

**BASE EDITING ENABLES SINGLE-STEP, HIGHLY MULTIPLEX GENOME
EDITING IN HUMAN IPSCS WITH NEGLIGIBLE GENOTOXICITY**

A DISSERTATION

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Dedication

This thesis is dedicated to my dad and my mom; they shaped me as a student, tutored me all through school, and instilled in me the importance of higher education. They grew up in hardship but put themselves through school and worked hard to provide the best for their family. Their study techniques, memorization lessons, discipline, and investment in my education made school life a breeze and laid the much-needed foundation for all my higher studies.

Abstract

Next-generation cancer therapies using genome-edited immune cells are a rapidly evolving yet promising avenue for personalized medicine. Human induced pluripotent stem cells (iPSCs) are a genetically tractable platform for scalable *in vitro* production of engineered immune cells; however, multiplex editing using Cas9 nucleases to enhance effector function and overcome the immunosuppressive tumor microenvironment (TME) poses significant risks for genotoxicity and chromosomal translocations. To mitigate risks associated with double-stranded breaks (DSBs) induced by CRISPR/Cas9 nucleases, we utilized base editing (BE) to install precise nucleotide edits without DSB induction, achieving highly efficient, single-step multiplexed genome editing in human iPSCs with minimal genotoxic side effects. Comparing Cas9 nuclease and adenosine base editor (ABE8e) delivered as synthetic mRNA, we simultaneously edited 9 genes previously shown to enhance function in patient-derived immune cells. Cas9 and ABE8e achieved >90% editing efficiencies in single plex at a single target (*B2M*). Conversely, in the multiplex setting, ABE8e achieved high editing rates (70% +/- 30%) compared to only 9% (+/- 8%) for Cas9. Cell toxicity was nearly undetectable with ABE8e but was notably higher with Cas9, as evidenced by lower cell recovery and a (5-35x fold) induction in levels of p21 transcription, a protein involved in DNA damage response and cell cycle arrest. This result confirmed substantial impacts on genomic stability, stress, and DNA damage with Cas9 that are absent with ABE8e. Furthermore, cytogenetic analysis of Cas9-edited populations revealed chromosomal abnormalities, inversions, and random loss events linked to targeted loci that were notably absent in cells edited with ABE8e. These findings confirm that base editing allows single-step, highly multiplexed editing of human iPSCs without the genotoxicity associated with nuclease-induced DSBs. Our platform supports the

rapid production of highly multiplex-engineered iPSCs using ABE8e, which is ideal for the in vitro characterization of diverse engineered immune cells with enhanced function for cancer immunotherapy and beyond. After the differentiation of these cells, we observed better Haemopoietic (CD34+CD43+) and improved NK cell fate (CD56+CD7+CD3-), along with significantly higher cytotoxicity.

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Chapter 1: Introduction

Induced Pluripotent Stem cells:

The ability to reprogram somatic cells into a pluripotent state, [1], has ushered in a new era in stem cell research and regenerative medicine. The discovery of induced pluripotent stem cells (iPSCs) has revolutionized our understanding of cellular plasticity and created opportunities for innovation in personalized medicine, disease modeling, and drug discovery [2].

The generation of iPSCs involves the introduction of a defined set of transcription factors, known as the Yamanaka factors (Oct4, Sox2, Klf4, and c-Myc), into somatic cells, such as skin fibroblasts or blood cells [1, 3]. This reprogramming process induces de-differentiation of the somatic cells, reverting them to a pluripotent state highly similar to ESCs. The resulting iPSCs possess hallmark characteristics of pluripotency, including the ability to self-renew and differentiate into cells of all three germ layers (ectoderm, mesoderm, and endoderm) [4, 5].

One of the most significant advantages of iPSCs over ESCs is their derivation from somatic cells, eliminating the need for the destruction of human embryos, which has been a major ethical concern in stem cell research [6]. Additionally, iPSCs can be generated from virtually any individual, enabling the creation of patient-specific cell lines that carry the unique genetic background and disease-associated mutations [7]. This personalized approach holds immense potential for studying the molecular mechanisms underlying genetic disorders, developing tailored therapeutic interventions, and advancing the field of precision medicine.

Applications in Disease Modeling and Drug Discovery

iPSCs have emerged as a powerful tool for modeling a wide range of human diseases, including neurological disorders [8], cardiovascular diseases [9], metabolic disorders [10], and various forms of cancer [11]. By reprogramming somatic cells from patients with specific genetic

conditions, researchers can generate iPSC-derived cell types that recapitulate the disease phenotype, enabling the study of disease pathogenesis in a controlled *in vitro* environment. These disease-specific iPSC models have proven invaluable for elucidating the molecular and cellular mechanisms underlying disease progression, identifying potential therapeutic targets, and evaluating the efficacy and toxicity of candidate drugs [12, 13].

One significant advantage of iPSC-based disease modeling is the ability to capture the genetic complexities of individual patients, allowing for the investigation of disease mechanisms in a context-specific manner [14]. This approach has the potential to unveil novel pathways and molecular signatures that may have been overlooked in traditional animal models or cell culture systems.

Furthermore, iPSC-derived cells can be used for high-throughput screening of small molecule libraries, facilitating the discovery of new therapeutic compounds and accelerating the drug development process [15]. By evaluating the effects of potential drug candidates on patient derived cells, researchers can gain valuable insights into the efficacy, toxicity, and mechanism of action of these compounds, ultimately improving the chances of successful clinical translation [16].

Challenges and Future Perspectives

While the potential of iPSC technology is undeniable, several challenges remain to be addressed before its full potential can be realized. One of the primary concerns is the risk of genetic and epigenetic abnormalities that may arise during the reprogramming process or subsequent differentiation steps [17]. These aberrations can potentially affect the fidelity of disease modeling and the safety of iPSC-derived cells for therapeutic applications.[18]

Additionally, the precise control of differentiation into specific cell types remains a significant challenge, as the molecular mechanisms governing cell fate determination are not fully understood [18]. Researchers are actively exploring various strategies, such as the use of small molecules, growth factors, and three-dimensional culture systems, to improve the efficiency and reproducibility of directed differentiation protocols [19, 20].

Another area of active research is the development of gene editing technologies, such as CRISPR/Cas9, to correct disease-causing mutations in patient-derived iPSCs [21]. This approach holds promise for generating isogenic controls and creating genetically corrected cell lines for therapeutic applications, further enhancing the utility of iPSCs in regenerative medicine.

To ensure the safe and effective translation of iPSC-based therapies into clinical practice, it is essential to establish robust quality control measures and regulatory frameworks [22]. Collaborative efforts between the scientific community, regulatory agencies, and industry partners have been established to develop standardized protocols for the generation, characterization, and long-term preservation of iPSCs, as well as guidelines for their clinical application [23, 24].

The discovery of iPSC has ushered in a new era of personalized medicine, offering unprecedented opportunities for understanding human biology, modeling complex diseases, and developing novel therapeutic strategies [25]. While challenges remain, ongoing research efforts and interdisciplinary collaborations are paving the way for the realization of the full potential of iPSC technology, ultimately leading to improved health outcomes and personalized treatments for patients worldwide.

Non-Viral Genome Engineering of Induced Pluripotent Stem cells:

Non-viral genome engineering techniques have emerged as promising alternatives to viral vectors for modifying the genome of iPSCs. These methods mitigate the risks associated with viral vectors, such as random insertional mutagenesis and potential immunogenicity, while offering precise and efficient genome editing capabilities. Among the non-viral approaches, the CRISPR/Cas9 system and its variants, including base editors, have gained significant attention for their versatility and ease of use.

By utilizing iPSCs, researchers can generate genetically modified immune cells with improved functionality within the tumor microenvironment (TME)[25]. CRISPR/Cas9 can be employed to disrupt the expression of inhibitory receptors and checkpoint molecules that are often upregulated in the TME, thereby overcoming the suppressive signals that dampen immune cell cytotoxicity [26, 27]. Additionally, iPSCs can be engineered to express chimeric antigen receptors (CARs) or other therapeutic transgenes, enhancing the targeting specificity and potency of the derived immune cells against tumor cells [28, 29].

CRISPR/Cas9: A Versatile Genome Editing Tool for iPSCs

The CRISPR/Cas9 system has revolutionized genome editing by providing a powerful and flexible tool for introducing targeted genetic modifications[30]. In the context of iPSCs, CRISPR/Cas9 has been widely adopted for various applications, including gene knockout, gene correction, and targeted integration of transgenes [30, 31].

One of the primary advantages of using CRISPR/Cas9 for iPSC genome engineering is its ability to precisely target specific genomic loci with high efficiency. The system relies on a guide RNA (gRNA) that directs the Cas9 nuclease to the desired target sequence, where it creates a double-strand break (DSB) [32]. The resulting DSB can then be repaired through either non-

homologous end joining (NHEJ) or homology-directed repair (HDR) pathways, enabling gene knockout or precise gene editing, respectively [33].

Numerous studies have demonstrated the successful application of CRISPR/Cas9 for engineering iPSCs, including the correction of disease-causing mutations [34], the generation of reporter cell lines [35], and the targeted integration of transgenes for cellular therapies [36]. Moreover, the CRISPR/Cas9 system has been used to generate isogenic iPSC lines, which are invaluable for studying the effects of specific genetic alterations in a controlled manner [37].

Base Editors: Precise Single-Base Modifications in iPSCs

While CRISPR/Cas9 is a powerful tool for introducing DSBs, base editors offer an alternative approach for precise single-base modifications without the need for DSB formation or donor DNA templates. Base editors are fusion proteins that couple a Cas9 nickase (Cas9n) to a deaminase enzyme, enabling the direct conversion of one base to another [38]. CRISPR-Cas9 genome editing, while revolutionary, faces several significant challenges and potential drawbacks. Off-target effects, where unintended genomic sites may be modified, can cause unforeseen consequences. Cas9- DSBs can result in large deletions and complex chromosomal rearrangements, potentially disrupting gene function beyond the targeted site. The cellular response to Cas9-induced DSBs presents another major issue. Additionally, p53-mediated DNA damage response can lead to cell cycle arrest or apoptosis in edited cells. This not only reduces editing efficiency but also risks selecting for p53-deficient cells, which could promote cancer development. Additionally, there is a potential for translocations, rearrangements, and other associated forms of genomic instability [21,60,67,119].

Two main classes of base editors have been developed: cytosine base editors (CBEs) and adenine base editors (ABEs). CBEs catalyze the deamination of cytosine (C) to uracil (U), which

is subsequently repaired to a thymine (T), effectively converting C base pairs to T [39]. Conversely, ABEs deaminate adenine (A) to inosine (I), which is recognized as guanine (G) during DNA replication, resulting in the conversion of A base pairs to G [40].

The application of base editors in iPSCs has gained significant traction due to their ability to introduce precise single-nucleotide modifications without the potential risks associated with DSB formation, such as unwanted indels or large deletions [41]. One major concern is the activation of p53-mediated responses due to Cas9-induced double-strand breaks (DSBs), which can lead to cell cycle arrest or apoptosis, reducing editing efficiency and potentially promoting cancerous transformations in p53-deficient cells [86]. Additionally, DSBs can trigger chromothripsis, causing unpredictable genomic rearrangements and instability, particularly in cells with compromised DNA repair mechanisms. Off-target effects pose another critical issue, where Cas9 may induce unintended edits at non-target sites, potentially disrupting critical genes involved in cancer regulation [38]. Repair of DSBs can also result in large deletions, chromosomal translocations, and mitochondrial DNA alterations, further complicating therapeutic applications [60,67]. Base editors have been successfully employed in iPSCs for correcting disease-causing point mutations [42].

In a recent report, Liu's team reported prime editor (PE), further expanding our ability to precisely engineer DNA without inducing DSBs [58]. PE uses a reverse transcriptase fused to dCas9 and Prime editing guide RNA (pegRNA) contains a sequence to be introduced. PE is capable of introducing all possible transversion and transition mutations, as well as small insertions and deletions [58]. At its current stage, PE has only been tested in a very limited number of mammalian cells, including 293T and K562, with up to 70% and 30% editing efficiency, respectively. Moreover, designing pegRNAs for prime editing can be complex and requires careful

consideration of sequence specificity and efficiency. Unlike CRISPR/Cas9 and base editors, which have established protocols and tools for guide RNA design, pegRNA design for prime editing demands meticulous optimization and validation, which can limit its widespread adoption and utility in different cell types, including iPSCs. Furthermore, the delivery efficiency of PE components into target cells, especially iPSCs and other difficult-to-transfect cell types, remains a significant hurdle. Ensuring uniform uptake and sufficient expression of PE components within the cells is critical for achieving reliable editing outcomes without off-target effects.[59]. However, sharing a similar mechanism as BE, it is worth testing PE in iPSCs.

In summary, non-viral genome engineering techniques, particularly CRISPR/Cas9 and base editors, have emerged as powerful tools for precisely modifying the genome of iPSC. These approaches offer several advantages over viral vectors, including reduced risks of insertional mutagenesis, transient expression, and the ability to multiplex genetic modifications. The successful application of non-viral genome engineering in iPSCs has enabled various applications, including disease modeling, gene therapy, and cellular engineering for cancer immunotherapy. While challenges remain, ongoing research efforts are continuously refining and optimizing these techniques, paving the way for safer and more efficient genome editing in iPSCs for translational applications.

Induced Pluripotent Stem Cell Transfection Strategies

Transfection is a crucial technique for delivering genome editing tools and exogenous genetic materials into iPSCs. Non-viral transfection methods offer several advantages over viral vectors

including a reduced risk for random integration into the host genome, thereby minimizing the potential for insertional mutagenesis and associated safety concerns [45]. Additionally, non-viral delivery systems typically result in transient expression of the genome editing components, reducing the likelihood of sustained off-target effects or unwanted long-term expression [46]. CRISPR/Cas9 and base editors can be easily multiplexed to target multiple loci simultaneously, enabling more complex genetic modifications in a single step [47]. Moreover, these techniques can be applied to a wide range of cell types, including difficult-to-transfect cells, extending their utility beyond iPSCs [48].

However, several challenges and limitations must be considered when employing non-viral genome engineering in iPSCs. Achieving high delivery efficiency and uniform uptake of the genome editing components can be challenging, particularly in difficult-to-transfect cell types [49]. While CRISPR/Cas9 and base editors are generally more specific than previous genome editing technologies, careful design and validation of guide RNAs are necessary to minimize off-target effects [50]. Incomplete editing can lead to a mosaic population of edited and unedited cells, requiring additional screening or enrichment steps [51]. Although non-viral delivery methods are generally less immunogenic than viral vectors, potential immune responses against the delivered components should be evaluated, especially in therapeutic applications [52]. Addressing these considerations will be crucial for harnessing the full potential of non-viral genome engineering in iPSCs while ensuring safety and efficacy in clinical and research settings. Despite these challenges, the continuous development of improved delivery methods, optimized gRNA design, and advanced screening techniques address many of the limitations associated with non-viral genome

engineering in iPSCs, the two commonly employed non-viral transfection methods for iPSCs are lipofection and electroporation.

Lipofection involves encapsulating nucleic acids or proteins within cationic liposomes, which can fuse with the cell membrane and release their cargo into the cytoplasm [53]. Several studies have successfully utilized lipofection for delivering CRISPR/Cas9 components and other genetic materials into iPSCs. For instance, Chen et al. [85] employed lipofection to deliver CRISPR/Cas9 ribonucleoproteins (RNPs) into iPSCs, achieving efficient gene editing with minimal off-target effects. Similarly [86] used lipofection to introduce CRISPR/Cas9 RNPs and donor templates for homology-directed repair, enabling precise gene correction in iPSCs.

Electroporation, on the other hand, relies on the application of electrical pulses to create transient pores in the cell membrane, allowing the entry of nucleic acids or proteins into the cytoplasm [83,84]. This technique has been widely used for delivering CRISPR/Cas9 components and other genetic materials into iPSCs. Mandegar et al. utilized electroporation to introduce CRISPR/Cas9 RNPs and single-stranded oligodeoxynucleotide donors for gene editing in iPSCs, enabling the generation of isogenic disease models and genetically corrected cell lines. Similarly, Kwart et al. employed electroporation to deliver CRISPR/Cas9 RNPs and donor templates for precise gene editing in iPSCs, demonstrating efficient gene correction and transgene integration. [83,84]

While both lipofection and electroporation have been successfully employed for genetic engineering of iPSCs, electroporation generally offers higher transfection efficiencies and better control over the delivery of genetic materials [54]. However, electroporation can also be more

cytotoxic, and optimization of parameters is crucial to maintain cell viability and pluripotency [55].

It's important to note that while transfection methods enable efficient genetic engineering of iPSCs, careful consideration must be given to the potential off-target effects, mosaicism, and potential epigenetic changes introduced during the process. Rigorous screening and characterization of genetically modified iPSC lines are essential to ensure their safety and efficacy for downstream applications, such as disease modeling and cell therapy [56, 57].

iPSC Hematopoietic Differentiation

iPSCs possess remarkable potential to differentiate into various hematopoietic cell types, offering promising avenues for understanding immune system development and therapeutic applications. Differentiation of iPSCs into hematopoietic stem and progenitor cells (HSPCs) typically involves multi-step processes mirroring embryonic hematopoietic development. Various protocols have been devised that involve modulating signaling pathways and employing specific growth factors and cytokines to guide differentiation.

Crucial to iPSC hematopoietic differentiation is mimicking the developmental stages of *in vivo* hematopoiesis [87]. Recent advancements incorporate modulation of Wnt activation and/or activin inhibition during early mesoderm formation, yielding cells akin to erythro-myeloid progenitors (EMPs) formed during fetal liver hematopoiesis and with similarity to adult hematopoiesis [88]. Among these methods, the improved 2D-multistep approach developed by members of our laboratory stands out for its efficiency and robustness. This method has been shown to produce significantly higher numbers of CD34⁺ progenitor cells, CD34⁺CD45⁺ hematopoietic progenitors, and functional hematopoietic progenitors, as assessed by colony-

forming unit (CFU) assays, compared to other commonly used methods. Additionally, the 2D-multistep approach is more time- and cost-effective, making it a practical choice for large-scale research and potential therapeutic applications.

The ability to generate HSPCs from patient-derived iPSCs has significant implications for studying genetic hematological disorders. While significant progress has been made in the differentiation of iPSCs into HSPCs, several challenges remain [89]. These include achieving higher purity and yield of HSPCs, ensuring long-term engraftment potential, and faithfully recapitulating the complex regulatory mechanisms involved in hematopoietic development. Ongoing research efforts are focused on addressing these challenges and further refining the differentiation protocols to generate HSPCs with improved functional properties and clinical relevance.

improved 2D-multistep method has proven to be a robust and efficient approach for generating HSPCs. Additionally, standardization and direct comparative reporting of iPSC hematopoietic differentiation methods will facilitate stepwise improvements and ensure the faithful recapitulation of normal and aberrant hematopoietic development [90], increasing the versatility and sensitivity of iPSC-based models for medical research and future therapeutic applications.

Induced Pluripotent Stem Cells: Differentiation into Immune Cells

One of the most intriguing applications of iPSCs is their ability to differentiate into various immune cell types, including natural killer (NK) cells, T cells, macrophages, and B cells. This has opened up new opportunities for understanding the development and function of the immune system, as well as providing potential therapeutic opportunities.

Differentiation of iPSC to natural killer cells

The ability to generate induced NK (iNK) cells from iPSCs has been a significant breakthrough in the field of immunotherapy. iNK cells possess the cytotoxic properties of their primary counterparts, but while primary NK cells are effective, their use is limited by several factors. iNK cells offer distinct advantages over primary NK cells, including the potential for large-scale production, enhanced consistency in cell quality, and the ability to genetically modify the cells more easily. Additionally, iNK cells can be derived from patient-specific iPSCs, potentially reducing the risk of rejection and allowing for more personalized treatments., making them attractive candidates for cancer immunotherapy and viral infections.[26,91]

Several studies have developed protocols for the efficient differentiation of iPSCs into iNK cells. One such study by Knorr et al. (2013) [91] described a three-stage protocol involving the formation of embryoid bodies, co-culture with stromal cells, and cytokine stimulation. The resulting iNK cells exhibited phenotypic and functional characteristics similar to primary NK cells, including cytotoxicity against cancer cell lines.

