development and characterization of many subsequent CBEs have revealed that several factors influence which nucleotides within the protospacer comprise this "editing window", and include the Cas homolog that is used, the linker length and composition between the deaminase and Cas protein, the overall architecture of the BE, and the deaminase enzyme used (discussed later). For BE1, the deamination activity window is between positions 4-8 within the protospacer (**Figure 1**). Processing of the U•G intermediate by the cell, using the U-containing strand as a template, results in an overall C•G to T•A conversion. However, the presence of the U•G mismatch intermediate triggers the cell's native base excision repair (BER) pathway to excise the uracil and revert the intermediate back to the original C•G base pair [4]. Consequently, editing activity by BE1 in live mammalian cells was quite low, and C•G to non-T•A conversions were observed as well (discussed later). To address this, a second-generation CBE was developed, BE2, which incorporated a uracil glycosylase inhibitor (UGI) peptide to temporarily block BER, thus preventing uracil excision and increasing C•G to T•A conversion efficiencies. One last modification to the system was to exchange dCas9 for a nickase version of the enzyme (nCas9), and produced the final original CBE, named BE3. BE3 installs a DNA nick on the strand opposite the uracil-containing strand. This in turn manipulates the cell's native DNA repair processes to preferentially replace this strand and use the uracil-containing strand as a template, thus increasing editing efficiency even more (**Figure 1**). Shortly after the development of BE3, an additional CBE (named Target-AID) was described, which included similar components (a cytidine deaminase, nCas9, and UGI), but utilized the more active cytidine deaminase pmCDA1 (cytidine deaminase 1 sourced from sea lamprey) and fused together in a different orientation, resulting in a slightly shifted editing window compared to BE3 [5]. Target-AID demonstrated the robustness of this general strategy for targeted, programmable point mutation introduction.

b. Adenine Base Editors (ABEs)

Using CBEs as a model, researchers sought to expand the BE toolbox to include adenine base editors (ABEs), which would use adenosine deamination chemistry to install A•T to G•C base pair conversions using an inosine-containing intermediate. ABEs would be capable of correcting the most common pathogenic single nucleotide variant (SNV), making them a vital tool for therapeutic genome editing [6,7]. While the general approach of replacing rAPOBEC1 for an ssDNA-specific adenosine deaminase enzyme was simple and elegant, unfortunately no such naturally occurring enzyme existed, and it therefore needed to first be created.

As a first step, several RNA adenosine deaminase enzymes were installed into the CBE architecture in place of rAPOBEC1 and assessed for A•T to G•C activity levels. With no activity observed, researchers began the arduous process of using directed evolution to create a ssDNA-specific adenosine deaminase enzyme to produce the first ABE [8].

Directed evolution facilitates the enhancement or alteration of the activity of a given protein [9–11]. The protein of interest is mutagenized to produce a library of members, and active members are screened or selected to identify those with the new or enhanced activity of interest. To generate the first ABE, TadA, a tRNA adenosine deaminase sourced from *Escherichia coli*, which shares partial structural homology with the rAPOBEC1 enzyme employed by CBEs, was selected as a starting point. Over the course of seven rounds of directed evolution, ecTadA accumulated fourteen mutations to

produce ABE7.10, which demonstrated on average 58% A•T to G•C editing efficiency across a variety of target sites with various sequence contexts [8]. It is important to note that adenine base editing did not require any BER inhibition components (such as the UGI of the CBE), presumably due to a lower efficiency of inosine excision by BER glycosylase enzymes. Consequently, no A•T to non-G•C editing was observed by ABE7.10.

c. Limitations and Modifications

We focus here on the limitations of BE tools from a therapeutic perspective, and the corresponding modifications to the original ABE and CBE constructs that have been engineered to overcome these limitations. The most obvious and major restriction of BE technologies is the limited types of base pair conversions (C•G to T•A and A•T to G•C only) achievable with CBEs and ABEs. Expansion of the base editor toolbox in this area has been via the development of "glycosylase base editors", which utilize the basic CBE architecture with additional enzyme components that facilitate excision of the uracil intermediate. Specifically, a suite of "CGBEs" (C•G to G•C base editors) has been developed, which exclude the UGI component of the CBE architecture and instead incorporate a uracil glycosylase enzyme and/or error-prone polymerases [12–16]. In these editors, the uracil intermediate is efficiently excised by either the endogenous uracil glycosylase enzyme of the cell, or that included in the CGBE architecture, to produce an abasic site. The resulting abasic site is then processed by the translesion synthesis pathway of the cell, or the polymerase included in the CGBE architecture, to mutagenize the target base, with a C•G to G•C base pair as the most common overall outcome. One such glycosylase base editor is currently being used in a clinical trial by Bioray Laboratories (discussed below). This same strategy was recently applied to ABEs as well, where an engineered hypoxanthine glycosylase enzyme (derived from N-methylpurine DNA glycosylase, MPG) was fused to an ABE, resulting in an adenine transversion base editor (AYBE) that mutagenizes target adenines, with an A•T to C•G base pair as the most common overall outcome [17].