Another study by Zeng et al. (2017) [92] employed a feeder-free, serum-free system to generate iNK cells from iPSCs. The protocol involved the use of defined cytokines and small molecules, resulting in a highly pure population of functional iNK cells capable of targeting cancer cells *in vitro* and *in vivo*.

Additionally, there are other methods for generating iNK cells from iPSCs. For instance, a study by Post M et al. (2017) [93] utilized a lentiviral transduction system to introduce NK cell-specific transcription factors into iPSCs, resulting in the direct conversion of iPSCs into iNK cells. This approach bypasses the intermediate stages of differentiation and may offer advantages in terms of efficiency and purity.

Differentiation into iT cells

The generation of induced T cells (iT cells) from iPSCs has been an area of active research, as it holds promise for developing cell-based therapies for various immune disorders and cancers.

One notable study by Nishimura et al. (2013) [94] demonstrated the successful differentiation of iPSCs into functional iT cells. The protocol involved the co-culture of iPSCs with OP9 DLL stromal cells and the addition of specific cytokines and growth factors (SUCH AS?). The resulting iT cells exhibited a diverse T cell receptor (TCR) repertoire and were capable of cytokine production and cytotoxic activity against target cells.

Similarly, Mohtashami M, et al. (2013) [95] developed a protocol for generating iT cells from iPSCs using Notch signaling modulation and cytokine stimulation. The iT cells displayed characteristics of mature T cells, including TCR expression, cytokine production, and cytotoxic activity against cancer cell lines.

Differentiation of iPSC into Macrophages

Cao et al. (2019) [96] reported a highly efficient protocol for generating iMacrophages from iPSCs. The protocol involved the sequential addition of cytokines and small molecules, resulting in the production of iMacrophages with phagocytic activity, cytokine secretion, and functional responses to polarizing stimuli.

Another study by Haenseler et al. (2017) [97] demonstrated the successful differentiation of iPSCs into iMacrophages using a serum-free, feeder-free system. The iMacrophages exhibited characteristics of primary macrophages, including phagocytic activity, cytokine secretion, and responses to inflammatory stimuli (cite).

Differentiation into iB cells

Sturgeon et al. (2015) [88] reported the successful differentiation of iPSCs into iB cells using a stepwise protocol involving the co-culture with stromal cells and exposure to specific cytokines and growth factors. The resulting iB cells exhibited B cell-specific surface markers, immunoglobulin gene rearrangements, and the ability to undergo class-switch recombination.

Similarly, Kwon et al. (2019) [98] developed a feeder-free, serum-free system for generating iB cells from iPSCs. The protocol involved the sequential addition of cytokines and small molecules, resulting in the production of functional iB cells capable of antibody production and antigen-specific responses.

In conclusion, the differentiation of iPSCs into various immune cell types, including iNK cells, iT cells, iMacrophages, and iB cells, has Created Opportunities for studying the immune system, disease modeling, and developing novel therapeutic approaches. These advances have been facilitated by the development of efficient differentiation protocols and the characterization of the functional properties of the resulting immune cell types. However, further research is needed to refine these protocols and ensure the faithful recapitulation of primary immune cell functions.

Chapter 2: Methods

Production of editing reagents

ABE8e plasmid was obtained from Addgene (<https://www.addgene.org/138489/>) and cloned into a pmRNA vector. ABE8e mRNA was produced by Trilink Biotechnologies. CD19 CAR were synthesized by gen script technologies

Guide RNA design

Guide RNAs (sgRNAs) for knockout (KO) were designed using the base editing splice-site disruption sgRNA design program SpliceR (<https://z.umn.edu/splicer>), which is implemented in the R statistical programming language (v. 3.4.3). This tool utilizes a target Ensembl transcript ID, a base editor PAM variant, and a species as inputs. Initially, SpliceR extracts exon and intron sequences from Ensembl and then identifies regions surrounding splice sites within a user-specified window. The N20-NGG motif is matched to the antisense strand of these extracted sequences. The matched patterns are subsequently scored based on the position of the target motif within the editing window, leveraging insights from prior publications. sgRNAs are further scored based on their position within the transcript, with those earlier in the transcript receiving higher scores. For ncCD16a, a specific sgRNA was manually designed to introduce the S197P mutation. This mutation was chosen due to its known functional impact on the target protein. The manual design process ensures that the sgRNA efficiently targets the desired site and minimizes off-target effects. By focusing on the specific genomic context and optimizing the sgRNA sequence, the design aims to maximize editing efficiency while maintaining genomic integrity. This approach integrates computational predictions with manual curation to achieve precise genome editing outcomes.

Cell lines and tissue culture

Two commercially available iPSC lines were used in this study, one derived from a fibroblast, 1210 iPSCs (ATCC) and an $\alpha\beta$ T IPS line (Stem Cell Technologies) were cultured on geltrex coated plates maintained in MTeSR plus media (Stem Cell Technologies), and were passaged every 3 days (70% confluency).

iNK production from human iPSC

On day 1, a 24-well plate was coated with the lymphoid differentiation coating material (Stem Cell Technologies). On day 0, fifty thousand iHPCs (CD34+/CD43+) were transferred to each well of the 24-well plate. The NK differentiation medium was prepared by supplementing IMDM-based medium (ThermoFisher) with human platelet lysate (5%) (Biomedical EliteCell Corp.), glutamate (1%) (ThermoFisher), and penicillin/streptomycin antibiotic solution (1%) (Gibco). Additionally, IL-3 (5 ng/mL) was added for the first week of differentiation, along with SCF (20 ng/mL), IL-7 (20 ng/mL), IL-15 (10 ng/mL), and Flt3 ligand (Flt3L) (10 ng/mL) (PeproTech). The medium was refreshed every 2-3 days, replacing half with fresh NK differentiation medium. Weekly assessments were conducted to monitor NK cell differentiation and viability (CD56+/CD7+/CD3-), adjusting the medium volume and supplement concentrations as necessary. On day 14, all the cells were harvested and plated onto plastic at a density of one hundred thousand cells. We have found that this re-plating of the cells prevents overcrowding of these iNK progenitors and leads to a better NK-like phenotype. The differentiation was continued in the same media with the same assessment strategy. The endpoint of the protocol involved harvesting the differentiated iNK cells after 35 days of initial seeding and expanding them with feeder-based approaches for downstream applications and functional analysis of these iPSC-derived mature NK cells. It's worth noting that

a seeding density of fifty thousand iPSCs per well in the 24-well plate was found to be optimal for efficient differentiation.

Antibodies and flow cytometry

The following antibodies and dyes were used: APC-, FITC-, or BV650-conjugated anti-CD56 (clone 5.1H11, BioLegend; clone REA196, Miltenyi Biotec; or clone HCD56, BioLegend), FITC-conjugated anti-CD3 (clone OKT3; BioLegend), PE/Cy7-conjugated anti-PD-1 (clone EH12.2H7; BioLegend), eFluor 450- or Alexa Fluor™ 700-conjugated anti-TIGIT (clone MBSA43; eBioscience), PE/Dazzle™ 594- or FITC-conjugated anti-human CD45 (clone 14C2A07, BioLegend; clone SA231A2, BioLegend), PE/Cy7 anti-human CD16 (clone 3G8, BioLegend), BV421-conjugated anti-human CD45 (clone 2D1; BioLegend), BV605-conjugated anti-mouse CD45 (clone 30-F11; BioLegend), Brilliant violet 421-conjugated anti-IFN γ (clone 4S.B3; BioLegend), Alexa Fluor™ 700-conjugated anti-TNF- α (clone MAb11; BioLegend), PE-conjugated anti-CD34 (clone QBEnd-10, Invitrogen), APC-conjugated anti-PD-L1 (clone B7-H1, BioLegend), FITC-conjugated anti-CD155 (clone SKII.4, BioLegend), FITC-conjugated anti-DNAM-1 (clone 11A8, BioLegend), Brilliant violet 510-conjugated anti-CD161 (clone HP-3G10, BioLegend), PE/Cyanine7-conjugated anti-Tim3 (clone F38-2E2, BioLegend), APC-conjugated anti-NKG2D (clone 1D11, BioLegend), PE-conjugated anti-NKG2A (clone S19004C, BioLegend), Alexa Fluor™ 700-conjugated anti-NKp46 (clone 9E2, BioLegend), PE/Dazzle™ 594-conjugated anti-NKp30 (clone P30-15, BioLegend), FITC-conjugated anti-CD107a (clone H4A3; BD Biosciences), SYTOX Blue dead cell stain (ThermoFisher), and Fixable viability dye eFluor 780 (eBioscience). Flow cytometry was performed on a CytoFLEX S flow cytometer (Beckman Coulter), and all data were analyzed using FlowJo version 10.4 software (FlowJo LLC).

Derivation of CD34⁺ HSPC from human iPSC:

To derive CD34⁺ hematopoietic stem and progenitor cells (HSPCs) from human iPSCs, we initiated hematopoietic differentiation by plating cells onto Matrigel-coated plates in mTeSR1 medium on Day 1. The following day, iPSCs were washed and transferred to basal hematopoietic differentiation medium supplemented with SCF, BMP4, VEGF, and FGF2. From Days 5 to 13, a Minimal Cytokine media with bFGF. After 13 days of differentiation, we harvested cells by gently washing them with a spent differentiation medium to collect floating cells. Flow cytometry analysis showed an increase in the number of CD34⁺CD43⁺ and CD34⁺CD45⁺ cells. Subsequently, Day 13 iPSCs were used for further experiments or cultured in a colony forming unit (CFU) assay for 10-14 days to assess their potential to form hematopoietic colonies.

iPSC electroporation

Electroporation was performed using the Neon Transfection System (ThermoFisher) with iPSCs resuspended in the provided electroporation buffer. For each condition, 1×10^6 cells were combined with the designated amounts (1 million) of unincubated plasmid DNA and transposase enzyme in a total volume of 100 μ L. The cell/DNA/enzyme mixture was electroporated with two 20 ms pulses at 1200 V. Immediately after electroporation, the cells were transferred to 6-well plates pre-coated with Geltrex basement membrane matrix (ThermoFisher) and cultured in mTeSRTMPlus medium (STEMCELL Technologies) supplemented with rock inhibitor Y-27632 (Selleck Chemicals). The electroporated cells were cultured for 48 hours before analysis or further differentiation.

Primer and sequence

Primer Name	Primer Sequence
AHR Exon 2 Splice Donor forward	TAC CAT GCA TCA TTT CAG TG
AHR Exon 2 Splice Donor reverse	TTT CAG AGT AAA GCC AAT CC
CISH Exon 2 Splice Donor forward	AAT TAG CTG GGG TAA CCA AT
CISH Exon 2 Splice Donor reverse	CCT TCT AGA CCT CGT CCT TT
TIGIT Exon 1 Splice Donor forward	GTC TGC AAA GTC CTT CAT CT
TIGIT Exon 1 Splice Donor reverse	ATA TGG TTT TTG CCA AAC TT
KLRG1 Exon 4 Splice Donor forward	CCC AAA CAG CAG AAG AAT TA
KLRG1 Exon 4 Splice Donor reverse	TAG GAA TCC AAT GTG GAA AG
PD-1 Exon 1 Splice Donor forward	ACC CTC CCT TCA ACC TGA CC
PD-1 Exon 1 Splice Donor reverse	AAG CCA CAC AGC TCA GGG TA
CD16A S197P mutation forward	TAA AGG ATA TAC GAG ATT AA
CD16A S197P mutation reverse	AAC TGG GTA ATT TAT AAC
B2M ex. 1 SD forward	CCTAGAATGAGCGCCCGGTG
B2M ex. 1 SD reverse	CTTCCCCGAGATCCAGCCCT
TGFbR2 DN forward	AAGGCAGCTCTGGGGTTTGG
TGFbR2 DN reverse	ACCCCTGGAATAATGCTCGAAG
Fas DN forward	GTGTTATGTATTGTGGCCCAGGT
Fas DN reverse	TGCCAATTACGAAGCAGTTGAAC

Genomic DNA analysis

The DNA isolation was performed on iPSCs harvested 10 days after electroporation, followed by PCR amplification of CRISPR-targeted loci using accuprime Taq DNA polymerase (Thermofisher). Base editing efficiency at the genomic level was assessed by Sanger sequencing of the PCR amplicons. The Sanger sequencing traces were analyzed using the web app EditR (baseeditr.com) and ICE by synthego to determine the editing outcomes.

NK cell cytotoxicity assays:

Cancer cell lines (Burkitt's Lymphoma cell line Raji) were seeded into a black round-bottom 96-well plate at a density of 50,000 cells per well. NK cells were added to the wells in triplicate at the

indicated effector-to-target (E:T) ratios. Wells containing only target cells served as a negative control for spontaneous cell death, while wells with target cells treated with 1% Triton X-100 served as a positive control for maximum killing. Co-cultures were then incubated at 37°C for 48 hours. After incubation, D-luciferin (potassium salt; Gold Biotechnology, St. Louis, MO) was added to each well at a final concentration of 25 µg/mL and incubated for 5 minutes with gentle shaking. Luminescence was measured in endpoint mode using a BioTek Synergy microplate reader.

Chapter 3: Results

Highly Efficient Single Gene Beta-2-Microglobulin (B2M) Knockout in Human iPSC's Using ABE and CRISPR/CAS9 Technology

Our initial investigation aimed to determine if single gene KOs could be effectively achieved using BEs in iPSCs, as BE offer the ability to achieve highly efficient gene KOs without inducing DSBs seen in Cas9 [60-65].

while assessing any subsequent impacts on cell functionality. Enhancing iPSC-based therapies often involves the suppression of inhibitory signals. This approach aims to optimize the function and efficacy of iPSC-derived cells in therapeutic applications. Inhibitory signals can include checkpoint molecules, such as PD-1 or CTLA-4, which normally regulate immune responses. In the context of cell therapies, particularly those targeting cancer, suppressing these inhibitory pathways can unleash the full potential of the engineered cells. This may involve genetic modifications to knock out genes encoding inhibitory receptors, or to enhance the expression of stimulatory molecules. Additionally, suppression of inhibitory signals can extend to modifying signaling pathways that limit cell persistence, proliferation, or effector functions. By carefully tuning these molecular brakes, more potent and durable cellular therapies could be generated while maintaining safety and specificity. Therefore, we sought to validate the editing of Beta-2-microglobulin (*B2M*) in iPSCs using both BEs and CRISPR/Cas9, as previous studies, such as those by Lotfi M et al. [99] have demonstrated the use of CRISPR/Cas9 for human iPSC editing. For this purpose, we used a ratio of 1 μ g:1 μ g ABE8e/Cas9: sgRNA.

We designed single guide RNAs (sgRNAs) targeting a splice donor (SD) site, found to be the most efficient for gene disruption, using SpliceR, an in-house-developed online BE sgRNA design and prediction tool [65]. The sgRNA was co-transfected with ABE8e mRNA and in tandem with CRISPR/Cas9. KO efficiency was assessed at the genomic level through flow cytometry and

Sanger sequencing. The quantification of A-to-G conversion rates was performed using EditR software (**Figure 3**) [66], while Cas9 indels were quantified using Synthego Performance Analysis, ICE Analysis. 2019. v3.0. Synthego [115], which directly correlates to the editing efficiency. We found the editing efficiency using BEs was significantly higher compared to CRISPR/Cas9-mediated editing (**Figure 3B, C**). This highlights the potential of BEs as a versatile tool for gene editing in iPSCs, offering lower incidences of deleterious DSBs and superior editing outcomes.

Following the validation of *B2M* editing with both BEs and CRISPR/Cas9, we compiled a panel of targets primarily consisting of intracellular and extracellular inhibitory proteins expressed in NK and T cells (**Table 1**). The panel of targets encompasses a diverse array of inhibitory proteins expressed in natural killer (NK) cells and T cells, which play crucial roles in regulating immune cell functions, each possessing distinct mechanisms of action with significant therapeutic implications.

The intracellular checkpoints include the Aryl Hydrocarbon Receptor (AHR) and Cytokine-Inducible SH2-Containing Protein (CISH). AHR is a ligand-activated transcription factor that negatively regulates NK cell cytotoxicity upon binding to specific agonists like kynurenine, commonly present in the tumor microenvironment (TME)[99]. By modulating AHR signaling, it is possible to enhance NK cell-mediated anti-tumor responses [100]. CISH acts as a negative regulator of interleukin-15 (IL-15) signaling, which is crucial for NK cell development, survival, and effector functions. Targeting CISH could potentially augment NK cell responses by enhancing IL-15 signaling pathways [101].

The targeted extracellular checkpoints include proteins such as Killer Cell Lectin-like Receptor G1 (KLRG1), T-cell Immunoreceptor with Ig and ITIM Domains (TIGIT), Programmed Cell Death Protein 1 (PD-1), and CD16a (Fc γ RIIIa). KLRG1 is an inhibitory immune checkpoint receptor expressed on various immune cells, including NK cells and T cells. It plays a role in suppressing immune responses and has been implicated in tumor immune evasion [100]. Blocking KLRG1 signaling may restore anti-tumor immunity and enhance the efficacy of cancer immunotherapies [102,103]. TIGIT, another inhibitory immune checkpoint receptor, is expressed on NK cells, T cells, and other immune cell subsets. It suppresses immune responses by binding to its ligands, such as CD155 and CD112, which are often upregulated in various cancers[104]. Targeting TIGIT has shown promising results in enhancing anti-tumor immunity and improving clinical outcomes in cancer immunotherapy [104,105]. PD-1 is a well-established immune checkpoint receptor expressed on T cells, NK cells, and other immune cells. It plays a crucial role in regulating immune responses and preventing autoimmunity. However, in the context of cancer, PD-1 signaling can be exploited by tumors to evade immune surveillance. Blocking PD-1 or its ligands (PD-L1/PD-L2) has been a successful strategy in cancer immunotherapy, leading to improved clinical outcomes [106,107]. CD16a is a low-affinity Fc receptor expressed on NK cells and other immune cells, playing a crucial role in antibody-dependent cell-mediated cytotoxicity (ADCC). This mechanism enables NK cells to recognize and eliminate antibody-coated target cells. Modulating CD16a expression or function could potentially enhance ADCC-mediated anti-tumor responses [108,109].

In addition to these checkpoints, the panel includes genetic modifications such as mutations in *FAS* Enhance resistance to apoptosis to prolong NK persistence, *TGF β R2* Block inhibitory cytokines

to boost proliferation and function, and β 2-microglobulin (*B2M*) responsible for Disruption of MHC-I interaction to prevent inhibition by tumor cells and cloaking in the autologous setting through removal of HLA-I [110-114]

Highly Efficient Single Gene Knockout in Human iPSCs Using ABE and CRISPR/Cas9 Technology

We next assessed the editing efficacy of BE and CRISPR/Cas9 across nine target genes representing inhibitory proteins that can be modified to enhance immune cell function. Our findings revealed that BE provided higher single gene KO editing efficiencies than CRISPR/Cas9 (**Figure 4B-E**). During our initial optimization efforts [60], we titrated the amounts of the enzymes (ABE8e/Cas9) and their respective guides (sgRNA). Through this process, we determined that a ratio of 1.5 μ g:1 μ g and 7.5 μ g:1 μ g for ABE8e/Cas9: Guides was optimal for achieving these edits. Each sgRNA was co-transfected with either ABE8e mRNA or Cas9 mRNA, leading to significant editing efficiency across all targeted genes.

Specifically, the editing efficiencies for both Cas9-edited and BE-edited cells of 1.5 μ g:1 μ g (ABE8e/Cas9): (sgRNA) were assessed across multiple gene targets. Generally, BE-edited cells demonstrated higher and more consistent editing efficiencies compared to Cas9-edited cells. Notably, BE-editing achieved 100% efficiency for the *B2M* target, while most other targets showed efficiencies above 80%. In contrast, Cas9-editing efficiencies were more variable, ranging from approximately 57% to 89% (**Figure 4B, C**). Comparing Cas9 and BE efficiencies demonstrated that BE achieved higher and more consistent editing efficiencies across most genes, specifically *KLRG1*, *TIGIT*, *AHR*, *CD16a*, *CISH Ex.2 SD*, and *PD1*. Notably, the BE cells achieved significantly higher editing efficiency for *B2M*, with zero variability, marking an exceptional

improvement over the Cas9 edited Group. Conversely, *TGFBR2* BE editing efficiency displayed increased variability despite similar mean values, suggesting the need for further optimization. Additionally, *FAS DN* showed slightly lower mean efficiency and higher variability using BE compared to Cas9. Of note the BE-engineered cells displayed greater health and performance, likely a direct result from the lack of DSB that are present in Cas9 edited populations (**Figure 4B, C**).

Building on this, we further assessed the editing efficiencies of the same target genes using a concentration ratio of 7.5 μg ABE8e/Cas9 to 1 μg sgRNA; For the Cas9-treated cells, the observed editing efficiencies were: KLRG1 (82 ± 3.06), TIGIT (62.33 ± 9.29), AHR (83.67 ± 0.47), CD16a (77.33 ± 0.47), CISH Ex.2 SD (56.33 ± 2.49), PD1 (71.67 ± 3.06), B2M (98.67 ± 1.25), TGFB DN (76.33 ± 1.25), and FAS DN (80.67 ± 0.94). In comparison, BE-treated cells demonstrated higher efficiencies for most genes: KLRG1 (92 ± 1.25), TIGIT (94.33 ± 0.47), AHR (87 ± 0.47), CD16a (94 ± 1.41), CISH Ex.2 SD (97 ± 2.36), PD1 (89.33 ± 2.05), B2M (100 ± 0), TGFB DN (78 ± 1), and FAS DN (77.67 ± 1.70).

These findings further highlight the superior performance of BE over Cas9, even in the presence of a higher concentration of the Cas9 enzyme. Specifically, genes such as TIGIT, CD16a, CISH Ex.2 SD, PD1, and AHR showed markedly higher mean BE editing efficiencies, along with consistently lower standard deviations, underscoring the enhanced precision and reliability of BE. Moreover, the higher editing efficiency achieved for *B2M* in the BE cells, maintained across both concentrations, underscores the robustness of the BE. (**Figure 4D, E**) These results reinforce the conclusion that BE offers substantial improvements in editing efficiency and consistency, making it a more effective and reliable approach (**Figure 4F**).

Since all experiments were conducted using the 1210 iPSC line derived from adult fibroblasts, we aimed to replicate these findings in a different cell line, specifically an alpha-beta T cell-derived iPSC line. The objective was to validate the results observed in the 1210 iPSC line across this new cell type.

Comparing Cas9 and ABE8e-mediated editing in the alpha-beta T cell-derived iPSC line revealed distinct editing efficiency patterns across the target genes. Cas9-mediated editing efficiencies revealed increased variability, with *KLRG1* exhibiting relatively lower efficiencies (61% KO), while *CD16a* and *B2M* showed higher efficiencies (83% and 76% respectively). Notably, TGFB DN had the lowest editing efficiency among the genes tested, approximately 50%.

In contrast, ABE8e-mediated editing exhibited higher and more consistent editing efficiencies. Specifically, *TIGIT*, *CD16a*, and *B2M* demonstrated robust editing efficiencies, ranging from 89% to 94% KO. Additionally, ABE8e-mediated engineering improved efficiency for *KLRG1* and *AHR* compared to Cas9, approximately 83-85% and 81-88%, respectively (**Figure 4G, H**).

Collectively, these findings demonstrate that ABE8e-mediated engineering is more effective than Cas9 in achieving higher and more consistent editing efficiencies across our nine target genes in the alpha-beta T cell-derived iPSC line. This demonstrates BE-mediated engineering ability to efficiently produce robust editing regardless of the iPSC line.

Highly efficient multiplex base editing in iPSCs using ABE

Future advancements in cellular therapies will likely necessitate numerous tailored gene edits to optimize efficacy, especially for tackling challenging solid tumor cancers. Previous studies

have shown that multiplex base editing techniques can improve the functionality of CAR-T cells [67] and NK cells [68] in this context.

Here, we investigated the feasibility of applying a similar approach to develop iPSC-based cell therapies using ABE8e or CRISPR/Cas9 for multiplex editing. We incorporated all nine targeted sgRNAs into a single electroporation master mix, including *ncCD16a*, *AHR*, *KLRG1*, *CISH*, *PDCD1*, *B2M*, *TIGIT*, *TGFB*, and *FAS DN* sgRNAs (**Figure 5A**). Multiplex editing efficiencies at the genomic level were evaluated by Sanger sequencing to quantify A to G conversions at the target sites using EditR for ABE8e-mediated editing (**Figure 5C, D**) and Synthego ICE tool for CRISPR/Cas9-edited groups to quantify indels (**Figure 5E, F**). We used the optimized ratios of 1.5 μg (ABE8e/Cas9):1 μg (sgRNA) and 7.5 μg (ABE8e/Cas9):1 μg (sgRNA) to achieve highly efficient editing.

Robust editing was achieved across all nine targets with ABE8e (**Figure 5**). Comparing ABE8e and CRISPR/Cas9 for multiplex base editing of 1210 adult fibroblast-derived iPSCs at a ratio of 1.5 μg :1 μg (ABE8e/Cas9 sgRNA) reveals significant differences in editing efficiencies across the targeted genes (**Figure 5**). Specifically, ABE8e showed robust editing efficiencies in genes such as *AHR*, *PD1*, and *CD16a*, ranging from approximately 60% to over 90% (**Figure 5C-F**). In contrast, CRISPR/Cas9-mediated editing showed generally lower editing efficiencies, ranging from 0 to 16.2% across all targeted genes (**Figure 5 E,F**). CRISPR/Cas9-mediated engineering did not produce any *TGFbR2 DN* and *CISH Ex.2 SD KO* (**Figure 5E**). Furthermore, in the 7.5 μg :1 μg ABE8e/Cas9: sgRNA group, ABE8e consistently demonstrated higher editing efficiencies. Genes such as *KLRG1*, *TIGIT*, *AHR*, *CD16a*, and *PD1* showed significantly higher mean editing efficiencies, ranging from 65% to 93%.

ABE8e achieved higher editing efficiency for B2M ex. 1 SD, with minimal variability ($99 \pm 1.7\%$). This represents a substantial improvement over Cas9, which showed editing lower than 10% for most of our genes of interest. Specifically, for B2M, Cas9 editing was approximately $16.2 \pm 3.8\%$, the highest editing seen across all nine targets for the Cas9 group. This significantly limits the use of Cas9 in a multiplex setting (**Figure 5C-F**).

Multiplex Base Editing in iPSCs Reduces p53 Response

CDKN1A (p21), a crucial cell cycle regulator, plays a pivotal role in maintaining cell cycle integrity at the G1/S checkpoint. [68] It functions as a potent inhibitor of cell cycle progression in response to various stress signals, including DNA damage and unfavorable growth conditions. [68,69] High levels of p21 lead to cell cycle arrest, a critical process for DNA repair and preventing the propagation of damaged cells. The p21 gene encodes a potent cyclin-dependent kinase inhibitor that plays a crucial role in regulating cell cycle progression and mediating cellular responses to various stress signals. The regulatory role of p21 in cell cycle control makes it significant in understanding diseases with cell cycle dysregulation, particularly cancer. [69]

Initially recognized for its role in cell cycle arrest under the transcriptional regulation of p53, p21 has emerged as a multifunctional protein with diverse roles in cellular processes. Besides its classic tumor-suppressor function, p21 exhibits oncogenic potential and anti-apoptotic effects, which are influenced by its subcellular localization and the status of p53. P53 directly binds to the promoter region of the CDKN1A gene, stimulating p21 expression in response to DNA damage and other stress signals. This dual role of p21 in both inhibiting cell cycle progression and promoting cell survival underscores its complexity and its potential as a target for therapeutic interventions in diseases associated with abnormal cell proliferation and survival [69] (**Figure 6A**).

We wanted to investigate the modulation of p21 expression levels in response to various treatment conditions involving CRISPR-associated components. We ran RT-qPCR to quantify p21 expression. Treatment with the Cas9 only, ABE only, and ABE8e + sgRNA resulted in virtually no change in p21 expression compared to the Pulse-only condition where the cells didn't get any sgRNAs or BE/Cas9 (**Figure 6B**). Strikingly, the combination treatments involving sgRNAs With Cas9 led to a significant upregulation of p21 expression. The most potent induction of p21 expression was observed for Cas9 + gRNA, where p21 expression increased 70-fold compared to the control condition, which is likely attributed to the cellular stress response triggered by the DNA damage and repair mechanisms activated by the CRISPR-Cas9 system (**Figure 6B**). These findings suggest that Cas9-mediated engineering elicits a strong stress response, leading to a significant upregulation of p21 expression. The p21 induction may serve as a molecular brake, arresting cell cycle progression and allowing cells to repair potential genomic lesions or undergo apoptosis in response to the genetic perturbations induced by the CRISPR-associated components.

Karyotypic Characterization of Multiplex Edited iPSC Populations

Karyotyping is a fundamental technique in cytogenetics that plays a crucial role in analyzing the chromosomal composition within cells. The term "karyotype" refers to the complete set of chromosomes found in an individual or species, arranged in a standardized format [71,72]. Karyotyping involves preparing metaphase chromosomes from cells and staining them with specific dyes, such as Giemsa stain. This staining process reveals unique banding patterns that help identify and distinguish chromosomes under a microscope. Through visual examination, cytogeneticists can determine the number, size, and shape of chromosomes within a cell or organism [71]. Thus, karyotyping provides essential insights into genetic health and abnormalities by studying the structure, number, and arrangement of chromosomes. Conventional karyotyping

identifies major chromosomal defects, including aneuploidy, insertions, deletions, duplications, inversions, and reciprocal translocations. It is instrumental in diagnosing conditions characterized by specific chromosomal abnormalities, such as Down syndrome (trisomy 21) and Turner syndrome (monosomy X), as well as in assessing mosaicism in tissues other than blood, such as fibroblasts [71].

We wanted to elucidate any karyotypic abnormality that might be caused by the multiplex engineering of these cells and wanted to analyze since its widely known that CRISPR based mRNA editing is infamous for Chromosomal Instability and Rearrangements in Cell Lines, and is Detectable by Cytogenetic Methods (72)

Upon careful examination of the karyotype data, distinct aberrations were observed in the CRISPR/Cas9-treated group, while the karyotype of the ABE-treated group remained unchanged (**Figure 7 C,D**), indicating that the cells maintained a healthy chromosomal structure. Specifically, the CRISPR/Cas9-edited cells exhibited significant chromosomal inversion events. Chromosomal inversion is a structural rearrangement where a segment of a chromosome is reversed in orientation. Inversions can disrupt gene expression and lead to functional consequences, depending on the genes affected and the size of the inverted segment. (73)

In the 1.5 μg :1 μg Cas9 group, a chromosomal inversion was observed in one of the chromosomes (**Figure 7E**). Similarly, the group edited with 7.5 μg :1 μg Cas9 displayed one distinct chromosomal inversion on chromosome 5—identified by their characteristic banding patterns (**Figure 7F**). Chromosomal inversions are deleterious structural aberrations that can arise from various factors, including exposure to radiation, certain chemicals, or genome editing techniques such as CRISPR-Cas9. These inversions can have significant phenotypic consequences, disrupting gene expression, promoting the formation of fusion genes, or altering the

overall genomic architecture. Such alterations can impact cellular functions and contribute to disease development or developmental abnormalities. (74)

Since we observed chromosomal inversion events in the Cas9 edited populations we next carried out a more in-depth 60-cell cytogenetic screen (**Figure 7G**). Pulse control cells exhibited a normal karyotype without any detectable chromosomal abnormalities. Chromosomal aberrations were observed across different editing conditions. In the ABE-edited populations, approximately 5% of chromosomal deletion events and 1% of chromosomal addition events were detected. However, in populations edited with CRISPR/Cas9, the frequency of these aberrations was notably higher, with about 32% chromosomal deletion events and 6.5% chromosomal addition events. The co-occurrence of both deletion and addition events within a subset of the analyzed Cas9-edited population suggests a more pronounced genomic instability in this group.

The higher frequency of chromosomal aberrations in the CRISPR/Cas9-edited group raises concerns about potential deleterious effects on cell function and viability. As a result of these findings, particularly the abnormal karyotypes observed in CRISPR/Cas9-engineered conditions, we excluded these populations from further downstream applications to maintain the integrity of our study and ensure the safety of potential therapeutic applications.

Differentiation of Multiplex edited iPSCs to iHPCs:

Hematopoietic stem progenitors possess the intrinsic capacity to generate all major blood and immune cell types in vitro, including lymphoid, myeloid, and erythroid lineages. To leverage

this capability and establish a more relevant source of these cells, we utilized a developed two-dimensional monolayer differentiation protocol for multiplex-edited iPSCs, aiming to generate a sustainable supply of iHPCs (Figure 8B). This approach allowed us to explore and optimize the differentiation process, ensuring efficient and reproducible production of iHPCs.

Upon differentiation, we characterized the multiplex-edited iPSC-derived iHPCs, focusing on the CD34⁺CD43⁺, a transmembrane protein expressed on most leukocytes subset contained within the supernatant. (**Figure 8A**). Interestingly, multiplex-edited iPSCs exhibited a higher yield of supernatant cells, indicating that our multiplex edits may enhance downstream hematopoietic differentiation(**Figure 8C**). Further characterization using flow cytometry revealed that the multiplex-edited iPSCs were highly efficient in generating HSPCs as evidenced by robust expression of CD34⁺ and CD43⁺,(**Figure 8D**). Notably, iPSCs edited with 7.5 µg ABE8e:1 µg sgRNA showed a significantly increased presence of CD34⁺CD45⁺, indicating a favorable HSPC fate and broader pan-leukocyte expression across all hematopoietic cells (**Figure 8E**).

These findings underscore the utility of multiplex editing in iPSCs to enhance their differentiation potential towards hematopoietic lineages, highlighting the therapeutic potential of these cells in regenerative medicine and disease modeling. Future studies will further explore the mechanisms underlying these enhanced differentiation capabilities and their implications for clinical applications.

Downstream Differentiation of Multiplex edited iPSCs into NK cells:

NK cells play a crucial role in innate immunity, defending against pathogens and malignant cells. Dysregulation of NK cell function is linked to immune disorders, including immunodeficiencies and autoimmune diseases [98]. Additionally, NK cells are effective in cancer

immunotherapy, inducing cytotoxicity against tumors [93]. Differentiating engineered iPSCs into NK cells offers advantages for developing "off-the-shelf" cell therapies. Unlike autologous approaches, iPSC-derived NK cells can be generated from a genetically defined starting population, enhancing product consistency and reducing variability [91]. iPSCs can self-renew indefinitely, enabling large-scale production of NK cells from a single cell line, which is advantageous for clinical applications and screening efforts [95].

The multiplex editing capabilities of iPSCs allow precise genetic modifications to study specific genes involved in NK cell development, activation, or effector functions. This approach can lead to enhanced or engineered NK cell products for therapeutic applications [92]. Leveraging iPSC technology's scalability, reproducibility, and genetic manipulability, researchers can advance NK cell biology and develop immunotherapeutic strategies for diseases like cancer and immune disorders. Differentiating multiplex-edited iPSCs into NK cells holds promise for translating research into clinical applications, improving patient outcomes, and advancing regenerative medicine and immunotherapy. Thus, we sought to differentiate iPSCs into NK cells and evaluate the impact of multiplex editing on their functionality

We attempted to differentiate Day 13 CD34+ iHPCs into CD56+CD3- iNK cells. Day 13 CD34+ iHPCs were plated on a lymphoid differentiation coating material (DLL4, VCAM1) and supplemented with IMDM human platelet lysate (5%) and IL-3 for the first week of differentiation. SCF, IL-7, IL-15, and Flt3L ligand were also added. On day 14, the cells were harvested, plated onto plastic, and differentiation continued in NK media for an additional 21 days. On day 35 post-seeding, iNK progenitors were harvested for final maturation and expanded with C9 feeder cells (**Figure 9A**).

Weekly flow cytometry was performed throughout the differentiation to analyze the phenotype. The multiplex-edited cells exhibited a superior phenotype and increased proliferation compared to unedited 1210 iNKs (**Figure 9B**). The multiplex-edited cells showed approximately 200-fold and 187-fold expansion from day 0 to day 14 in the 7.5 μg :1 μg and 1.5 μg :1 μg conditions, respectively (**Figure 9C**). On day 14, cells were reseeded at a density of 100,000 cells per well, and significant growth was observed from day 14 to day 35, with the iNK progenitors displaying an exceptional NK phenotype. Approximately 95% of the cells were CD56+CD7+ at the end of the 35-day differentiation (**Figure 9D**). Analysis of CD56+CD3- cells showed nearly 100% CD56+CD3- cells (**Figure 9F**). We also see that during our differentiation our cells are morphologically similar to the PBNKs and they attain this morphology within 14 days of Differentiation (**figure 9G**).

Feeder Expansion of Multiplex engineering of iNK Progenitors Produces Mature and pure NK cell population

After differentiating induced hematopoietic stem cells (iHSCs) into induced NK (iNK) progenitors, the cells were matured by expanding them using irradiated K562 feeder lines (Clone9.mbIL21 [C9]) in co-cultures supplemented with IL-2 at a ratio of 1:2 iNK progenitor to feeder. Initially, 1 million progenitor cells were seeded [76,77], and every 7 days the cells were harvested, characterized with flow cytometry, and reseeded at a density of 1 million iNK cells per flask, marking the start of the next round of expansion (**Figure 10A**). Progenitor cell populations from Day 28 and Day 35 exhibited a similar phenotype with nearly identical CD56+CD7+ expression levels. Despite undergoing three rounds of expansion, we did not observe a significant difference in the proportion of cells exhibiting the CD56+CD7+ phenotype between progenitors

that were expanded from Day 28 or Day 35 (**Figure 10B**). Multiplex-edited iNKs and unedited iNKs showed comparable cell proliferation and yield in the first two rounds of expansion. However, after a third round of expansion, there was a noticeable decrease in cell proliferation across all groups, regardless of editing status (**Figure 10C**). This suggests that iNK cells mature most effectively after two rounds of expansion, and the third round may induce signaling cascades that impede further growth. Further research is necessary to explore this phenomenon. Despite reduced proliferation, all groups were 100% CD56⁺ CD3⁻ after feeder expansion showing the Purity of the NK cell population (**Figure 10D**). Interestingly, all iPSC-derived groups showed similar morphology to primary PBNK under brightfield microscopy at the end of the third round of expansion, suggesting efficient production of mature Production of NK cell Population (**Figure 10E**).

Feeder-Expanded Multiplex Engineered iNK Cells Are Highly Functional and Cytotoxic Against Burkitt's Lymphoma in-vitro

After expanding the iNK progenitors for 2 rounds, they were functionally evaluated, by assessing their iNK cytotoxicity via luciferase-based co-culture killing assay against Raji cells at different E:T ratios (effector-to-target) ratios: 2:1, 1:1, 1:2, and 1:4 (68,77) We observed that during the first 24 hours, at 2:1 E:T, the multiplex-engineered iNK cells exhibited robust killing, reaching over 90% cytotoxicity against Raji cells. This potent cytotoxic activity was maintained across lower E:T ratios of 1:1, 1:2, and 1:4, with the killing percentage remaining above 80% in all cases. Notably, the unedited iNK displayed a lower level of killing, ranging from approximately 50-60% across the different E:T ratios. (**Figure 11A**) This suggests that the multiplex engineering of the

iNK cells significantly enhanced their cytotoxic potential against the Raji cells, even at lower E:T ratios.