An additional major limitation of early BEs was their targeting scope. Due to the restrictive editing window (positions 4 through 8 in the most widely used editors), many times a requisite PAM sequence could not be located at the necessary location. After establishing the architectural framework of the first CBE, subsequent efforts found that replacing the Cas9 enzyme with Cas9 variants with relaxed or altered PAM requirements, or Cas homologs from different species, resulted in editors with high editing efficiencies and significantly increased the targeting scope [18,19]. With the advent of extremely PAM-relaxed Cas9 variants, such as Cas9-NG and SpRY-Cas9, BE targeting scope issues have been largely alleviated [20,21]. ABE7.10 was not as compatible with alternative Cas proteins, but this issue was resolved with the development of next-generation ABEs (discussed next).

An important characteristic of a therapeutic genome editor is high editing efficiency. Additional directed evolution efforts have been undertaken on both CBEs and ABEs to improve their overall efficiencies and remove sequence context biases that the deaminases possessed. Architectural engineering efforts on the original BE3 construct produced BE4, which has higher editing efficiencies and product purities than BE3 [22]. In fact, BE4 is currently being used in a clinical trial by Great Ormond Street Hospital for Children (discussed below). Directed evolution efforts have also produced optimized CBEs via the improvement of deaminase kinetics and/or solubility [23,24]. Additionally, codon optimization has been found to be crucial for optimizing expression of BEs in different cell types, which is an important consideration therapeutically [25,26]. The further directed evolution of ABE7.10, resulting in various ABE8 and ABE9 constructs, was particularly important from a therapeutic context, as the resulting ABE8 variants are being used in the current clinical trials [25,27,28]. As mentioned previously, these ABE8 variants were also found to be compatible with additional Cas homologs, which in effect expanded the targeting scope of these editors significantly. In fact, ABE8 variants are currently being used in two clinical trials (discussed below).

Finally, arguably the most important limitation of BEs from a therapeutic perspective are unintended edits. Unintended edits include any modification to the cell's genome other than the intended edit. These may include "bystander edits" (which occur within the same protospacer as the

intended edit), the wrong type of edit being installed at the target nucleotide (such as C•G to non-T•A conversions by CBEs) or “off-target edits” (which occur at other genomic loci in the cell), and it is important to note that these unintended editing events aren’t necessarily deleterious, and in fact many times can be benign. Bystander editing occurs as a consequence of deaminase processivity; if multiple target Cs or As are accessible within the ssDNA window, the deaminase will modify some or all. However, extensive deaminase engineering efforts have resulted in less active deaminases that have narrower activity windows. Additionally, alteration of the overall architecture has been shown to manipulate the activity window. Finally, with the development of PAM-relaxed Cas9 variants, many times multiple gRNAs can be designed for a given target base, some of which will “push” the bystander bases outside of the editing window. These efforts are more thoroughly outlined in several key publications [22,25,29–31]. An additional type of bystander editing was observed with ABEs; namely, cytidine deamination activity by the mutant TadA protein, which would result in undesired bystander C•G to T•A mutations in addition to the desired A•T to G•C mutation [32]. This activity was then significantly reduced through engineering efforts, resulting in more precise ABE variants [33].

Extensive work has been done to characterize the off-target editing efficiencies of BEs, and three different types have been observed: gRNA-dependent off-targets, gRNA-independent DNA off-targets, and gRNA-independent RNA off-targets [34–38]. gRNA-dependent off-target editing occurs when Cas9 binds to a homologous genomic locus despite mismatches between the protospacer and spacer. The use of “high fidelity” Cas variants which have lower tolerance for mismatches can be incorporated into the BE architecture to eliminate these [39]. Additionally, judicious choice of the gRNA can sometimes eliminate potential off-targets. gRNA-independent off-target editing occurs when the deaminase has access to ssRNA (both ABE and CBE) or ssDNA (CBEs only) within the cell (such as mRNA and transcription or replication bubbles) and deaminates cytosines or adenines within these bubbles. Several key publications have reported engineering of the deaminase domain (in both ABEs and CBEs) to reduce or eliminate RNA off-target editing events [40–43]. To reduce DNA off-target editing events, researchers have mutated the rAPOBEC1 protein to reduce its catalytic activity, as well as identified APOBEC homologs that naturally have lower gRNA-independent off-target editing activities [44,45]. Additionally, researchers have leveraged the previously undesired cytidine deamination activity observed with ABEs to engineer TadA-derived CBEs that have no gRNA-independent off-target DNA editing activity, like their ABE counterparts [46–48]. Finally, we will also note that delivery of BEs as mRNA rather than plasmid DNA has been shown to significantly reduce all forms of off-target editing [28]. A relationship between genome editing specificity and delivery modality/dosage was discovered prior to the development of BEs [49–52]. Genome editing agents typically modify the on-target locus first, and, will then modify off-target loci if their intracellular lifetime is long enough. To balance high on-target editing with minimal off-target editing, a short burst of a high level of active editor complex is therefore desired. Delivering DNA encoding for the editor will result in long-term expression, increasing chances of off-target editing. The lifetime of RNA is shorter than that of DNA, and transcription is not required to produce active editor when delivering mRNA encoding for the editor and gRNA. This results in a shorter timeframe between delivery and editing for mRNA and gRNA versus DNA, as well as shorter term expression of active editor. Both mRNA and gRNA can be chemically modified to extend their half-lives as well. In a recent example of ex vivo base editing in hematopoietic stem cells (HSCs), chemically modified mRNA encoding BE3 and synthetic gRNA were electroporated, and BE3 protein expression peaked at 12 hour post-electroporation, and was nearly entirely gone by 24 hour post-electroporation [53]. Finally, delivery of genome editing agents as purified protein:gRNA complexes (discussed below) results in the shortest overall lifetime of active editing agents. However, large-scale production of BEs at the purity required for therapeutic applications has been challenging, and thus mRNA delivery of BEs is generally preferred [54]. While off-target DNA edits are a therapeutic concern (particularly if they happen to occur in oncogenes or tumor suppressor genes), the quick turnover of mRNA within the cell alleviates some concerns regarding RNA off-targets.

3. Base Editor Therapeutics

Translating the broad efforts of BE development, mentioned above, into the clinical space requires an influx of support. To this end, many biotechnology companies have been founded or have sublicensed key BE intellectual property since the development of the inaugural CBE to accomplish this lofty goal, with Beam Therapeutics and Verve Therapeutics dominating the BE clinical trial space in the United States. (**Figure 2** and **Supplementary Table 1**). In the following discussions, we will detail the first cohort of BE clinical trials, examining the targeted indications, delivery methods, and reported rates of success.

Figure 2: Profiling Beam Therapeutics and Verve Therapeutics. Starting with the inner most ring and moving outward, these profiles include the year each company was established, key scientific founders, location of headquarters and number of employees, last reported total funding and round acquired, technology specialization, and targeted indications addressed with the company's specialized technology. Defined clinical trial candidates are denoted in parentheses. ***Left***, profile on Beam Therapeutics. REPAIR is RNA editing for programmable A-to-I replacement and RESCUE is RNA editing for specific C-to-U exchange. ***Right***, profile on Verve Therapeutics. Additional profiles can be found within **Supplementary Table 1**.

a. Delivery Options

Translating optimized BE tools to the clinic requires viable delivery strategies, which has long been a bottleneck in the field of gene therapy. A variety of delivery strategies exist, with the choice of which one to use entirely dependent on the disease that is being treated. Delivery modalities can be roughly broken down by whether treatment will occur *in vivo* or *ex vivo*. In the case of *in vivo* delivery, the BE is delivered directly into the target tissue(s) of the patient, while in the case of *ex vivo* delivery, cells are extracted from the patient, treated with the desired BE, and subsequently re-delivered into the patient via autologous transfer. Both strategies have a unique set of risks, challenges, and advantages. We expand on several relevant delivery avenues below.

In vivo gene editing must be used in cases where the treatment is designed to address a genetic disease afflicting an internal organ (i.e., the lung or liver). Given that genetic modification takes places within the body, *in vivo* therapies are subject to metabolic clearance and native immune responses [55]. Given these considerations, *in vivo* BE treatments must be dosed such that editing efficiencies are high, yet toxicity and undesired immune responses are avoided/minimized. Given these requirements, viral vectors have historically been considered attractive delivery vehicles, despite their strict cargo packaging capacity limits. This has led to additional BE modifications and optimizations to minimize their size. Specifically, split-intein BEs have been generated in which the BE is split into two separate constructs (each packaged within its own virus), which are reassembled via intein chemistry when the separate halves are translated within the same cell [56–58]. Further, BEs have been engineered using small Cas proteins to reduce the size of the full BE construct [59–61]. These advances have leveraged adeno-associated viruses (AAVs) as the delivery vehicle, as AAVs posit the lowest immunological profile out of the suite of viral candidates for human *in vivo* delivery [62]. Unfortunately, one of the first reported *in vivo* clinical trials using an AAV resulted in a fatal immune response [63,64].

Circumventing potentially dangerous immuno-side effects can be achieved using non-viral delivery vehicles such as a lipid-, inorganic-, or polymer-nanoparticles [65]. In addition to an increased safety profile, these vehicles do not have restrictive size limitations, and can be synthetically produced with relative ease compared to viral production [29,66,67]. These nanoparticles can be packaged with DNA or mRNA encoding the BE and gRNA, or purified BE:gRNA ribonucleoprotein (RNP) complex, which provided some flexibility. However, systemic treatment is difficult to achieve using nanoparticles, which is quite limiting. Typically, systemic delivery of lipid nanoparticles (the most commonly used nanoparticle for *in vivo* nucleic acid delivery) results in preferential accumulation in the liver and spleen [68]. This biodistribution profile has been leveraged in Verve's BE clinical trial (discussed below). Nanoparticles can also be locally injected into certain organs, such as the inner ear [69,70].

Ex vivo genome editing is particularly well-suited for treating blood disorders, such as hemoglobinopathies and leukemias. In addition to largely bypassing immune response issues, as genetic modification occurs outside of the patient, with *ex vivo* therapies, cells can be quality checked for accuracy before autologous transplantation [71]. While viral vectors can be used to deliver BEs *ex vivo*, nucleic acid or RNP electroporation is also an option. This method is quite efficient and similar to *in vivo* nanoparticles, does not have payload size restrictions. Unsurprisingly, these advantages are leveraged in three of the four clinical studies discussed below.

b. Framework for a Clinical Trial

Once a proposed therapeutic has been put through rigorous testing and optimization, the transition from the preclinical to clinical phase (I-IV) begins (**Figure 3**). It is important to recognize the strict demands these companies face in bringing a drug candidate to the clinical trial phase. The United States Federal Food and Drug Administration (USFDA) oversees all clinical trials in the US to ensure the safety and welfare of trial participants. Strict regulations are put in place for each phase of a clinical trial to maintain the integrity of the study. These regulations are for all levels of trial involvement: design, management and handling, data analysis, data reporting, and overall good practice.