A similar trend is observed, with the multiplex engineered iNK cells exhibiting robust and consistent killing activity against Raji cells across all E:T ratios at the 48-hour time point. At the highest E:T ratio of 2:1, the killing percentage exceeded 90%, comparable to the 24-hour time point. (**Figure 11A, B**). As the E:T ratio decreased, the killing efficiency remained high, with values above 80% for the 1:1 and 1:2 ratios, and approximately 70% for the lowest 1:4 ratio. Interestingly, the unedited iNK cells showed increased cytotoxic activity compared to the 24-hour time point, indicating that their killing potential may have improved with extended co-culture time. More importantly at both the timepoints, we saw improved cytotoxicity compared to the PBNK control (**Figure 11A, B**). Given the robust response observed, we conducted a serial-killing assay with all cell groups. The multiplex-engineered cells demonstrated significantly higher cytotoxicity compared to unedited iNK cells through three rounds of serial killing. Notably, after four rounds, the cytotoxicity of the multiplex-engineered cells exceeded that of the PBNK group, while the unedited iNK cells exhibited diminished functionality. These results highlight the superior efficiency of multiplex-engineered cells in sustained cytotoxic activity. (**Figure 11 C-H**).

After four rounds of cytotoxic killing assays, Multiplex-edited iNK cells demonstrated significantly higher efficiency in killing target cells compared to both unedited and PBNK counterparts. This finding underscores our ability to successfully generate functional Multiplex-engineered iNK cells with enhanced cytotoxicity.

Concurrent multiplex base editing and non-viral transposon engineering

Chimeric antigen receptors (CARs) have emerged as highly promising tools for enhancing cell-based cancer immunotherapy over the past decade (78). However, the delivery of CAR constructs into iPSC-derived cells and NK cells has been predominantly limited to viral vectors [79-83]. The high cost and extended manufacturing process associated with viral vectors have underscored the need for a faster and more cost-effective alternative method for stable transgene delivery.

To address this, we developed non-viral engineered CAR-iPSC-derived cells using a TcBuster transposon plasmid encoding an insulated CAG CD19 CAR (**Figure 12A**). iPSCs were co-transfected with CD19 CAR, TcBuster transposase mRNA, sgRNAs, and ABE8e mRNA through electroporation (**Figure 12B**). Following recovery, CAR expression was confirmed by quantifying GFP and CAR presence on the cells (**Figure 12C**). Approximately 48% of the cells were GFP-positive and about 42% were CAR-positive using FMC63 anti-idiotypic antibody (**Figure 12C**). Recognizing the need to enrich our cell population, we performed FACS-based sorting to achieve approximately 99% GFP-positive and 87% CAR-positive cells (**Figure 12C**). Following this, the cells underwent assessment for editing, confirming significant modifications across all nine targeted genes. Consistent with previous observations, an increase in editing efficiency (A-G conversion) was evident across all experimental conditions (**Figure 12D**). This establishes a co-transfection strategy with TcBuster transposase mRNA and ABE8e mRNA for simultaneous knockout and knock-in of cargo.

CONCLUSIONS AND FUTURE DIRECTIONS

The findings presented in this study represent a significant stride towards the development of next-generation cancer immunotherapies utilizing genetically engineered immune cells derived from human iPSCs. By harnessing the precise and efficient capabilities base editing we successfully demonstrated the feasibility of introducing multiplex genetic modifications in iPSCs while mitigating the genotoxic risks and chromosomal aberrations associated with conventional nuclease-based approaches.

One of the pivotal discoveries of this study is the remarkable performance of ABEs in achieving high and consistent editing efficiencies across multiple target genes simultaneously. Specifically, the ABE8e editor consistently outperformed CRISPR/Cas9 in the multiplex editing setting, often exceeding 90% editing efficiency at target loci. This finding underscores the versatility and robustness of ABEs in addressing complex genetic engineering maneuvers, where the simultaneous modulation of multiple genes is essential for enhancing cellular functions and overcoming therapeutic barriers.

The high editing efficiencies achieved with ABEs were accompanied by minimal cellular stress and genotoxicity, as evidenced by the low induction of p21 expression, a crucial marker of DNA damage response and cell cycle arrest [116]. In contrast, CRISPR/Cas9 nucleases elicited a substantial upregulation of p21 expression, indicating the activation of cellular stress pathways and potential genomic instability. This observation aligns with previous studies that have reported the involvement of p21 in mediating cellular responses to DNA damage and regulating cell cycle progression [60,117,118]. The ability of ABEs to circumvent these stress responses and minimize off-target effects represents a critical advantage over conventional nuclease-based approaches,

particularly in the context of developing cell-based therapies, where preserving genomic integrity is paramount for ensuring safety and efficacy.

Furthermore, the cytogenetic analysis revealed a striking absence of chromosomal abnormalities, inversions, and random loss events in ABE-edited cells, in stark contrast to the significant aberrations observed in CRISPR/Cas9-edited populations. These findings corroborate previous reports that have linked CRISPR/Cas9-mediated double-strand breaks (DSBs) to the induction of chromosomal rearrangements and genomic instability [119, 120]. The absence of such aberrations in ABE-edited cells further reinforces the safety profile of base editing and its potential for minimizing the risk of adverse genomic events that could compromise the therapeutic potential of engineered cells.

The robust and consistent editing capabilities of ABEs, combined with their minimal genotoxic impact, position base editing as a powerful tool for the development of next-generation cell therapies. The ability to simultaneously engineer multiple genes implicated in immune cell function and tumor microenvironment (TME) resistance opens up new avenues for enhancing the efficacy of cancer immunotherapies. By modulating a diverse array of intracellular and extracellular checkpoints, such as AHR, CISH, KLRG1, TIGIT, PD-1, and CD16a, we can potentially overcome the immunosuppressive barriers posed by the TME and unleash the full potential of engineered immune cells against cancer.

In addition to the exceptional editing performance, our study demonstrated the successful differentiation of multiplex-edited iPSCs into induced hematopoietic progenitor cells (iHPCs) and subsequently into induced NK (iNK) cells. Remarkably, the multiplex-edited cells exhibited enhanced differentiation potential towards the hematopoietic lineage, as evidenced by the higher yield of supernatant cells and robust expression of CD34+ and CD43+ markers, indicative of bona

vide hematopoietic progenitor cells. These findings suggest that the precise genetic modifications introduced by base editing may contribute to improving the differentiation efficiency and lineage commitment of iPSCs, further enhancing their therapeutic potential.

The functional characterization of the multiplex-edited iNK cells revealed their superior cytotoxic activity against target cells compared to unedited and primary NK cell counterparts. The robust and consistent killing ability observed across multiple effector-to-target ratios and in serial killing assays underscores the potential of this approach in generating highly potent and effective NK cell products for cancer immunotherapy. These findings align with previous studies that have demonstrated the potential of genetically engineered NK cells in enhancing anti-tumor responses and overcoming immunosuppressive mechanisms [129,130].

Moreover, the successful integration of non-viral transposon engineering with concurrent multiplex base editing demonstrated in this study paves the way for the efficient delivery of chimeric antigen receptors (CARs) or other transgenes into engineered iPSC-derived cells. This approach addresses the limitations associated with viral vectors, such as high costs and extended manufacturing processes, providing a faster and more cost-effective alternative for stable transgene delivery. The utilization of non-viral transposon systems for CAR delivery has been explored in various studies, demonstrating their potential for clinical translation [131,132].

While the findings of this study are highly promising, several future directions and considerations should be studied for further explorations. Mechanistic insights into enhanced differentiation and functionality are needed, as the observed enhancement in hematopoietic differentiation and cytotoxic potential of multiplex-edited cells warrants further investigation into the underlying molecular mechanisms. Elucidating the interplay between the edited genes and their

impact on cellular signaling pathways, transcriptional regulation, and epigenetic modulation could provide valuable insights into optimizing the engineering process and developing more potent cell therapies. Transcriptomic and proteomic profiling, as well as functional assays, could shed light on the molecular determinants governing the improved differentiation and functional outcomes observed in this study. In vivo validation and preclinical studies are essential, as the in vitro characterization of multiplex-edited iNK cells demonstrated promising results. Validating their safety and efficacy in relevant in vivo models is crucial. Preclinical studies using appropriate tumor models would be crucial to assess the antitumor activity, biodistribution, and potential off-target effects of these engineered cells. Additionally, evaluating their ability to overcome immunosuppressive TME barriers and their persistence in vivo would be critical for translational applications. These studies could involve xenograft models or patient-derived xenograft (PDX) models, which have been widely used in preclinical cancer research. Expansion and scale-up strategies must be developed as the field progresses towards clinical applications. Developing scalable and cost-effective strategies for the expansion and manufacturing of multiplex-edited iPSC-derived cells will be essential. Optimizing bioreactor systems, exploring alternative culture conditions, and implementing automation and process control measures could facilitate large-scale production while maintaining product consistency and quality. Strategies such as fed-batch culture systems, microcarrier-based bioreactors, or the integration of advanced monitoring and control systems could be explored. Exploration of alternative base editing systems is another consideration. While this study focused on the use of ABE8e, the rapidly evolving field of base editing offers a plethora of new tools and variants. Exploring alternative base editors, such as cytosine base editors (CBEs) or newly engineered variants like Prime editor with improved editing windows, target specificity, or reduced off-target effects, could further enhance the precision and

versatility of multiplex engineering in iPSCs. Combinatorial approaches and additional genetic modifications beyond the nine target genes investigated in this study could further enhance the functionality of engineered immune cells. Integrating base editing with other gene editing tools, such as CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa), could enable fine-tuning of gene expression levels and provide an additional layer of control over cellular functions. Immune cell engineering beyond NK cells is also a potential avenue, as the potential applications of base editing extend to other immune cell types. Exploring the multiplex editing of iPSC-derived T cells, macrophages, or other immune cell subsets could open new avenues for developing personalized immunotherapies targeting a wide range of diseases, including autoimmune disorders, infectious diseases, and cancer. Finally, integration with other therapeutic modalities could be explored. The engineered iPSC-derived immune cells developed through base editing could potentially be integrated with other therapeutic modalities, such as small molecule inhibitors, checkpoint inhibitors, or targeted therapies, to create synergistic and multi-pronged treatment strategies. This combinatorial approach could potentially overcome resistance mechanisms and enhance therapeutic outcomes.

In conclusion, the findings presented in this study represent a significant step forward in the development of next-generation cancer immunotherapies using genetically engineered immune cells derived from human iPSCs. By leveraging the precise and efficient capabilities of base editing technology, we have successfully demonstrated the ability to introduce multiplex genetic modifications while minimizing genotoxic risks and chromosomal aberrations associated with conventional nuclease-based approaches.

The superior editing performance, enhanced differentiation potential, and improved cytotoxic activity of multiplex-edited iNK cells highlight the transformative potential of this approach in overcoming the immunosuppressive barriers posed by the tumor microenvironment and unleashing the full potential of engineered immune cells against cancer.

While significant progress has been made, this study also underscores the need for continued research and exploration in areas such as mechanistic insights, in vivo validation, scalable manufacturing strategies, and the integration of base editing with other therapeutic modalities. By addressing these challenges and leveraging the rapidly evolving toolbox of gene editing technologies, the field of personalized cancer immunotherapy can continue to advance, bringing us closer to realizing the promise of highly potent and precisely engineered cell-based therapies for improved patient outcomes.

Figures

Figure 1:

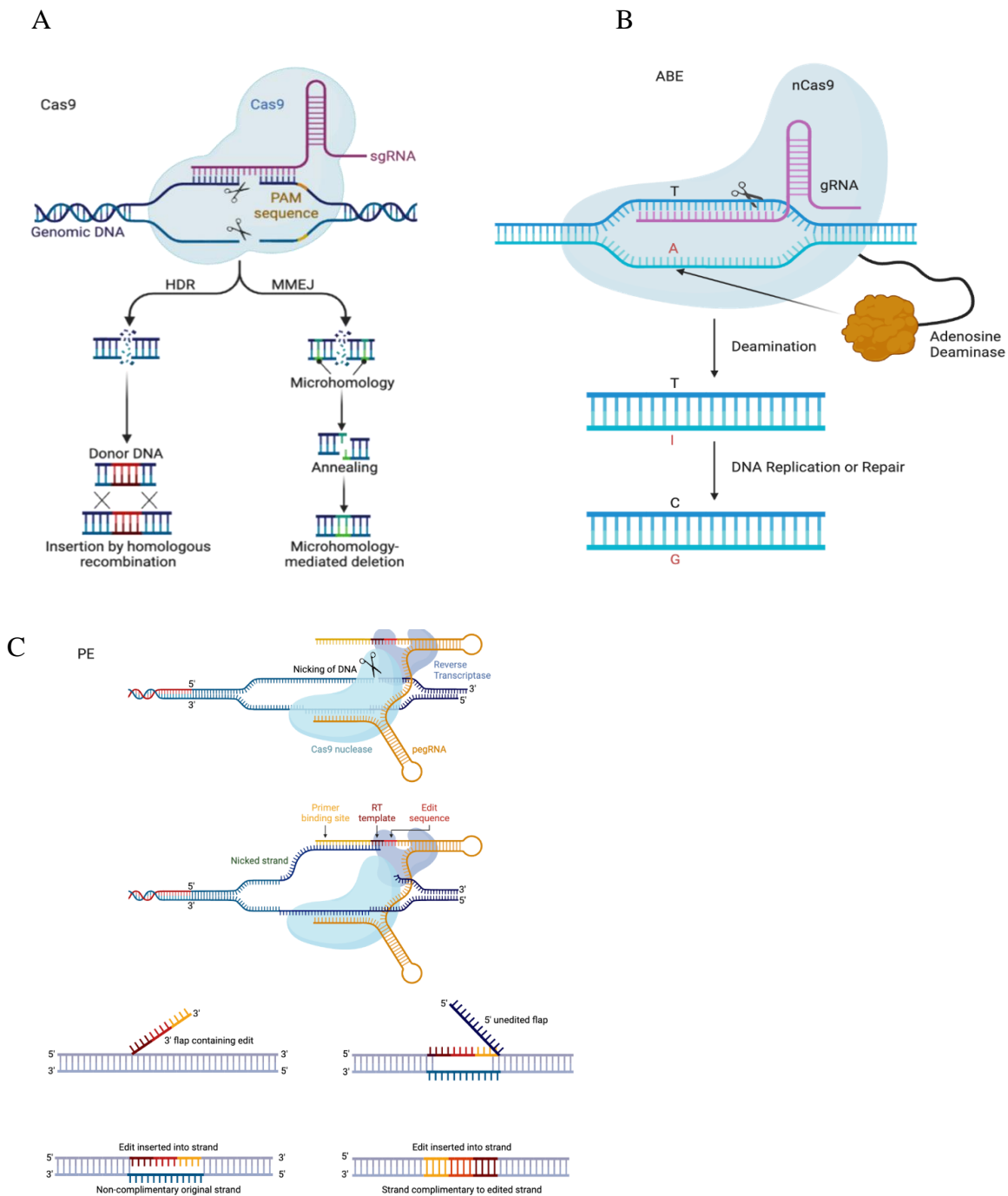


Figure 1. Schematic Representations of CRISPR/Cas9, Base Editing, and Prime Editing Systems. (A) CRISPR/Cas9 System: The Cas9 protein cleaves a specific DNA sequence guided

by a single guide RNA (sgRNA). **(B)** Adenine Base Editing Mechanism: Conversion of A•T base pairs to G•C base pairs using a Cas9 nickase. **(C)** Prime Editing Mechanism: Cas9 nickase fused to a reverse transcriptase and a prime editing guide RNA (pegRNA).

Figure 2: IPSC Pathway

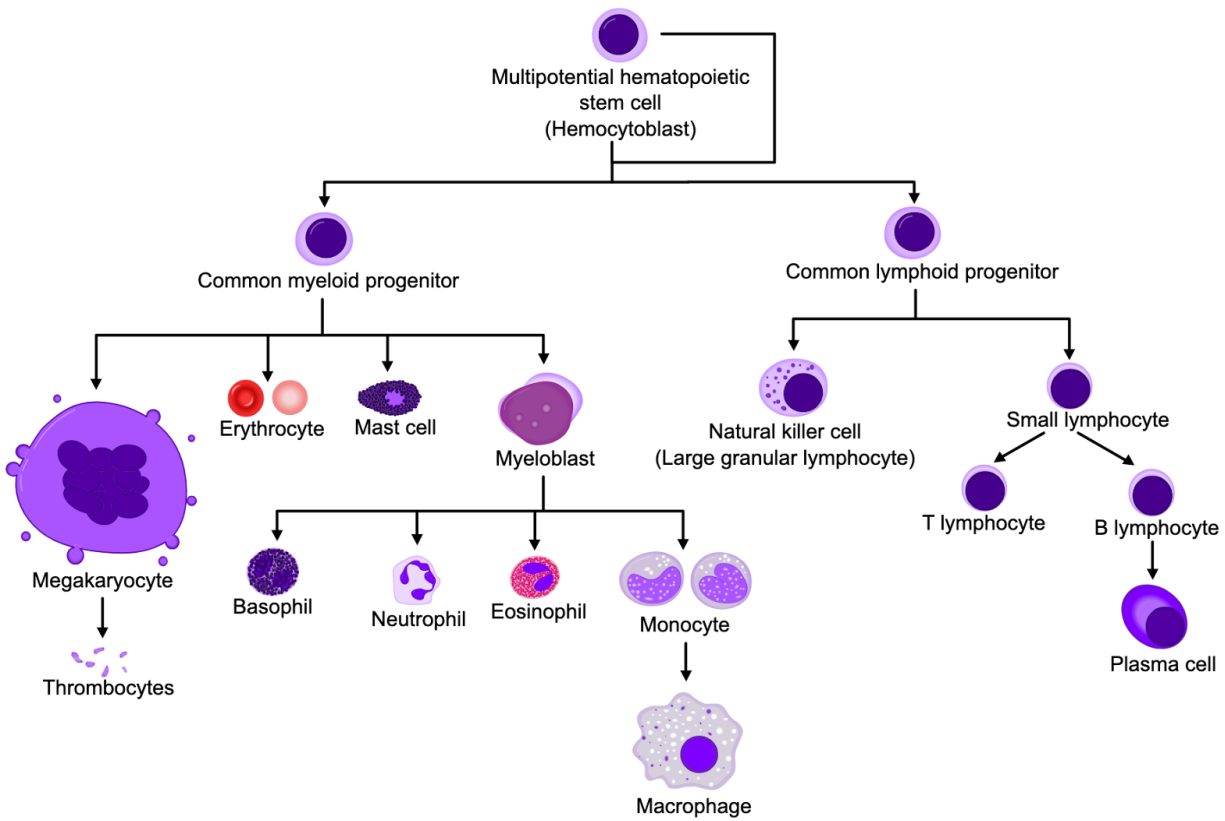


Figure 2: The differentiation and development of myeloid and lymphoid lineages

FIGURE 3:

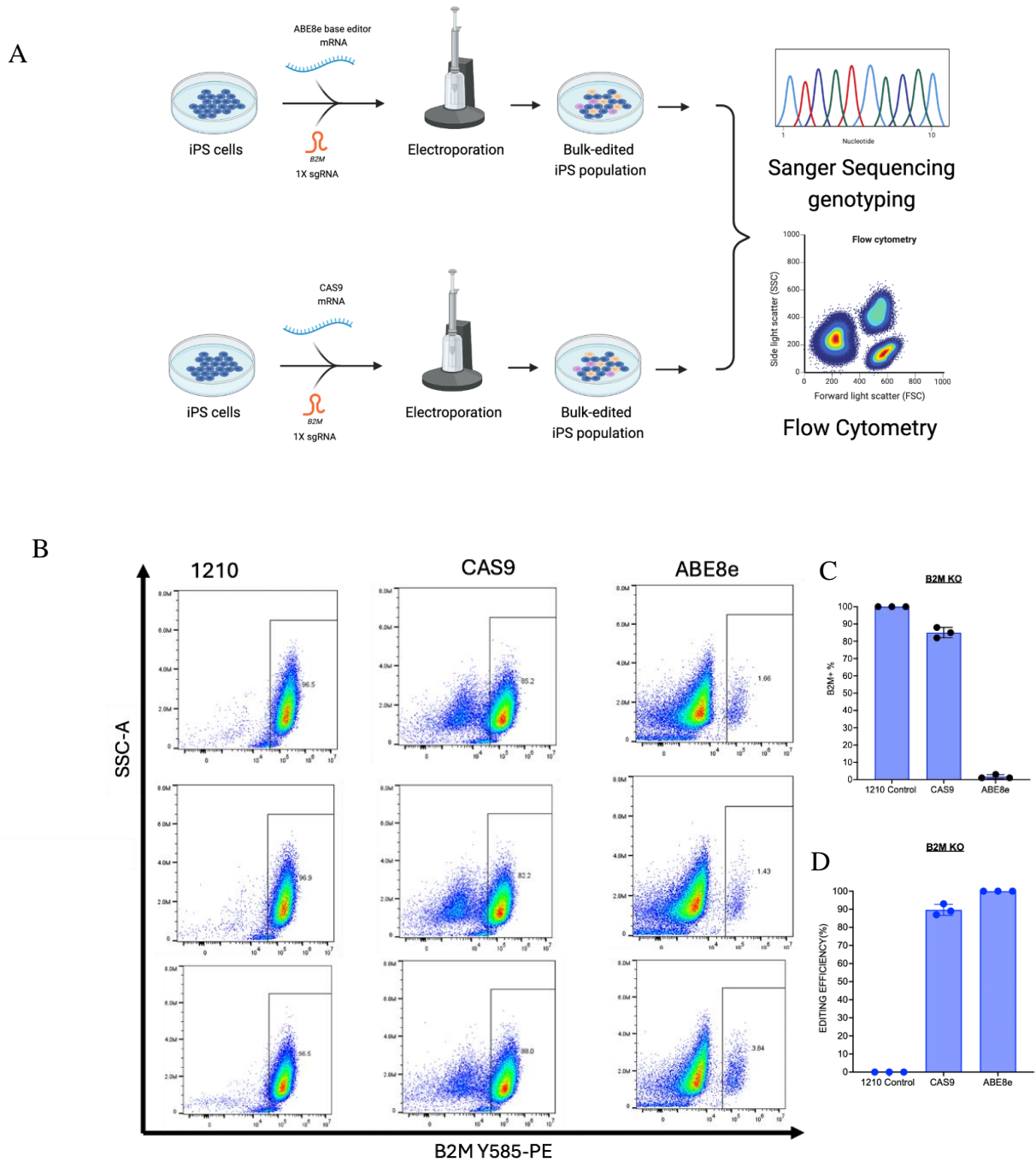


Figure 3. Beta-2-Microglobulin (B2M) Knockout in Human iPSCs Using ABE and CRISPR/Cas9 Technology. (A) Experimental setup and workflow for B2M knockout. (B) Flow

cytometric plots for B2M knockout: 1210—unedited human adult fibroblast-derived line, Cas9—iPSCs edited with the enzyme, ABE—cells edited with Adenine Base Editor (n=3, data from 3 independent electroporation's). **(C)** Quantification of the flow phenotype of the cells. **(D)** Editing efficiency at the genomic level quantified by A-to-G conversion at the target base for each gene locus (n=3).

Table 1 : Panel of Target Inhibitory Genes

Gene Names	Protein Name	Subcellular Location	SgRNA Squence	Target Site	PAM
KLRG1	Killer cell lectin like receptor G1	Cell membrane	CCTTACCTTGAGAAGTTTAG	Chromosome 12: 8,950,044-9,010,760 forward strand.	NGG
TIGIT	T cell immunoreceptor with ig and ITIM domains	Cell membrane	CAGGCCTTACCTGAGGCGAG	Chromosome 3: 114,276,913-114,310,298 forward strand.	NGG
AHR	Aryl hydrocarbon receptor	Intracellular	CTTACCATCAAAGAAGCTCT	Chromosome 7: 16,916,359-17,346,152 forward strand.	NGG
PDCD1	Programmed cell death protein 1	Cell membrane	CACCTACCTAAGAACCATCC	Scaffold HSCHR2_3_CTG15: 61,979-71,006 reverse strand.	NGG
CD16a Mutation	Fc fragment of igG receptor IIIa	Cell membrane	TTGACACTGCCAAACCTATT	chromosome 1q23 encoded by the FCGR3A gene	NGG
Fas DN	Fas cell surface death receptor	Cell membrane	AAATATATCACCACTATTGC	Chromosome 10: 88,953,813-89,029,605 forward strand.	NGG
TGFBR2 DN	Transforming growth factor beta receptor 2 Cell membrane	Cell membrane	GTAGACATCGGTCTGCTTGA	Chromosome 3: 30,605,601-30,694,142 forward strand.	NGG
B2M	Beta-2-microglobulin	Extracellular	ACTCACGCTGGATAGCCTCC	Chromosome 15: 44,711,358-44,718,851 forward strand.	NGG
CISH	Cytokine inducible SH2 containing protein	Intracellular	CTCACCAGATCCCGAAGGT	Chromosome 3: 50,606,489-50,611,774 reverse strand.	NGG

FIGURE 4:

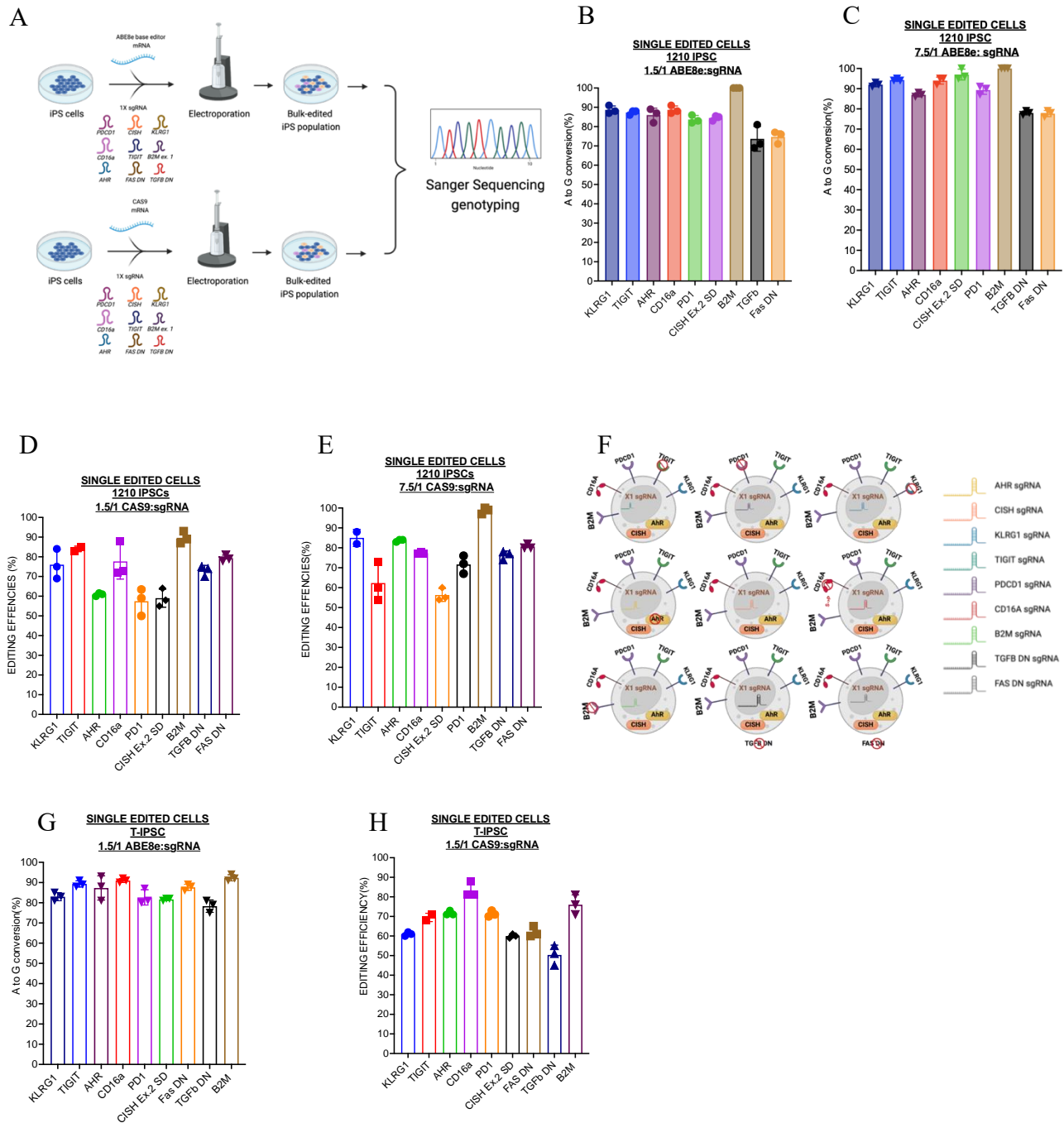


Figure 4. Single Gene Knockout in Human iPSCs Using ABE and CRISPR/Cas9 Technology Across 9 Targeted Genes. (A) Experimental setup and workflow for single knockout of 9 targeted genes. **(B)** Editing efficiency at the genomic level in the 1210 human fibroblast-derived iPSC line,

quantified by A-to-G conversion of the target base for each gene locus using an adenine base editor with 1.5 μ g:1 μ g, ABE8e: Guides (n=3; data from 3 independent electroporations). **(C)** Editing efficiency at the genomic level in the 1210 human fibroblast-derived iPSC line, quantified by A-to-G conversion of the target base for each gene locus using an adenine base editor with 7.5 μ g:1 μ g, ABE8e: Guides (n=3; data from 3 independent electroporations). **(D)** Editing efficiency at the genomic level in the 1210 human fibroblast-derived iPSC line, quantified by ICE Synthego, indicated by Indels for each gene locus with an adenine base editor with 1.5 μ g:1 μ g, Cas9: Guides (n=3; data from 3 independent electroporations). **(E)** Editing efficiency at the genomic level in the 1210 human fibroblast-derived iPSC line, quantified by ICE Synthego, indicated by Indels for each gene locus with an adenine base editor with 7.5 μ g:1 μ g, CAS9: Guides (n=3; data from 3 independent electroporations). **(F)** Schematic of single gene editing strategy. **(G)** Editing efficiency at the genomic level in alpha-beta T cell-derived iPSC line, quantified by A-to-G conversion of the target base for each gene locus using an adenine base editor with 1.5 μ g:1 μ g, ABE8e: Guides (n=3; data from 3 independent electroporations). **(H)** Editing efficiency at the genomic level in alpha-beta T cell-derived iPSC line, quantified by ICE Synthego, indicated by Indels for each gene locus with an adenine base editor with 1.5 μ g:1 μ g, CAS9: Guides (n=3; data from 3 independent electroporations).

Figure 5:

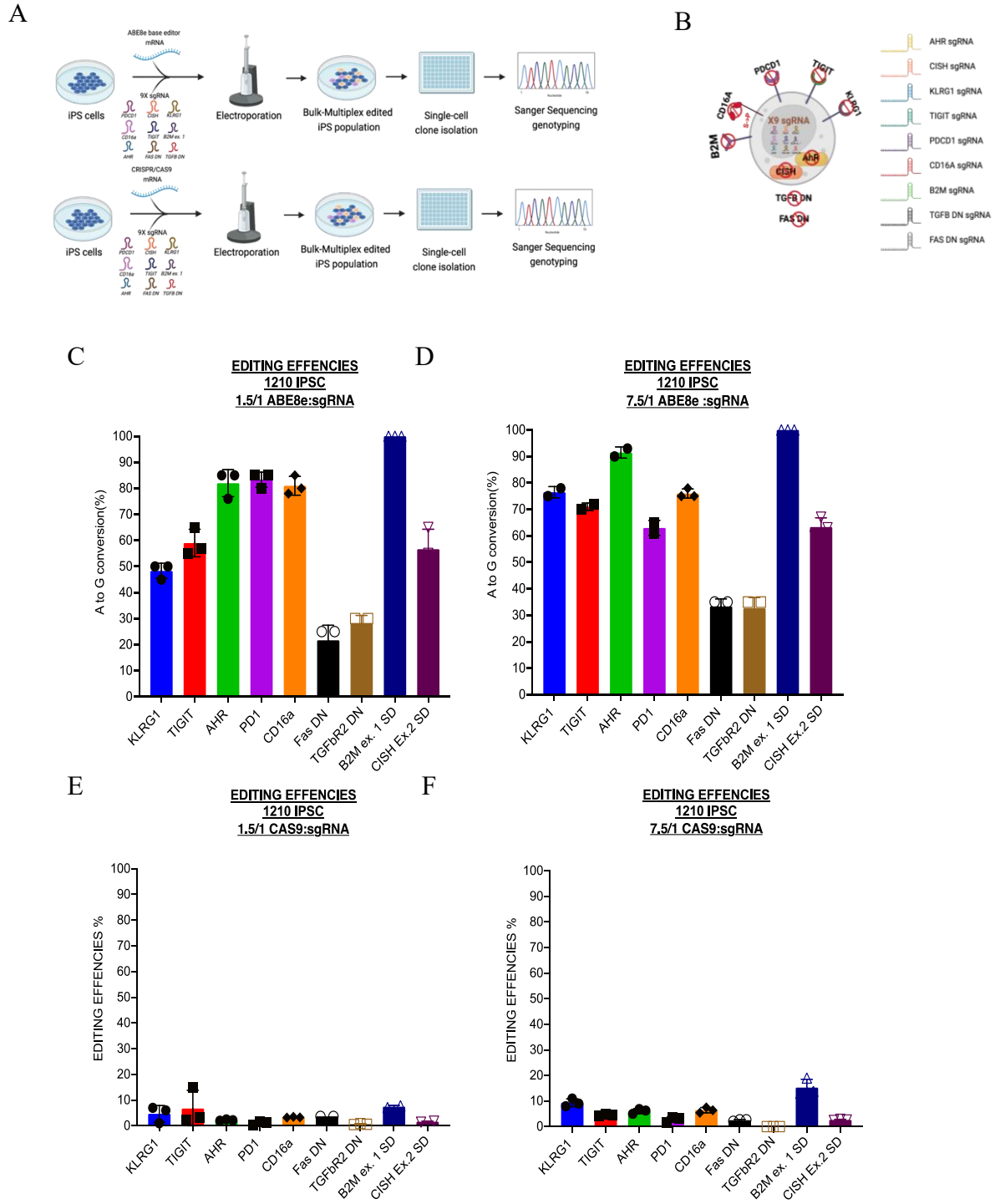


Figure 5. Highly Efficient Multiplex Base Editing in iPSCs Using ABE. (A) Schematic of the experimental setup and workflow for multiplexing 9 targeted genes with CAS or ABE8e. (B) Schematic of single gene editing strategy. (C) Multiplex editing efficiency at the genomic level quantified by A-to-G conversion of the target base for each gene locus, using 1.5 μ g:1 μ g ABE8e: Guides (n=3). (D) Multiplex editing efficiency at the genomic level quantified by A-to-G conversion of the target base for each gene locus, using 7.5 μ g:1 μ g ABE8e: Guides (n=3). (E) Multiplex editing efficiency at the genomic level quantified by ICE Synthego, indicated by Indels at the target base for each gene locus, using 1.5 μ g:1 μ g CAS9: Guides (n=3) (all data generated from 1210 iPSCs)

Figure 6:

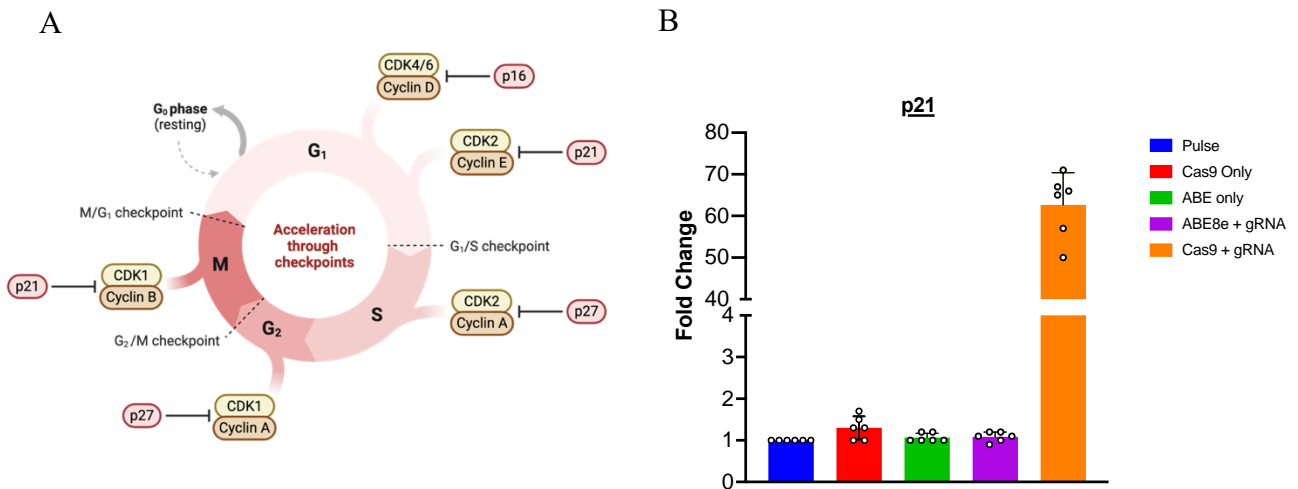


Figure 6. (A) Schematic of the cell cycle model involving cyclin/cdk complexes essential at each phase. CyclinD/cdk4-6 complexes play a crucial role by phosphorylating the Rb protein, thereby suppressing its function and enabling cells to transition through the restriction R-point. This regulation of cell cycle progression induces the expression of p21, a cyclin-dependent kinase inhibitor. p21 binds to and inactivates various cyclin/cdk complexes, contributing to the suppression of cell cycle progression. **(B)** Depiction of the fold change in expression levels of the p21 gene under various treatment conditions involving CRISPR-associated components: Pulse (control treatment or baseline condition), Cas9 Only (treatment with Cas9 nuclease alone), ABE Only (treatment with an adenine base editor, specifically ABE9e, alone), ABE8e + gRNA (combined treatment with ABE8e and guide RNAs targeting a specific genomic locus), and Cas9 + gRNA (combined treatment with Cas9 nuclease and guide RNAs targeting a specific genomic locus).