Figure 3: Overview of current BE clinical trials. ***Left***, a simplified timeline for the path of a drug from pre-clinical to FDA-approval. Current statuses of ongoing trials are represented by colored special characters: ^ are clinical candidates in development, while * are candidates currently undergoing clinical trials sponsored by Verve Therapeutics (forest), Beam Therapeutics (mauve), Great Ormond Street Hospital (mustard), and Bioray Laboratories (navy). ***Middle***, a visual representation of the target of each of the four clinical trials; a single *in vivo* treatment is delivered to the liver (forest) and three others function by *ex vivo* treatments which are then subsequently re-administered via bloodstream IV and will repopulate cells in the bone marrow (mauve, mustard, navy). ***Right***, detailed breakdown of the four on-going clinical trials. Shown are the delivery modality (mRNA (teal)/gRNA (purple) electroporation versus lipid nanoparticle delivery), as well as targeted indications.

4. Ongoing Base Editor Trials

a. VERVE-101

In July 2022, Verve Therapeutics announced the first patient had been dosed with VERVE-101, an investigational *in vivo* base editing medicine targeting the *PCSK9* gene (**Figure 3**). The clinical trial, which is taking place in New Zealand and the United Kingdom (NCT05398029), marks the first instance of a BE treatment in human patients. Another noteworthy aspect of the clinical trial is that the base editing occurs *in vivo* (rather than *ex vivo*), which is a significant milestone. VERVE-101 is an intended treatment for heterozygous familial hypercholesterolemia (HeFH), atherosclerotic cardiovascular disease (ASCVD), and uncontrolled hypercholesterolemia. In HeFH, the liver's ability to metabolize low density lipoprotein (LDL) is compromised [72]. The build-up of LDL within the body results in high cholesterol levels that in turn form plaques which over time, will cause arteries to harden and restrict blood flow. This often leads to coronary artery disease and potentially fatal myocardial infarctions. The *PCSK9* gene is a target to lower LDL levels and treat HeFH, as the PCSK9 protein degrades the LDL receptor, which is required for ingestion of LDL particles into hepatocytes. Additionally, naturally occurring loss-of-function mutations in *PCSK9* have been identified in healthy individuals [73,74].

VERVE-101 is a single-course treatment for HeFH that will permanently knock-out the *PCSK9* gene in the liver to reduce LDL levels. This is achieved using an ABE8 variant to mutate the G<u>T</u> (the target A is base paired with the underlined T) splice donor at the exon 1/intron 1 boundary in *PCSK9* [75]. Following A•T to G•C point mutation introduction, intron 1 is retained in the mature mRNA transcript, resulting in a premature stop codon and degradation of the mRNA through nonsense mediated decay [28,76,77]. VERVE-101 is administered via an intravenous infusion of an engineered lipid nanoparticle (LNP) containing ABE8-encoding mRNA and the *PCSK9*-targeting gRNA, resulting

in LNP delivery mainly to the liver of patients [75,78,79]. This approach was recently undertaken in cynomolgus monkeys, where 90% reduction of PCSK9 levels in the blood was observed [75]. In this phase 1b trial, Verve seeks to assess the safety and pharmacodynamic profile of VERVE-101.

b. BEAM-101

In July 2022, Beam Therapeutics announced patient enrollment had begun for its BEACON trial (NCT05456880), which aims to assess its BEAM-101 therapy as a treatment for three forms (HbSS, HbSβ^0, and HbSβ^+) of severe sickle cell disease (SCD) (**Figure 3**). These three types of SCD are all caused by mutations in the hemoglobin β subunit (*HBB*) gene, which encodes for the beta globin protein [80]. The most common form of hemoglobin in adults, hemoglobin A (HbA), is a tetramer comprised of two beta globin subunits and two alpha globin subunits. All three forms of SCD that BEAM-101 is intended to treat have the "HbS" mutation in one of the *HBB* alleles, which is an A•T to T•A mutation that causes a Glu6Val substitution in the beta globin protein. This hydrophobic amino acid substitution causes beta globin proteins to "stick" to each other and polymerize to form long fibers. These polymers in turn distort the shape of erythrocytes, causing "sickling" of the cells. Individuals with the HbSS form of SCD are homozygous for this mutation (this is known as "sickle cell anemia"). Individuals with the HbSβ^0 and HbSβ^+ forms of SCD have the HbS mutation on one allele, and another mutation in *HBB* on the other allele that impacts expression of the beta globin protein. Those with HbSβ^0 have no expression of beta globin from this second allele, and those with HbSβ^+ have reduced production of beta globin from this second allele [81]. In all three forms of SCD, the red blood cells become sickled, which causes blood flow clogs [82]. This consequently results in sickle cell crises (attacks of pain), infections, and stroke.

Beam's approach to treat SCD is to "reactivate" expression of fetal hemoglobin (HbF), which is comprised of two alpha globin subunits and two gamma globin subunits. HbF is involved in transporting oxygen in fetuses, and expression of gamma globin (encoded by the *HBG1* and *HBG2* genes, which encode for the same protein but have different regulatory sequences) naturally decreases to very low levels within a year of birth. Reactivation of HbF can compensate for low levels of beta globin and inhibit polymerization of HbS proteins [83]. Certain healthy individuals naturally have mutations that cause Hereditary Persistence of Fetal Hemoglobin (HPFH), in which HbF levels in adults exceed the normal level. BEAM-101 is an autologous cell therapy that seeks to introduce the "British" HPFH mutation (a T•A to C•G mutation in the *HBG1* and *HBG2* enhancers) into patient-derived hematopoietic stem and progenitor cells (HSPCs) *ex vivo* [84,85]. Specifically, CD34$^+$ HSPCs are harvested from the patient and electroporated with ABE8-encoding mRNA and *HBG1/2*-targeting gRNA. The resulting mutation prevents the BCL11A repressor from binding to the *HBG1/2* enhancers. To facilitate efficient engraftment of the edited cells, patients must be conditioned prior to reintroduction of the edited cells. Beam has previously reported the successful, high-efficiency editing and subsequent robust reactivation of HbF in *ex vivo*-edited patient-derived CD34$^+$ HSPCs [28]. In this phase 1/2 trial, Beam seeks to assess the safety and efficacy of BEAM-101.

c. BE-CAR7

In May 2022, Great Ormond Street Hospital for Children in collaboration with University College London (UCL) began patient enrollment for its BE-CAR7 trial (NCT05397184), which aims to assess the safety of this treatment for relapsed and refractory T cell leukemia in patients aged 6 months to 16 years (**Figure 3**). T cells (a type of white blood cell) are derived from hematopoietic stem cells in the bone marrow, and differentiate into T cells in the thymus. Certain genetic and epigenetic modifications can occur during this process and cause T cell acute lymphoblastic leukemia (T-ALL), which is an aggressive and quick-progressing leukemia [86,87]. Chimeric antigen receptor (CAR) T cell therapy has emerged as a promising treatment for such types of cancer. CAR T cell therapy involves collecting T cells from either a healthy donor (allogeneic CAR T cell therapy, which the BE-CAR7 trial is) or the patient (autologous CAR T cell therapy) and engineering the cells to express a CAR on the cell surface

(this is generally achieved using lentiviral transduction methods). The CARs are receptor proteins that both bind to a specific antigen that the leukemia cells are expressing and activate T cell function. In the case of BE-CAR7, the T cells are engineered to express a CAR that recognizes cluster of differentiation 7 (CD7), which is a transmembrane protein that is highly expressed on both normal and malignant T cells [88]. The resulting CAR7 cells can in theory then be infused into the patient where they will bind to CD7-expressing malignant T cells and destroy them.

Unfortunately, both the engineered CAR T cells and the malignant T cells express CD7, resulting in CAR T "fratricide," in which the CAR T cells target and destroy themselves. To prevent this, the endogenous *CD7* gene must first be knocked-out. Additionally, the T cell receptor α chain (*TRAC*) gene must also be knocked-out to prevent graft-versus-host disease (which occurs when donor T cells recognize the patient's cells as foreign and destroy host tissue). Finally, the *CD52* gene must also be knocked-out, to enhance the lifetime of the CAR T cells in the presence of the lymphocytic leukemia medication alemtuzumab (which is an antibody that binds to the CD52 protein). Therefore, in the BE-CAR7 trial, prior to lentiviral transduction of the CAR7, the T cells are electroporated with CBE-encoding mRNA (specifically, BE3) and three synthetic gRNAs, which target the *CD7*, *TRAC*, and *CD52* genes for knock-out. The *CD7*-targeting gRNA targets the CBE to a Gln codon (CAG codon) in the *CD7* gene and converts it to a premature stop codon (TAG) via C•G to T•A base editing, resulting in nonsense-mediated decay of the mRNA transcript and knock-out of the gene. It should be noted that bystander mutations are also concurrently introduced but are benign due to knock-out of the gene. *TRAC* and *CD52* knock-out is accomplished similarly. Multiplexing gene knock-outs using traditional, DSB-reliant genome editing methods is accompanied by large-scale chromosomal rearrangements and cytotoxicity, which are avoided when using BEs to install premature stop codons or splice site disruptions [89]. Therefore, future CAR T cell therapies requiring multiplexed knock-out strategies will greatly benefit from the use of BEs. The Great Ormond Street Hospital and UCL team recently reported specific cytotoxicity of engineered *CD7* knock-out CAR T cells against CD7+ T-ALL cells both *in vitro* and an *in vivo* humanized mouse model [53]. In this phase 1 trial, the team seeks to assess the safety of the BE CAR-7 treatment and assess if the CAR7 T cells can eliminate T cell leukemia. In exciting recent news, Alyssa, the first patient to be administered BE CAR-7, reported complete remission of T-ALL six months after her treatment.

d. BRL-103

In July 2022, Bioray Laboratories announced its BRL-103 clinical trial (NCT05442346), which is an autologous cell therapy for patients with β-thalassemia major (**Figure 3**). β-thalassemias, similar to SCD, are caused by mutations in the *HBB* gene that cause reduced or no expression of beta globin. β-thalassemia major is caused by mutations in both *HBB* alleles and symptoms typically include severe anemia. Without treatment, patient death typically occurs before age 20. Treatment includes periodic blood transfusions and chelation of iron overload that is caused by the repeated blood transfusions.