Figure 7

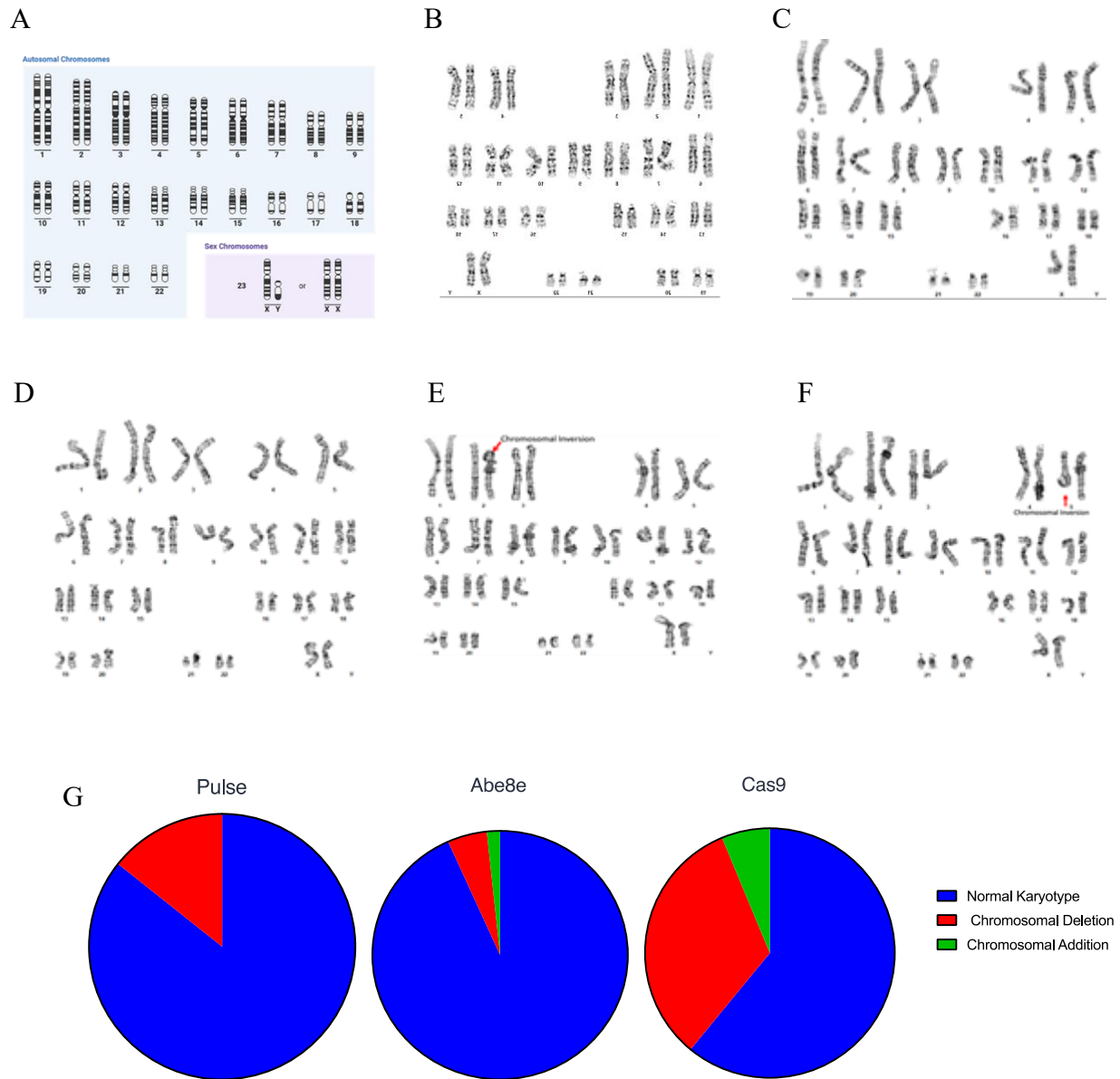


Figure 7. Karyotypic Analysis of Multiplex Engineered iPSCs. (A) Normal human karyotype. (B) Normal karyotype of unedited 1210 iPSC line. (C) Normal karyotype of multiplex base-edited cells with 1.5 μ g:1 μ g ABE8e: sgRNA. (D) Normal karyotype of multiplex base-edited cells with 7.5 μ g:1 μ g ABE8e: sgRNA. (E) Karyotypic abnormality of multiplex CAS9-edited cells with 1.5 μ g:1 μ g CAS9: sgRNA. (F) Karyotypic abnormality of multiplex CAS9-edited cells with 7.5 μ g:1 μ g CAS9: sgRNA. (G) Pie charts showing the proportion of Normal Karyotype (Blue), Chromosomal Deletion (Red), and Chromosomal Addition (Green) for Pulse, Abe8e, and Cas9 conditions.

μg CAS9: sgRNA. **(G)** In-depth karyotypic characterization of 60 cells, including chromosomal deletions and additions in unedited, base-edited, and CAS9-edited cells.

Figure 8.

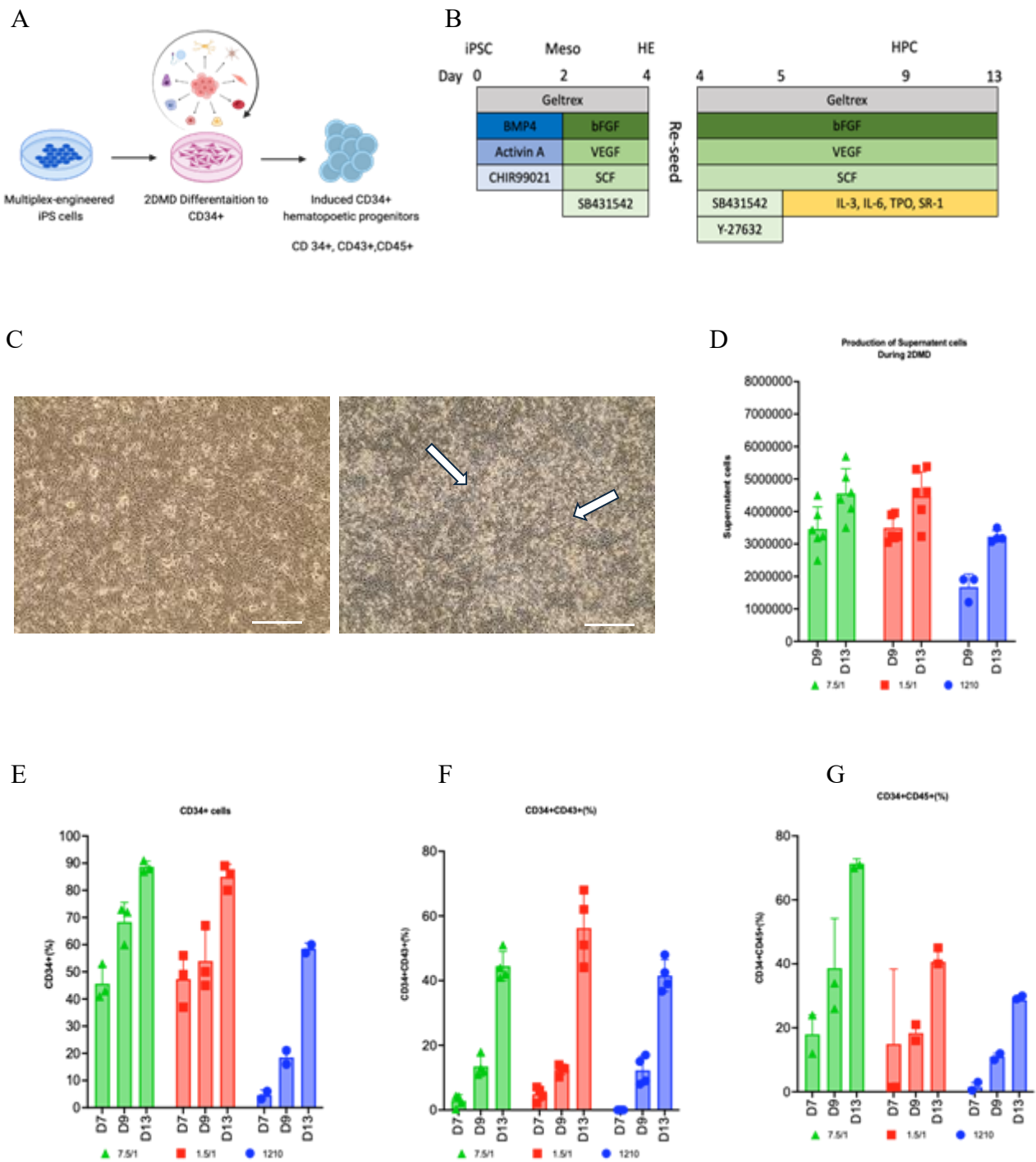


Figure 8: Downstream iHPCs Differentiation of Multiplex Edited iPSCs. (A) Schematic of iPSC differentiation to iHPCs workflow. (B) Schematic of the protocol to differentiate iPSCs to iHPCs. (C) Brightfield images of the iPSCs through differentiation, with day 5 on the left and day 13 on the right, showing suspension cells detaching from the monolayer. (D) Total supernatant cells produced during the differentiation process. (E-G) Flow phenotype of supernatant cells.

Figure 9:

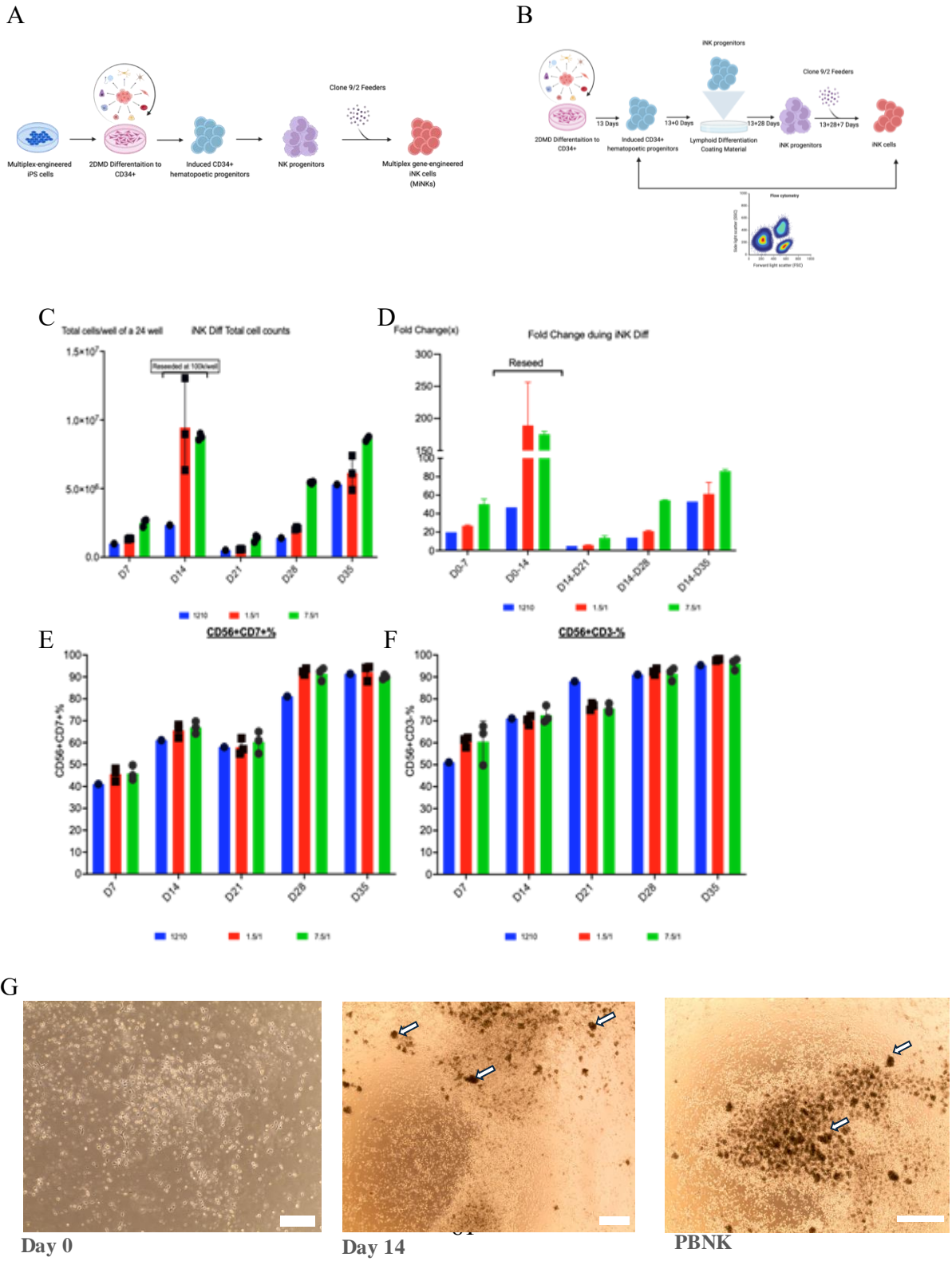


Figure 9. Downstream Differentiation of Multiplex Edited iPSCs into NK Cells. (A) Schematic of workflow from iPSC to NK stage. (B) NK differentiation protocol and the time points for NK cell fate analysis. (C) Total NK cell count throughout the NK differentiation (n=3; all experiments were done with 3 technical replicates). (D) Fold change of NK cells throughout the differentiation. (E) NK cell phenotype (CD56+CD7+) throughout the NK differentiation. (F) NK cell phenotype (CD56+CD3-) throughout the NK differentiation. (G) Brightfield images at day 0 and day 14 show similar morphology to PBNKs.

Figure 10:

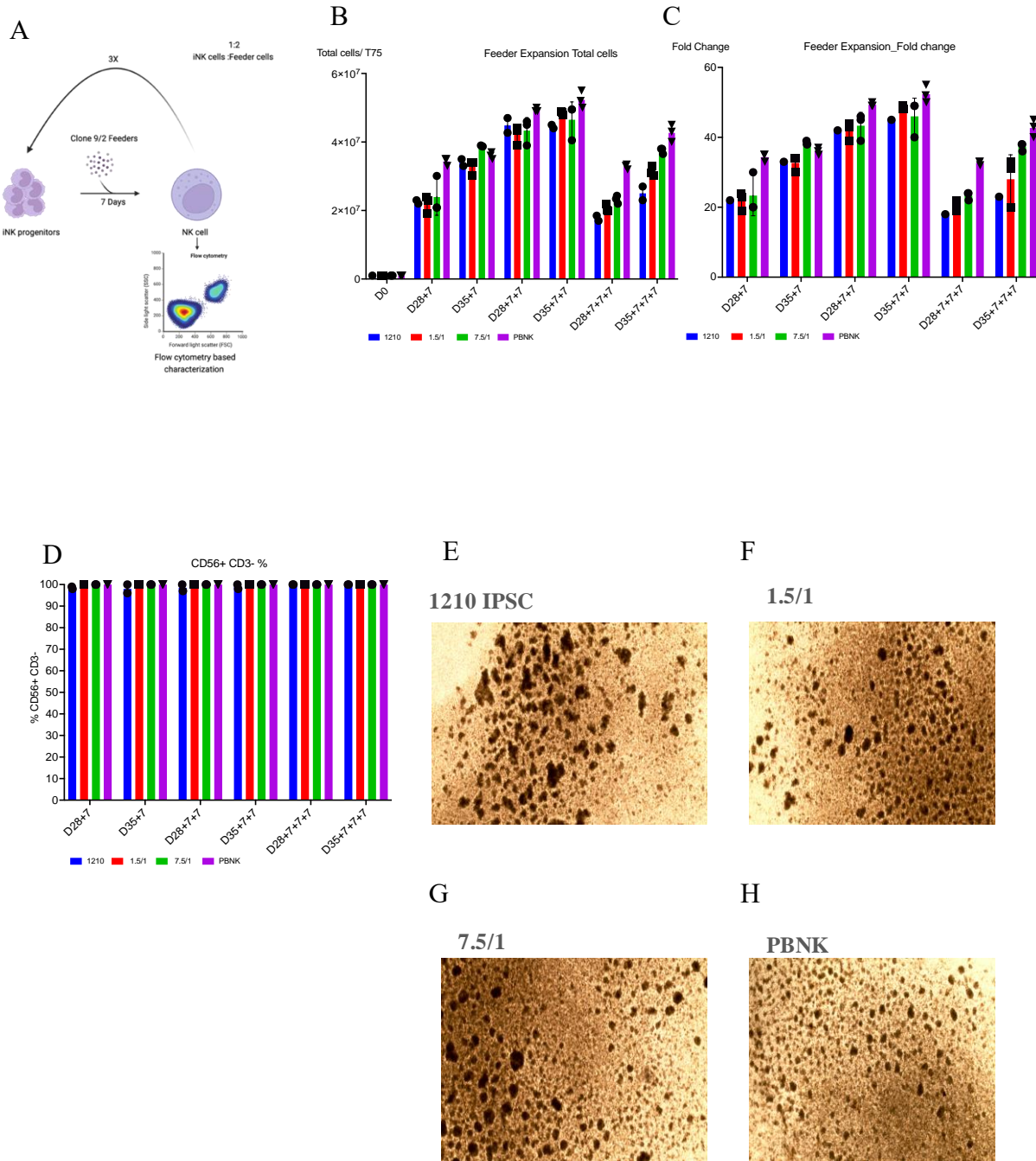
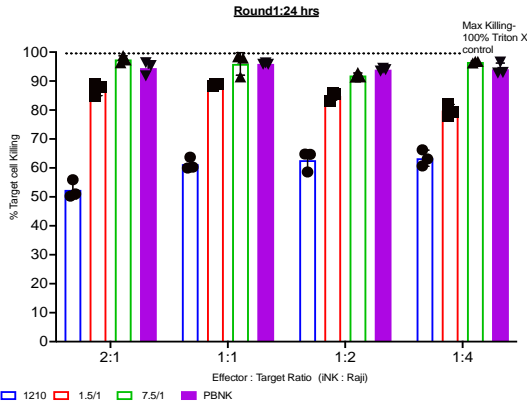


Figure 10. Feeder expansion and multiplex engineering of iNK progenitors. (A) Schematic depicting the experimental workflow employed during feeder expansion. **(B-C)** Quantitative analysis showing total cell count and total fold change observed throughout the feeder expansion

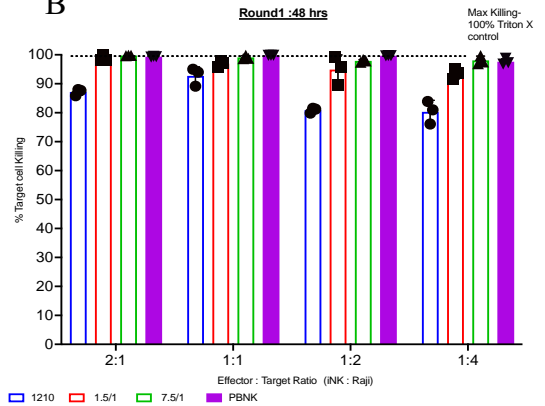
process. **(D)** Flow cytometric analysis of CD56⁺CD3⁻ cells at various timepoints during feeder expansion. **(E-H)** Representative brightfield microscopy images illustrating the progression from iNK progenitors to mature NK cells during the third round of expansion (Scale bar = 100 μ m).