BRL-103 is similar to BEAM-101, and involves harvesting HSCs from patients, reactivating HbF using base editing, and reintroducing the edited cells into the patients after conditioning. A major distinction from the BEAM-101 trial is BRL-103's use of a glycosylase base editor, which presumably is used to mutate the *BCL11A* enhancer (based on similarities to their NCT04211480 clinical trial) [90]. As mentioned previously, the BCL11A repressor silences *HBG1/2* expression. Disruption of *BCL11A* expression would therefore reactivate *HBG1/2* expression. The use of a base editor to mutate the *BCL11A* enhancer rather than wtCas9 has a variety of benefits including fewer genotype outcomes, lower risk of chromosomal rearrangements due to DSBs, and lower cytotoxicity. While no publications have been reported on BRL-103 yet, preliminary results from their phase 1/2 clinical trial NCT04211480 in which Cas9 was used to mutate the *BCL11A* enhancer have been published and showed increased hemoglobin production and a high persistence of edited cells in the bone marrow [90]. In this phase 1/2 trial, Bioray seeks to assess the safety and efficacy of BRL-103.

e. Clinical Expansions of Current Trials

In addition to the VERVE-101 and BEAM-101 clinical trials, other BE-based therapies are earlier in the clinical pipeline from both companies. For Verve, their second drug, VERVE-201, targets the *ANGPTL3* gene in the liver for permanent silencing. This treatment is for individuals suffering from homozygous familial hypercholesterolemia, HoFH. In theory, this treatment could also be used for HeFH patients who do not receive sufficient results from the *PCSK9* therapy. VERVE-201 is still preclinical in the IND (investigational new drug) enabling phase but is expected to be rolled out in the clinic in 2024. The news of this development accompanied reporting that the VERVE-101 clinical trial in the US has been put on hold, however studies are still ongoing in New Zealand and the UK.

Similarly, Beam has forged ahead on several new drugs: ESCAPE-1 in which *ex vivo* multiplexed editing of *HBG1/2* and *CD117* can treat SCD and β-thalassemia with less toxic conditioning of the patient, BEAM-201 in which multiplexed gene knock-out will be used for T-cell leukemia and lymphoma treatment, BEAM-301 in which *in vivo* correction of the R83C mutation in the glucose-6-phosphatase (*G6PC1*) gene in the liver will be used to treat glycogen storage disease 1a (GSD1a), and BEAM-302 in which *in vivo* gene correction of the E342K mutation in the alpha-1-antitrypsin (*SERPINA1*) gene will be used to treat alpha-1 antitrypsin deficiency (Alpha-1). ESCAPE-1, BEAM-201, and BEAM-301 are all in the IND enabling phase, while BEAM-302 is still relatively early in the optimization phase. It is important to note additional therapies are also in development at both Verve and Beam, however for proprietary reasons, further details on these technologies are not presently available.

5. Ethical Implications and Future of the Field

Given the fast pace of genome editing therapeutics, and the quick turnaround time from the development of the first BE to BE clinical trials, ethical discussions and considerations are imperative. This is particularly timely given the events of 2018, when CRISPR/Cas9 was used to perform germline genome editing on two embryos, causing members of the general public to feel mistrust and apprehension about therapeutic genome editing in general. Therefore, transparency and open discussions among scientists, bioethicists, policy makers, clinicians, and patient advocacy groups is necessary to ensure productive progress forward and avoid the dissemination of misinformation. Given the short timespan from BE discovery in 2016 to the initiation of clinical trials now, it is also important to expand our basic understanding of how BEs function, which will ultimately aid BE drug development and potentially clinical approval.

In addition to the candidate therapies in clinical trials underway, more work is being done to expand the host of potentially curative genomic medicines. These efforts are both inside and outside the BE field. For example, prime editors (PEs) are one such next step in the evolution of genomic medicine and have addressed some of the limitations of BEs [91]. This new technology, like BEs, avoids the use of DSBs and therefore installs genomic modifications with high precision. PEs perform genome editing using a completely different mechanism than BEs, and the two technologies are therefore complementary to each other. PEs employ a reverse transcriptase (RT) fused to nCas9 and an extended gRNA, called a prime editing gRNA (pegRNA) that has a 3' extension. The pegRNA encodes both the location of editing (via the spacer sequence), and the edit to be introduced (via the 3' extension). Following DNA binding and nicking of the PAM-containing strand, the RT directly appends a portion of the 3' extension of the pegRNA sequence onto the broken DNA end. In this manner, PEs can install any type of small modification into the genome in a programmable and precise manner. The quick establishment of Prime Medicine to develop PEs into therapeutics is a sign of additional exciting clinical trials in the future. Despite the uncertainty and ambiguity of scientific research, one thing is certain: BEs are a staple of genomic medicine and will clearly have a real impact on society and human health.

Exciting new work within the BE field has yielded mitochondrial genome editing agents, which have the potential to cure genetic disorders caused by mitochondrial mutations [92]. Mitochondrial genome editing had been unfeasible until recently, for reasons related to mitochondrial DSB repair and

delivery of nucleic acids to the mitochondria. Reliable nucleic acid delivery to the mitochondria has not yet been established [93], thus CRISPR-based genome editing agents (which require gRNAs) cannot be used for mitochondrial genome editing. CRISPR-free programmable nucleases such as transcription activator-like effector nucleases (TALENs) and zinc finger nucleases (ZFNs) can be delivered to the mitochondria via the use of mitochondrial targeting signals (MTSs). However, cleaved mitochondrial DNA is degraded rather than repaired, thus precision mitochondrial genome editing cannot be performed by TALENs and ZFNs [94–96]. Therefore, TALE- and ZF-derived CBE and ABEs were developed to enable mitochondrial genome editing [97–101]. As these editors are fully protein-based, they can be delivered to the mitochondria, and as they use uracil and inosine intermediates, they install point mutations into mitochondrial DNA rather than degrade it. This new class of BEs opens up new therapeutic opportunities in the mitochondrial disorder space.

6. Conclusions

The fast timeline (6 years) for the progression of BEs from bench to bedside was supported by the concurrence of several factors, including the robustness of the technology, an influx of support to both academic research on BEs as well as to the biotechnology sector, and the knowledge gained from therapeutic efforts on other genome editing agents, particularly in the area of delivery. Fervent research in the space of base editing uncovered limitations of the technology (including undesired editing events) almost as quickly as it developed solutions to these limitations, allowing for the evolution of BEs from research tools to therapeutic agents. We described here this development, and the four current examples of BE clinical trials. With several more on the horizon, we are excited to see additional creative applications of these technologies to human health.

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Supporting Information

S1 Table: Sampling of prominent biotechnology companies within the gene editing therapeutics field. Information presented is an extension of **Figure 2**, across a broader range of biotechnology companies. In the interest of space, the following set of exclusionary criteria was used to determine the final list of companies represented: non-CRISPR or BE based technology, not based in the United States, acquired by a larger company (larger, acquiring companies may be included), solely cell-therapy focused, large-scale pharmaceutical companies, and non-human based research applications.

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In the Business of Base Editors: Evolution from Bench to Bedside

Elizabeth M. Porto and Alexis C. Komor

S1 Table	Sampling of prominent biotechnology companies within the gene editing therapeutics field

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Company	Year Founded	Founders	Headquarters	No. of Employees	Amount and Source of Funding	Technology Specialization	Targeted Indications
Acrigen Biosciences	2019	Joe Bondy-Denomy & David Rabuka	San Francisco, CA	1-10	$1.2M Grant round (02/2022)	AcrTAIN software platform to identify new Cas enzymes and combine with engineered recombinant anti-CRIPSR (ErAcr) proteins to minimize off-target editing	No declared focus
Arbor Biotechnologies	2016	Feng Zhang, David R. Walt, David Scott, Winston Yan	Cambridge, MA	51-100	$230M Series B (11/2021)	Engineered nucleases (DNA, RNA, and transposases)	Primary hyper-oxaluria, liver diseases 1&2
Artisan Bio	2019	Tanya Warnecke & Ryan Gill	Denver, CO	1-10	$21M Series A (11/2020)	STAR-CRISPR platform comprised of an enhanced nuclease and STAR gRNAs	Non-small cell lung cancer, pediatric sarcomas, multiple myeloma
Capsida Biotherapeutics	2019	Nicholas Flytzanis, Nick Goeden, Viviana Gradinaru	Los Angeles, CA	11-50	$140M Corporate round (04/2021)	Tissue targeted gene therapies using optimally engineered AAV capsids	Central Nervous System (CNS) disorders
Caribou Biosciences	2011	Jennifer Doudna, James Berger, Martin Jinkek, Rachel E. Haurwitz	Berkeley, CA	101-250	$168M Series C (03/2021)	CRISPR hybrid RNA-DNA genome editing (Cas12a chRDNA) and CAR-T/CAR-NK cell therapies	Hematologic, solid tumor, non-Hodgkin lymphoma, 2 undisclosed CAR-T programs
CRISPR Therapeutics	2013	Emmanuelle Charpentier, Chad Cowan, Daniel Anderson, Matthew Porteus, Rodger Novak, Shaun Foy	Cambridge, MA	101-250	$130M Series B (12/2020)	Traditional CRISPR/Cas9 approach for geneteically-defined diseases and cellular engineering	Beta-thalassemia, Sickle Cell Disease (SCD)
Editas Medicine	2013	Feng Zhang, George Church, J. Keith Joung, Jennifer Doudna	Cambridge, MA	101-250	$657M Post-IPO (01/2021)	Traditional Cas9 and Cas12a (Cpf1) for *in vivo* gene edited and *ex vivo* gene edited cellular medicines	Solid tumors, SCD, Beta-thalassemia, Leber congenital amaurosis 10 (LCA10)
ElevateBio (a LifeEdit company)	2017	David Hallal, Mitchell Finer, Vikas Sinha	Cambridge, MA	101-250	$845M Series C (03/2021)	Proprietary novel RNA-guided nucleases (RGNs) and BEs derived from AgBiome's colelction of microbes with broad range of PAMs	Neurological conditions with high unmet need
Emendo Biotherapeutics	2015	Shilo Ben Zeev	New York City, NY	11-50	$61M Series B (01/2020)	Novel OMNI nucleases with non-NGG PAMs to maximize activity and allele-specificity while minimizing off-target activity	Severe Congenital Neutropenia (SCN)
Excision BioTherapeutics	2015	Rob Simmons & Thomas Malcolm	Philadelphia, PA	11-50	$78M Grant round (09/2022)	Dual gRNA system to force large DNA deletions and precent viral escape and reproduction	Human immuno-deficiency virus (HIV), Progressive multifocal leuko-encephalopathy (PML), Herpes simplex virus (HSV), Hepatitis B virus (HBV)