Figure 11:

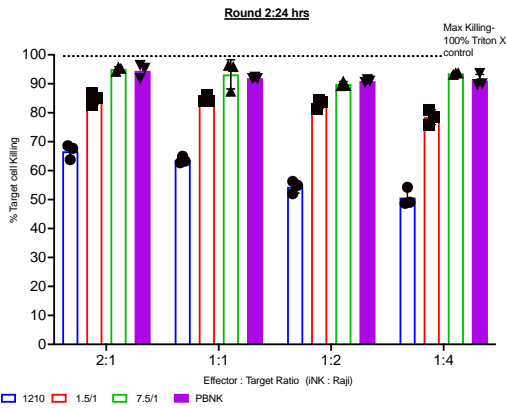
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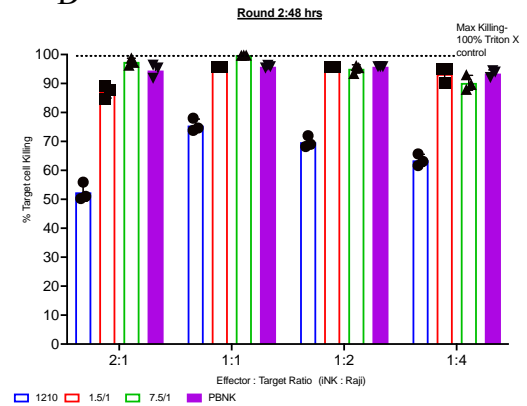
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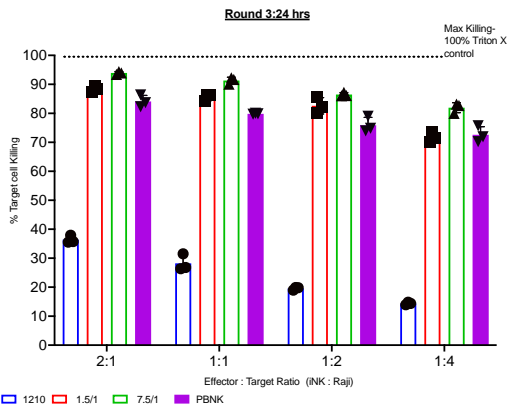
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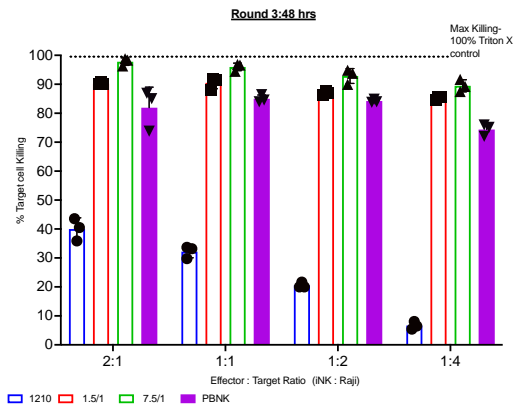
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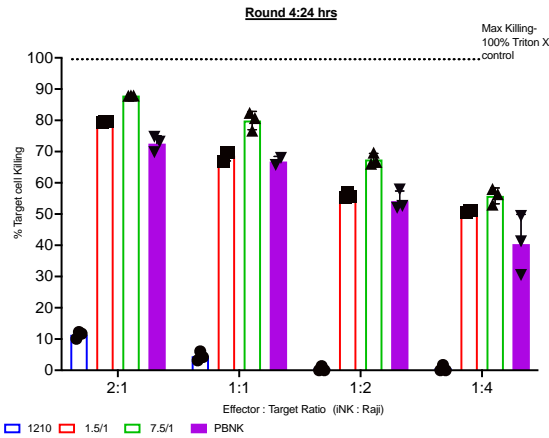
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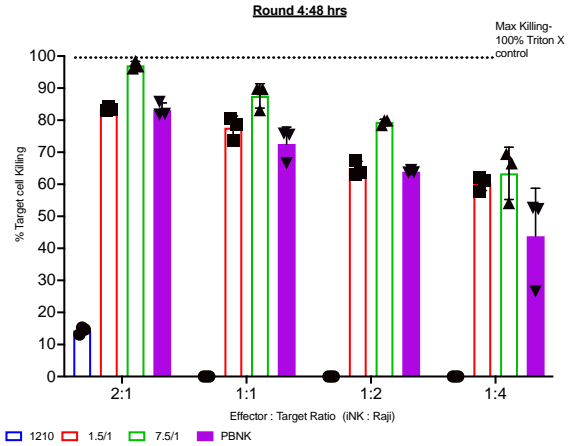


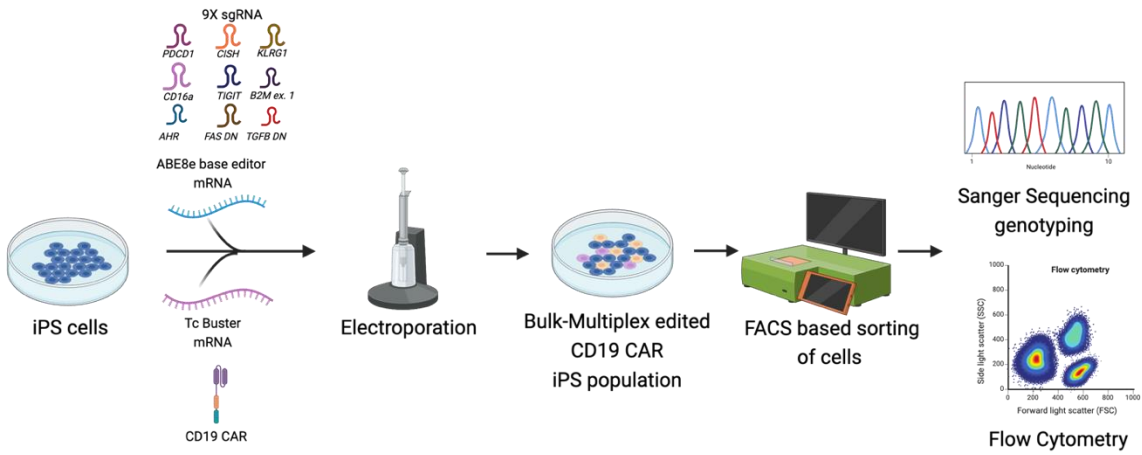
Figure 11. Serial killing assay demonstrating multiple rounds of target cell elimination. (A-B) Round 1 of the killing assay showing results at 24 hours (left) and 48 hours (right). (C-D) Round 2 of the killing assay with outcomes at 24 hours (left) and 48 hours (right). (E-F) Round 3 of the killing assay presenting results at 24 hours (left) and 48 hours (right). (G-H) Round 4 of the killing assay displaying outcomes at 24 hours (left) and 48 hours (right).

Figure 12:

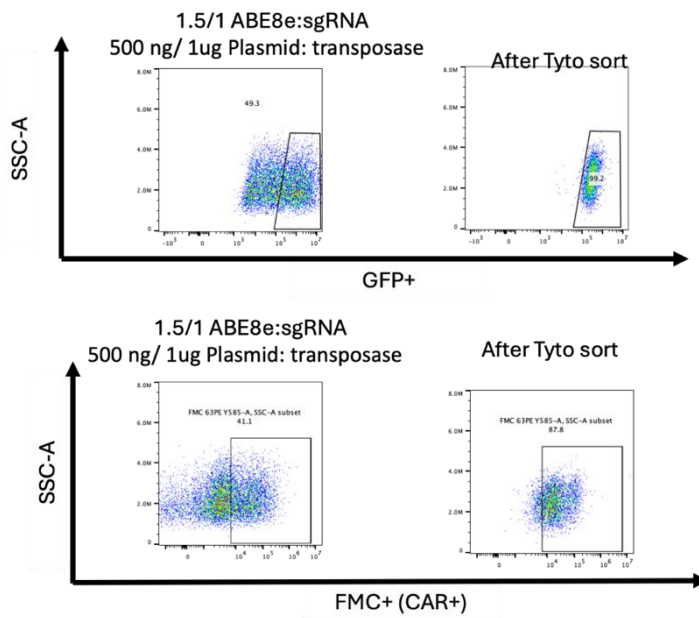
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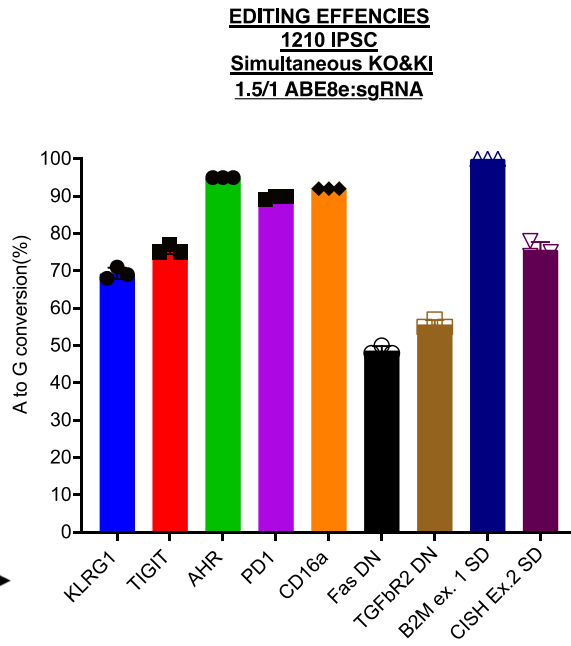


Figure 12. Concurrent multiplex base editing and non-viral transposon engineering. (A) Schematic representation of the insulated CAG CD19 CAR construct. (B) Schematic illustration of the concurrent multiplex and transposon engineering process. (C) Flow cytometry analysis showing GFP+ (top) and CAR (bottom) expression levels before and after Tyto sorting of the concurrent multiplexed and non-viral transposon-engineered cells. (D) Quantification of multiplex editing efficiency at the genomic level, measured by A to G conversion of target base for each gene locus, using a 1.5 μ g:1 μ g ratio of ABE8e to guides (n=3). (All editing data generated from 1210 iPSCs; error bars represent standard deviation).

References:

1. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 126, 663–676 (2006).
2. Robinton, D. A. & Daley, G. Q. The promise of induced pluripotent stem cells in research and therapy. *Nature* 481, 295–305 (2012).
3. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861–872 (2007).
4. Yu, J. et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318, 1917–1920 (2007).
5. Park, I.-H. et al. Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* 451, 141–146 (2008).
6. Lo, B. & Parham, L. Ethical issues in stem cell research. *Endocr. Rev.* 30, 204–213 (2009).
7. Rowe, R. G. & Daley, G. Q. Induced pluripotent stem cells in disease modeling and drug discovery. *Nat. Rev. Genet.* 20, 377–388 (2019).
8. Mertens, J. et al. Directly reprogrammed human neurons retain aging-associated transcriptomic signatures and reveal age-related nucleocytoplasmic defects. *Cell Stem Cell* 17, 705–718 (2015).
9. Lan, F. et al. Abnormal calcium handling properties underlie familial hypertrophic cardiomyopathy pathology in patient-specific induced pluripotent stem cells. *Cell Stem Cell* 12, 101–113 (2013).
10. Cayo, M. A. et al. A drug screen using human iPSC-derived hepatocyte-like cells reveals cardiac glycosides as a potential treatment for hypercholesterolemia. *Cell Stem Cell* 20, 478–489.e5 (2017).
11. Miyoshi, N. et al. Induced pluripotent stem cells from cancer cells: Induction, differentiation, and applications in cancer investigation. *Cancers* 13, 142 (2021).
12. Avior, Y., Sagi, I. & Benvenisty, N. Pluripotent stem cells in disease modeling and drug discovery. *Nat. Rev. Mol. Cell Biol.* 17, 170–182 (2016).
13. Shevelev, A. et al. Modeling therapeutic doses of pluripotent stem cell-derived human cardiac myocytes. *Nat. Commun.* 12, 4981 (2021).
14. Paull, D. et al. Utility of induced pluripotent stem cells for the study of human diseases. *Curr. Opin. Genet. Dev.* 51, 84–90 (2018).
15. Kitamura, S., Söderblom, E. J. & Forgacs, G. Drug discovery using induced pluripotent stem cells: Promises and challenges. *Curr. Opin. Pharmacol.* 48, 69–76 (2019).
16. Avior, Y., Sagi, I. & Benvenisty, N. Pluripotent stem cells in disease modeling and drug discovery. *Nat. Rev. Mol. Cell Biol.* 17, 170–182 (2016).
17. Wang, W. et al. CRISPR/Cas9 in pluripotent stem cells: Commentary on functional studies available for human disease research. *Curr. Protoc. Stem Cell Biol.* 38, 5B.4.1–5B.4.13 (2016).
18. Paull, D. et al. Utility of induced pluripotent stem cells for the study of human diseases. *Curr. Opin. Genet. Dev.* 51, 84–90 (2018).
19. Morizane, R. et al. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. *Nat. Biotechnol.* 33, 1193–1200 (2015).

20. Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science* 345, 1247125 (2014).
21. Ihry, R. J. et al. P53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat. Med.* 24, 939–946 (2018).
22. Fong, E. H. H. et al. Modeling Ewing sarcoma in human pluripotent stem cell-derived neural crest stem-like cells. *PLOS ONE* 13, e0198689 (2018).
23. Morizane, R. et al. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. *Nat. Biotechnol.* 33, 1193–1200 (2015).
24. Lancaster, M. A. & Knoblich, J. A. Organogenesis in a dish: Modeling development and disease using organoid technologies. *Science* 345, 1247125 (2014).
25. Avior, Y., Sagi, I. & Benvenisty, N. Pluripotent stem cells in disease modeling and drug discovery. *Nat. Rev. Mol. Cell Biol.* 17, 170–182 (2016)
26. Zhu, H., Blum, R. H., Bernareggi, D., et al. (2020). Metabolic reprogramming via deletion of CISH in human iPSC-derived NK cells promotes in vivo persistence and anti-tumor activity. *Cell Stem Cell*, 27(2), 198-211.
27. Li, Y., Hermanson, D. L., Moriarity, B. S., & Kaufman, D. S. (2018). Human iPSC-derived natural killer cells engineered with chimeric antigen receptors enhance anti-tumor activity. *Cell Stem Cell*, 23(2), 181-192.
28. Zeng, J., Tang, S. Y., Toh, T. C., & Wang, S. (2017). Generation of "off-the-shelf" natural killer cells from peripheral blood cell-derived induced pluripotent stem cells. *Stem Cell Reports*, 9(6), 1915-1929.
29. Ng, E. S., Davis, R. P., Gole, L., et al. (2019). Engineered CRISPR-Cas9 natural killer cells derived from induced pluripotent stem cells for anti-tumor therapy. *Cancer Immunology Research*, 7(11), 1835-1846.
30. Hockemeyer, D. & Jaenisch, R. Induced Pluripotent Stem Cells Meet Genome Editing. *Cell Stem Cell* 18, 573–586 (2016).
31. Gonzalez, F., Zhu, Z., Shi, Z.-D., Lelli, K. & Verma, N. A CRISPR repair protocol for treatment of Duchenne muscular dystrophy. *Nat. Commun.* 11, 4583 (2020).
32. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821 (2012).
33. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. *Nat. Protoc.* 8, 2281–2308 (2013).
34. Howden, S. E. et al. A Cas9 Variant for Efficient Gene Integration Coupled with Cis-Regulatory Targeting. *Mol. Ther.* 28, 112–122 (2020).
35. Shao, Y. et al. A Pluripotent Stem Cell-Based Multicolor Fluorescent Reporter Assay to Study CRISPR-Cas9 Induced Genomic Structural Variations. *Stem Cell Reports* 15, 293–309 (2020).
36. Rong, Z. et al. An effective approach to prevent immune rejection of human ESC-derived allografts. *Cell Stem Cell* 14, 121–130 (2014).
37. Merkle, F. T. et al. Efficient CRISPR-Cas9-Mediated Generation of Knockin Human Pluripotent Stem Cells Lacking Undesired Mutations at the Targeted Locus. *Cell Rep.* 23, 3262–3278 (2018).
38. Komor, A. C., Kim, Y. B., Packer, M. S., Zuris, J. A. & Liu, D. R. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 533, 420–424 (2016).

39. Komor, A. C. et al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 561, 149–153 (2018).
40. Gaudelli, N. M. et al. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. *Nature* 551, 464–471 (2017).
41. Ryu, S.-M. et al. Adenine base editing in mouse embryos and an adult mouse model of myelin regulatory factor-related neuropathy. *Nat. Biomed. Eng.* 2, 692–705 (2018).
42. Liang, P. et al. Correction of β -thalassemia mutant by base editor in human embryos. *Protein Cell* 8, 811–822 (2017).
43. Niu, D. et al. Induction of Oligodendrocyte Progenitor Cells from Cystic Fibrosis Patient-Specific iPSCs by Inhibiting the mTOR Pathway. *Sci. Rep.* 9, 9472 (2019).
44. Ryu, S.-M. et al. Adenine base editing in mouse embryos and an adult mouse model of myelin regulatory factor-related neuropathy. *Nat. Biomed. Eng.* 2, 692–705 (2018).
45. Wang, W. et al. Crispr/Cas9 in pluripotent stem cells: Commentary on functional studies available for human disease research. *Curr. Protoc. Stem Cell Biol.* 38, 5B.4.1–5B.4.13 (2016).
46. Liang, X. et al. Enhanced CRISPR/Cas9-mediated precise genome editing by improved design and delivery of gRNA, Cas9 nuclease, and donor DNA. *J. Biotechnol.* 241, 136–146 (2017).
47. Sentmanat, M. F. et al. A survey of successful CRISPR/Cas9 guides and their nucleic acid motifs. *bioRxiv*, 213512 (2018).
48. Glass, Z. et al. Nanoparticles for CRISPR–Cas9 delivery. *Nat. Biomed. Eng.* 1, 854–855 (2017).
49. Horii, T. et al. Generation of an ICF syndrome model by efficient genome editing of human induced pluripotent stem cells using the CRISPR system. *Int. J. Mol. Sci.* 14, 19115–19126 (2013).
50. Fu, Y. et al. High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells. *Nat. Biotechnol.* 31, 822–826 (2013).
51. Veres, A. et al. Low incidence of off-target mutations in individual CRISPR-Cas9 and TALEN-targeted human stem cell clones detected by whole-genome sequencing. *Cell Stem Cell* 15, 27–30 (2014).
52. Chew, W. L. et al. A multifunctional AAV–CRISPR–Cas9 and its host response. *Nat. Methods* 13, 868–874 (2016).
53. Felgner PL. et al. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A.* (1987)
54. Haridhassan et al.. A comprehensive understanding of genome editing technologies: Obstacles and their optimization for therapeutic applications. *Stem Cell Research & Therapy*, 12(1), 1-26.
55. Tanaka, A. et al. Generation of human induced pluripotent stem cells by simple transcription of short hairpin RNA targeting OCT4 pseudo-gene transcript. *Scientific reports*, 5(1), 1-9.
56. Shao, Y. et al. A pluripotent stem cell-based multicolor fluorescent reporter assay to study CRISPR-Cas9-induced genomic structural variations. *Stem Cell Reports*, 15(2), 293-309.