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Graphite Bio	2020	Daniel Dever, Maria-Grazia Roncarolo, Matthew Porteus	San Francisco, CA	101-250	$196M Series B (03/2021)	UltraHDR platform aimed at 'find and replace' gene editing with HiFi Cas9	SCD and Beta-thalssemia
Hunterian Medicine	2016	Vinod Jaskula-Ranga	Cambridge, MA	1-10	$3.8M Grant round (05/2021)	Bidirectional promoter aimed at shrinking Cas protein for packaging within a single AAV capsid	No declared focus
iECURE	2012	Jim Wilson	Philadelphia, PA	11-50	$50M Series A (09/2021)	Proprietary ARCUS nuclease used for duplicate healthy gene insertion	Genetic liver diseases with emphasis on early childhood patients
Inscripta	2015	Andrew Garst, Ryan T. Gill, Tanya Warnecke	Denver, CO	101-250	$460M Series E (04/2021)	Further development on MAD7 nuclease for optimized viral delivery	No declared focus
Intellia Therapeutics	2014	Jennifer Doudna, Andy May, Derrick Rossi, Erik Sontheimer, Luciano Marraffini, Nessan Bermingham, Rodolphe Barrangou	Cambridge, MA	251-500	$925M Post-IPO (06/2021)	Traditional CRISPR/Cas9 genome editing for *in vivo* and *ex vivo* therapies	Transthyretin amyloidosis, hereditary angiodema, acute myeloid leukemia, Alpha-1 antitryp-sin deficiency (A1D1), Hodgkin's lymphoma
Ligandal	2014	Andre Watson & Christian Foster	Brooklyn, NY	1-10	$4.6M Seed round (02/2019)	Platform for creating target cell population-specific gene therapy delivery vehicles	COVID-19
Locanabio	2016	David Nelles & Gene Yeo	San Diego, CA	11-50	$156M Series B (12/2020)	CORRECTX platform aimed to create modular RNA binding protein systems for multi-functional RNAs	Neuro-degenerative, neuromuscular, and retinal diseases
Locus Biosciences	2015	Charles Gersbach, Chase Beisel, David Ousterout, Nick Taylor, Paul Garofolo, Rodolphe Barrangou	Morrisville, NC	11-50	$129M Series B (05/2022)	Technology mergining bacteriophage targeting with CRISPR-Cas3 activity to combat bacterial pathogens	Antibiotic-resistant infections, microbiome-related diseases, inflammatory bowel disease (IBD)
Mammoth Biosciences	2017	Jennifer Doudna, Lucas Harrington, Ashley Tehranchi, Janice Chen, Trevor Martin	San Francisco, CA	101-250	$265M Series D (09/2021)	Specializes in miniature Cas enzymes for ease of delivery and precise edits with alternative PAMs	No declared focus
Metagenomi	2018	Jillian Banfield & Brian Thomas	San Francisco, CA	101-250	$357M Corporate round (11/2022)	Developing next-generation gene editing tools using optimized, novel nucleases	Liver diseases, Hemophilia A, oncological diseases
Poseida Therapeutics	2014	Eric Ostertag	San Diego, CA	251-500	$411M Post-IPO (08/2022)	Proprietary Cas-CLOVER homo-dimer nuclease system for gene editing in resting T-cells	Hemophilia A, Ornithine transcarbamylase deficiency (OTC)

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Prime Medicine	2019	David Liu, Andrew Anzalone, Jeremy Duffield	Cambridge, MA	101-250	$315M Series B (07/2021)	Leverages prime editing technology for 'search and replace' gene editing	No declared focus
Scribe Therapeutics	2017	Jennifer Doudna, Benjamin Oakes, Brett Staahl, David F. Savage	San Francisco, CA	11-50	$120M Series B (03/2021)	X-editing technology built on novel CRISPR foundation for *in vivo* genetic modification	No declared focus
Sherlock Biosciences	2019	Feng Zhang, David R. Walt, Deborah Hung, Jim Collins, Jonathan Gootenberg, Omar Abudayyeh, Pardis Sabeti, Rahul Dhanda, Todd Golub	Cambridge, MA	11-50	$136M Series B (03/2022)	CRISPR-utilizing specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) for single molecule detection of nucleic acid targets	No declared focus
Spotlight Therapeutics	2017	Alexander Marson, Jacob Corn, Patrick Hsu	San Francisco, CA	11-50	$81M Series B (03/2022)	Use of targeted active gene editors (TAGE) CRISPR effectors for direct delivery of cell-selective gene therapies	Opthalmic and HSC-mediated diseases and hemoglobino-pathies
Tessera Therapeutics	2018	Geoffrey von Maltzahn & Jacob Rubens	Cambridge, MA	101-250	$532M Series C (04/2022)	DNA- and RNA-based GENE WRITING technology	No declared focus
Tome Biosciences	2021	Omar Abudayyeh & Jonathan Gootenberg	Watertown, MA	51-100	$96M Series A (01/2022)	Licensed programmable addition via site-specific targeting elements (PASTE) technology, with future technology in development	No declared focus
Wave Life Sciences	2012	Takeshi Wada & Greg Verdine	Boston, MA	101-250	$174M Post-IPO (02/2018)	Stereopure oligonucleotides for range of modalities such as RNA editing	Huntington's disease, Duchenne muscular dystrophy (Dmd), A1D1