57. Merkle, F. T. et al. Efficient CRISPR-Cas9-mediated generation of knockin human pluripotent stem cells lacking undesired mutations at the targeted locus. *Cell Reports*, 11(6), 875-883. (2015).
58. Anzalone, A. V., Koblan, L. W. & Liu, D. R. Genome editing with CRISPR-Cas nucleases, base editors, transposases and prime editors. *Nat Biotechnol* 38, 824–844 (2020).
59. Anzalone, A. V. et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 576, 149–157 (2019).
60. Webber, B. R., et al. Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors. *Nat. Commun.* 10, 5222 (2019). [Google Scholar](#)
61. Liu, X. et al. CRISPR-Cas9-mediated multiplex gene editing in CAR-T cells. *Cell Res.* 27, 154–157 (2017). [CrossRefPubMedGoogle Scholar](#)
62. Kuscu, C. et al. CRISPR-STOP: gene silencing through base-editing-induced nonsense mutations. *Nat. Methods* 14, 710–712 (2017). [CrossRefPubMedGoogle Scholar](#)
63. Dang, L. et al. Comparison of gene disruption induced by cytosine base editing-mediated iSTOP with CRISPR/Cas9-mediated frameshift. *Cell Prolif.* 53, e12820 (2020). [Google Scholar](#)
64. Zhu, H., et al. Metabolic Reprogramming via Deletion of CISH in Human iPSC-Derived NK Cells Promotes In Vivo Persistence and Enhances Anti-tumor Activity. *Cell Stem Cell* 27, 224–237.e6 (2020). [CrossRefPubMedGoogle Scholar](#)
65. Kluesner, M. G. et al. CRISPR-Cas9 cytidine and adenosine base editing of splice-sites mediates highly-efficient disruption of proteins in primary and immortalized cells. *Nat. Commun.* 12, 2437 (2021). [Google Scholar](#)
66. Kluesner, M. G. et al. EditR: A Method to Quantify Base Editing from Sanger Sequencing. *CRISPR J* 1, 239–250 (2018). [Google Scholar](#)
67. Webber, B. R., et al. Highly efficient multiplex human T cell engineering without double-strand breaks using Cas9 base editors. *Nat. Commun.* 10, 5222 (2019). [Find Full Text at U of MGoogle Scholar](#)
68. Wang, Minjing, Joshua B. Krueger, Alexandria K. Gilkey, Erin M. Stelljes, Mitchell G. Kluesner, Emily J. Pomeroy, Joseph G. Skeate, et al. “Precision Enhancement of CAR-NK Cells through Non-Viral Engineering and Highly Multiplexed Base Editing.” *bioRxiv*, January 1, 2024, 2024.03.05.582637. <https://doi.org/10.1101/2024.03.05.582637>.
69. Shamloo B, Usluer S. p21 in Cancer Research. *Cancers (Basel)*. 2019 Aug 14;11(8):1178. doi: 10.3390/cancers11081178. PMID: 31416295; PMCID: PMC6721478.
70. Burger's Medicinal Chemistry and Drug Discovery Sixth Edition, Volume 5: Chemotherapeutic Agents Edited by Donald J. Abraham ISBN 0-471-37031-2 © 2003 John Wiley & Sons, Inc.
71. Paul J. Newey, Caroline M. Gorvin, Michael P. Whyte, Rajesh V. Thakker, Chapter 1 - Introduction to Genetics of Skeletal and Mineral Metabolic Diseases, Editor(s): Rajesh V. Thakker, Michael P. Whyte, John A. Eisman, Takashi Igarashi, Genetics of Bone Biology and Skeletal Disease (Second Edition), Academic Press, 2018, Pages 1-21, ISBN 9780128041826, <https://doi.org/10.1016/B978-0-12-804182-6.00001-0>. (<https://www.sciencedirect.com/science/article/pii/B9780128041826000010>)

72. Rayner E, Durin MA, Thomas R, Moralli D, O'Cathail SM, Tomlinson I, Green CM, Lewis A. CRISPR-Cas9 Causes Chromosomal Instability and Rearrangements in Cancer Cell Lines, Detectable by Cytogenetic Methods. *CRISPR J.* 2019 Dec;2(6):406-416. doi: 10.1089/crispr.2019.0006. Epub 2019 Nov 19. PMID: 31742432; PMCID: PMC6919265.
73. Kirkpatrick M. How and why chromosome inversions evolve. *PLoS Biol.* 2010 Sep 28;8(9):e1000501. doi: 10.1371/journal.pbio.1000501. PMID: 20927412; PMCID: PMC2946949.
74. Liu Y, Ma G, Gao Z, Li J, Wang J, Zhu X, Ma R, Yang J, Zhou Y, Hu K, Zhang Y, Guo Y. Global chromosome rearrangement induced by CRISPR-Cas9 reshapes the genome and transcriptome of human cells. *Nucleic Acids Res.* 2022 Apr 8;50(6):3456-3474. doi: 10.1093/nar/gkac153. PMID: 35244719; PMCID: PMC8989517.
75. Fowler, Jonas L., Sherry Li Zheng, Alana Nguyen, Angela Chen, Xiaochen Xiong, Timothy Chai, Julie Y. Chen, et al. "Lineage-Tracing Hematopoietic Stem Cell Origins in Vivo to Efficiently Make Human HLF+ HOXA+ Hematopoietic Progenitors from Pluripotent Stem Cells." *Developmental Cell* 59, no. 9 (May 6, 2024): 1110-1131.e22. <https://doi.org/10.1016/j.devcel.2024.03.003>.
76. Denman, C. J. et al. Membrane-bound IL-21 promotes sustained ex vivo proliferation of human natural killer cells. *PLoS One* 7, e30264 (2012).
77. Pomeroy, E. J. et al. A Genetically Engineered Primary Human Natural Killer Cell Platform for Cancer Immunotherapy. *Mol. Ther.* (2019) doi:<https://doi-org.ezp1.lib.umn.edu/10.1016/j.ymthe.2019.10.009>.
78. Rafei, H., Daher, M. & Rezvani, K. Chimeric antigen receptor (CAR) natural killer (NK)- cell therapy: leveraging the power of innate immunity. *Br. J. Haematol.* 193, 216–230 (2021).
79. Guven, H. et al. Efficient gene transfer into primary human natural killer cells by retroviral transduction. *Exp. Hematol.* 33, 1320–1328 (2005).
80. Zhang, L., Tian, L., Dai, X. *et al.* Pluripotent stem cell-derived CAR-macrophage cells with antigen-dependent anti-cancer cell functions. *J Hematol Oncol* 13, 153 (2020). <https://doi.org/10.1186/s13045-020-00983-2>
81. Ueda, T., Shiina, S., Iriguchi, S. *et al.* Optimization of the proliferation and persistency of CAR T cells derived from human induced pluripotent stem cells. *Nat. Biomed. Eng* 7, 24–37 (2023). <https://doi.org/10.1038/s41551-022-00969-0>
82. Lin, Xiaotong, Yao Sun, Xin Dong, Zishen Liu, Ryohichi Sugimura, and Guozhu Xie. "iPSC-Derived CAR-NK Cells for Cancer Immunotherapy." *Biomedicine & Pharmacotherapy* 165 (September 1, 2023): 115123. <https://doi.org/10.1016/j.biopha.2023.115123>.
83. Mandegar MA, Huebsch N, Frolov EB, Shin E, Truong A, Olvera MP, Chan AH, Miyaoka Y, Holmes K, Spencer CI, Judge LM, Gordon DE, Eskildsen TV, Villalta JE, Horlbeck MA, Gilbert LA, Krogan NJ, Sheikh SP, Weissman JS, Qi LS, So PL, Conklin BR. CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs. *Cell Stem Cell.* 2016 Apr 7;18(4):541-53. doi: 10.1016/j.stem.2016.01.022. Epub 2016 Mar 10. PMID: 26971820; PMCID: PMC4830697.

84. Kwart D, Gregg A, Scheckel C, Murphy EA, Paquet D, Duffield M, Fak J, Olsen O, Darnell RB, Tessier-Lavigne M. A Large Panel of Isogenic APP and PSEN1 Mutant Human iPSC Neurons Reveals Shared Endosomal Abnormalities Mediated by APP β -CTFs, Not A β . *Neuron*. 2019 Oct 23;104(2):256-270.e5. doi: 10.1016/j.neuron.2019.07.010. Epub 2019 Aug 12. Erratum in: *Neuron*. 2019 Dec 4;104(5):1022. PMID: 31416668.
85. Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA. Chemically defined conditions for human iPSC derivation and culture. *Nat Methods*. 2011 May;8(5):424-9. doi: 10.1038/nmeth.1593. Epub 2011 Apr 10. PMID: 21478862; PMCID: PMC3084903.
86. Liu JT, Corbett JL, Heslop JA, Duncan SA. Enhanced genome editing in human iPSCs with CRISPR-CAS9 by co-targeting *ATP1a1*. *PeerJ*. 2020 May 1;8:e9060. doi: 10.7717/peerj.9060. PMID: 32391204; PMCID: PMC7197401.
87. Ng, E. S., Azzola, L., Bruveris, F. F., Calvanese, V., Phipson, B., Vlahos, K., ... & Elefanty, A. G. (2016). Differentiation of human embryonic stem cells to HOXA+ hemogenic vasculature that resembles the precursor to definitive hematopoietic stem cells. *Nature communications*, 7(1), 1-16.
88. Sturgeon, C. M., Ditadi, A., Awong, G., Kennedy, M., & Keller, G. (2014). Wnt signaling controls the specification of definitive and primitive hematopoiesis from human pluripotent stem cells. *Nature biotechnology*, 32(6), 554-561.
89. Yang, C. T., French, D. L., Goh, P. A., Jonsson, T. J., & Polak, L. (2022). Enhancing the production and function of hematopoietic stem and progenitor cells from pluripotent stem cells. *Experimental Hematology*, 106, 1-14.8
90. Azzola, L., Vlahos, K., Elefanty, A. G., & Ng, E. S. (2022). Stepping towards standardized production of hematopoietic stem and progenitor cells from pluripotent stem cells. *Stem Cell Reports*, 17(2), 267-282.
91. Knorr, D. A., Ni, Z., Hermanson, D., Loh, K. M., Jang, I. J., Durham, N. M., ... & Kaufman, D. S. (2013). Clinical-grade derivation of natural killer cells from human pluripotent stem cells for cancer therapy. *Stem Cells Translational Medicine*, 2(4), 274-283.
92. Zeng, J., Tang, S. Y., Toh, T. C., & Wang, S. (2017). Generation of "natural killer" cells from human pluripotent stem cells. *Stem Cells and Development*, 26(9), 633-640.
93. Post M, Cuapio A, Osl M, Lehmann D, Resch U, Davies DM, Bilban M, Schlechta B, Eppel W, Nathwani A, Stoiber D, Spanholtz J, Casanova E, Hofer E. The Transcription Factor ZNF683/HOBIT Regulates Human NK-Cell Development. *Front Immunol*. 2017 May 15;8:535. doi: 10.3389/fimmu.2017.00535. PMID: 28555134; PMCID: PMC5430038.
94. Nishimura, T., Kaneko, S., Kawana-Tachikawa, A., Tajima, Y., Goto, H., Zhu, D., ... & Nakauchi, H. (2013). Generation of rejuvenated T cells by reprogramming to pluripotency and redifferentiation. *Cell Stem Cell*, 12(1), 114-126.
95. Mohtashami M, Shah DK, Kianizad K, Awong G, Zúñiga-Pflücker JC. Induction of T-cell development by Delta-like 4-expressing fibroblasts. *Int Immunol*. 2013 Oct;25(10):601-11. doi: 10.1093/intimm/dxt027. Epub 2013 Aug 29. PMID: 23988616.

96. Cao, J., Packer, J. S., Ramani, V., Cusanovich, D. A., Huynh, C., Daza, R., ... & Shendure, J. (2019). Comprehensive single-cell transcriptional profiling of a multicellular organism. *Science*, 365(6452), 428-432.fixed
97. Haenseler, W., Sansom, S. N., Buchrieser, J., Newey, S. E., Moore, C. S., Nicholson, F. J., ... & Cowley, S. A. (2017). A highly efficient human pluripotent stem cell microglia model displays a neuronal-co-culture-specific expression profile and inflammatory response. *Stem Cell Reports*, 8(6), 1727-1742.
98. Eric Vivier *et al.* Innate or Adaptive Immunity? The Example of Natural Killer Cells. *Science* **331**,44-49(2011).
99. Kwon, Y. D., Lee, W. J., Sekaran, S., Pineau, J., Shiba, Y., Furusawa, C., ... & Maki, K. (2019). Scalable and robust production of induced pluripotent stem cell-derived B lymphocytes. *Journal of Experimental Medicine*, 216(7), 1608-1624.
100. Lotfi M, Morshedi Rad D, Mashhadi SS, Ashouri A, Mojarrad M, Mozaffari-Jovin S, Farrokhi S, Hashemi M, Lotfi M, Ebrahimi Warkiani M, Abbaszadegan MR. Recent Advances in CRISPR/Cas9 Delivery Approaches for Therapeutic Gene Editing of Stem Cells. *Stem Cell Rev Rep*. 2023 Nov;19(8):2576-2596. doi: 10.1007/s12015-023-10585-3. Epub 2023 Sep 18. PMID: 37723364; PMCID: PMC10661828.
101. Opitz, C. A., Litzemberger, U. M., Sahm, F., Ott, M., Tritschler, I., Trump, S., ... & Platten, M. (2011). An endogenous tumour-promoting ligand of the human aryl hydrocarbon receptor. *Nature*, 478(7368), 197-203.
102. Delconte, R. B., Guittard, G., Baginska, J., Lamichhane, R., Herrmann, A., Shortman, K., ... & Hunt, D. F. (2016). CIS protein: an essential regulator of cell fate decisions in vivo. *Molecular and Cellular Biology*, 36(18), 2268-2284.
103. Baixeras, E., Huard, B., Miossec, C., Jitsukawa, S., Martin, M., Hercend, T., ... & Triebel, F. (1992). Characterization of the lymphocyte activation gene 3-encoded protein. A new ligand for human leukocyte antigen class II antigens. *Journal of Experimental Medicine*, 176(2), 327-337.
104. Henson, S. M., & Akbar, A. N. (2009). KLRG1--more than a marker for T cell senescence. *Age*, 31(4), 285-291.
105. Yu, X., Harden, K., Anlon, L. C., Yang, D., Miller, H., Bifferova, K., ... & Barker, B. (2009). Cellist-cell ligand interaction software for immunoglobulin structural modeling. *Nat Protoc*, 4, 2057-2090.
106. Stanietsky, N., Simic, H., Arapovic, J., Toporik, A., Levy, O., Novik, A., ... & Mandelboim, O. (2013). The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. *Proceedings of the National Academy of Sciences*, 110(41), 16589-16594.
107. Ishida, Y., Agata, Y., Shibahara, K., & Honjo, T. (1992). Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *The EMBO journal*, 11(11), 3887-3895.
108. Keir, M. E., Buggert, M., Linkevicius-Salčius, M., Ganesan, S., Kochenderfer, J. N., Blazars, W. L., ... & Haining, W. N. (2008). Tissue expression of PD-L1 mediates peripheral T cell tolerance. *The Journal of Experimental Medicine*, 205(4), 883-895.
109. Ravetch, J. V., & Perussia, B. (1989). Alternative membrane forms of Fc gamma RIII (CD16) on human natural killer cells and neutrophils. Cell type-specific expression of

- two genes that differ in single nucleotide substitutions. *Journal of Experimental Medicine*, 170(2), 481-497.
110. Koene, H. R., Kleijer, M., Algra, J., Roos, D., Von dem Borne, A. E. G. K., & De Haas, M. (1997). Fc gamma RIIIa-158V/F polymorphism influences the binding of IgG by natural killer cell Fc gamma RIIIa, independently of the Fc gamma RIIIa-48L/R/H phenotype. *Blood, The Journal of the American Society of Hematology*, 90(3), 1109-1114.
 111. Brint, E., O'Callaghan, G., Watson, M., Dorico, A., Gregory, C., & Sanjeev, G. (2021). The defective apoptosis pathway in cancer and its therapeutic implications. *Apoptosis*, 26(1-2), 1-22.
 112. Hahn, S. A., Schutte, M., Hoque, A. T., Moskaluk, C. A., da Costa, L. T., Rozenblum, E., ... & Kern, S. E. (1996). DPC4, a candidate tumor suppressor gene at human chromosome 18q21. 1. *Science*, 271(5247), 350-353.
 113. Batlle, E., & Massagué, J. (2019). Transforming growth factor- β signaling in immunity and cancer. *Immunity*, 50(4), 924-940.
 114. Cabrera, C. M., Jiménez, P., Cabrera, T., Esparza, C., Ruiz-Cabello, F., & Garrido, F. (2003). Total loss of MHC class I in colorectal tumors can be explained by two molecular pathways: beta2-microglobulin inactivation in MSI-positive tumors and LMP7/TAP2 downregulation in MSI-negative lesions. *Tissue Antigens*, 61(3), 211-219.
 115. Zaretsky, J. M., Garcia-Diaz, A., Shin, D. S., Escuin-Ordinas, H., Hugo, W., Hu-Lieskovan, S., ... & Ribas, A. (2016). Mutations associated with acquired resistance to PD-1 blockade in melanoma. *New England Journal of Medicine*, 375(9), 819-829.
 116. Synthego Performance Analysis, ICE Analysis. 2019. v3.0. Synthego
 117. Gartel, A. L., & Radhakrishnan, S. K. (2005). Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer research*, 65(10), 3980-3985.
 118. Gartel, A. L. (2005). The conflicting roles of the cdk inhibitor p21 CIP1/WAF1 in apoptosis. *Leukemia research*, 29(11), 1237-1238
 119. Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., ... & Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*, 282(5393), 1497-1501.
 120. Kosicki, M., Tomberg, K., & Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR-Cas9 leads to large deletions and complex rearrangements. *Nature Biotechnology*, 36(8), 765-771.
 121. Haapaniemi, E., Botla, S., Persson, J., Schmierer, B., & Taipale, J. (2018). CRISPR-Cas9 genome editing induces a PDCD5-associated G2 arrest in human cells. *BioRxiv*, 335919
 122. Mohammadi, S., Vafadari, B., & Zaker, F. (2022). The role of aryl hydrocarbon receptor (AHR) in cancer: New insights. *Pharmacological Research*, 176, 106064.
 123. Chang, Y. H., Ouyang, W., Ho, I. C., & Chen, Y. J. (2021). CISH: A negative regulator of cytokine signaling and potential therapeutic target. *Journal of Biomedical Science*, 28(1), 1-13.
 124. Mathewson, N. D., Ashenberg, O., Tirosh, I., Gritsch, S., Perez, E. M., Marx, S., ... & Yosef, N. (2021). Inhibitory CD161 receptor identified as a target of reversible NK cell dysfunction. *Science*, 373(6562), eabf5326.
 125. Rodriguez-Abreu, D., Bordoni, R., & Cortés, J. (2022). Targeting TIGIT in cancer immunotherapy. *Future Oncology*, 18(1), 11-20.

126. Myers, J. A. (2018). Emerging KLRG1 inhibitors play pivotal role in enhancing T cell immunity. *Cancer Discovery*, 8(2), 155-157.
127. Wolchok, J. D. (2015). PD-1 blockers. *Cell*, 162(5), 937.
128. Alderson, K. L., & Sondel, P. M. (2011). Clinical cancer therapy by NK cells via antibody-dependent cell-mediated cytotoxicity. *Journal of Biomedicine and Biotechnology*, 2011.
129. Zhou, J., Lee, S. Y., Kim, B. J., & Lee, J. W. (2021). Antibody-Fc engineering for enhancing cancer immunotherapy. *Biotechnology Progress*, 37(5), e3168.
130. Romee, R., Rosario, M., Berrien-Elliott, M. M., Wagner, J. A., Jewell, B. A., Schappe, T., ... & Felices, M. (2016). Cytokine-induced memory-like natural killer cells exhibit enhanced responses against myeloid leukemia. *Science translational medicine*, 8(357), 357ra123-357ra123.
131. Brehm, C., Michels, S., Dunkelmann, C., Kuehl, L., Scheel, C., Schrauben, M. R., ... & Becker, P. S. (2022). Engineered Natural Killer Cell Therapy by mRNA Electroporation for Anti-Tumor Treatment. *Pharmaceutics*, 14(5), 1064.
132. Li, L., Liu, L. N., Feller, S., Allen, O. G., Shivakumar, E. B., Radovich, M., ... & Gerson, S. L. (2010). Expression of chimeric antigen receptors in natural killer cells with a regulatory-compliant non-viral method. *Cancer Gene Therapy*, 17(3), 147-154.
133. Moye, Z. D., Voskuil, J. L., & Bourque, K. (2021). Improving the efficacy of chimeric antigen receptor engineered natural killer cells through genetic modifications and combination immunotherapy. *Frontiers in Immunology*, 12, 2177.
134. Lai, Y., Wei, X., Lin, S., Qin, L., Cheng, L., & Li, P. (2017). Current status and perspectives of patient-derived xenograft models in cancer research. *Cancers*, 9(5), 52.
135. Hidalgo, M., Amant, F., Biankin, A. V., Budinská, E., Byrne, A. T., Caldas, C., ... & Villanueva, A. (2014). Patient-derived xenograft models: an emerging platform for translational cancer research. *Cancer discovery*, 4(9), 998-1013.
136. Lipsitz, Y. Y., Woodford, C., Saper, A., Hotaling, N. A., Iyengar, R., Jones, K. S., ... & Zandstra, P. W. (2021). Process analytical monitoring in stem cell manufacturing using Raman spectroscopy. *Biotechnology Journal*, 16(1), 1900342.