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IMPROVING TARGETED GENE EDITING IN HEMATOPOIETIC STEM CELLS FOR CLINICAL TRANSLATION

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ACRONYMS AND ABBREVIATIONS

53BP1: p53-binding protein 1
7-AAD: 7-AminoActinomycin D
AAV: Adeno-Associated Virus
AAVS1: AAV integration Site 1
ADA: Adenosine DeAminase
Ad: Adenoviral Vector
ART: AntiRetroviral Therapy
ART: Ataxia telangiectasia and Rad3-related
ATM: Ataxia Telangiectasia Mutated
BCL11A: B-cell CLL/lymphoma 11A
BER: Base Excision Repair
BM: Bone Marrow
bp: base pair
BRCA: BReast Cancer
bNAbs: broadly Neutralizing Antibodies
CAR: Chimeric Antigen Receptor
CB: Cord Blood
CCR5: C-C Chemokine Receptor type 5
CD: Cluster of Differentiation
CD40L: CD40 ligand
CDK: Cyclin-Dependent Kinase
cDNA: complementary DNA
CFC: Colony-Forming Cell
CFU-C: Colony-Forming Units - Cell
CGD: x-linked Chronic Granulomatous Disease
ChIP: Chromatin ImmunoPrecipitation
CIRCLE-seq: Circularization for in vitro reporting of cleavage effects
CLP: Common Lymphoid Progenitor
CMEP (or CMP): Common Myeloid-Erythroid progenitor cells
CMV: CytoMegaloVirus
crRNA: CRISPR RNAs
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
CtIP: CtBP-Interacting Protein
CXCL12: C-X-C motif chemokine ligand 12
CXCR4: C-X-C chemokine Receptor type 4
DDR: DNA Damage Response
DIGENOME-seq: Digest Genome sequencing
DISCOVER-seq: discovery of in situ Cas off-targets and verification
DNA: DeoxyriboNucleic Acid
dmPGE2: 16,16-dimethyl-PGE2
DNA-PKcs: DNA-dependent Protein Kinase catalytic subunit
DSB: Double Strand Break
dsDNA: double stranded DNA
dsODN: double stranded OligoDeoxyNucleotide
dsRNA: double stranded RNA
ELISA: Enzyme-Linked ImmunoSorbent Assay
FLASH: Fast Ligation-based Automatable Solid-phase High-throughput
FLT3-L: FMS-related Tyrosine kinase 3 Ligand

G-CSF: Granulocyte-Colony Stimulating Factor
gDNA: genomic DNA
GFP: Green Fluorescent Protein
GLP: Good Laboratory Practise
GM-CSF: Granulocyte-Macrophage Colony Stimulating Factor
GMLP: Granulocyte-Macrophage-Lymphocyte Progenitor
GMP: Granulocyte-Macrophage Progenitor
gRNA (or sgRNA): (single)guide RNA
GUIDE-seq: Genome-wide Unbiased Identification of DSBs Enabled by NGS
GvHD: Graft-versus-Host Disease
GvL: Graft-versus-Leukemia
H2AX: H2A histone family member X
HA: Homology Arm
HbF: Hemoglobin Fetal
HbS: Hemoglobin Sickle
HCT: Haematopoietic stem Cell Transplantation
HDR: Homology-Directed Repair
HIV: Human Immunodeficiency Virus
HJ: Holliday Junction
HLA: Human Leukocyte Antigen
HR: Homologous Recombination
HS: Hypersensitive Site
HSC: Haematopoietic Stem Cell
HSCT: Haematopoietic Stem Cell transplantation
HSPC: Haematopoietic Stem and Progenitor Cell
IDLV: Integrase-Defective Lentiviral Vector
IFN: InterFeroN
IL: InterLeukin
IL2RG: InterLeukine 2 Receptor common Gamma-chain
iPSC: induced Pluripotent Stem Cell
IS: Integration Site
kb: kilobases (1000 bp)
KI: Knock-In
KO: Knock-Out
LAM: Linear Amplification Mediated
LCR: Locus Control Region
LT-HSC: Long-Term HSC
LTR: Long Terminal Repeat
LV: Lentivirus
MEP: Megakaryocyte-Erythrocyte Progenitor
MLP: Multipotent lymphoid progenitor
MMEJ: Microhomology-Mediated End Joining
MPP: MultiPotent Progenitor
mPB: Mobilized peripheral blood
mRNA: messenger RNA
MHC: Major Histocompatibility Complex
MSC: Mesenchymal Stem Cell
NER: Nucleotide Excision Repair
NHEJ: Non-Homologous End Joining
NK: Natural Killer

NLS: Nuclear Localisation Signal
NOD: Non-Obese Diabetic
NSG: Next Generation Sequencing
NSG: NOD SCID Gamma
PAM: Proto-spacer Adjacent Motif
PBMC: Peripheral Blood Mononuclear Cell
PCR: Polymerase Chain Reaction
PGE2: ProstaGlandin E2
PGK: PhosphoGlycerate Kinase
PID: Primary ImmunoDeficiency
polyA: polyAdenylation sequence
PPP1R12C: Protein PhosPhatase 1 Regulatory subunit 12C
crRNA: CRISPR RNA
PRR: Pattern-Recognition-Receptor
RNA: RiboNucleic Acid
RNP: RiboNucleoProtein
ROS: Reactive Oxygen Species
RPA: Replication Protein A
RV: Retrovirus
SA: Splicing Acceptor
SCD: Sickle-Cell Disease
SCF: Stem Cell Factor
SCID-X1: X-linked Severe Combined ImmunoDeficiency
SCID: Severe Combined ImmunoDeficiency
SD: Splicing Donor
SDSA: Synthesis-Dependent Strand Annealing
SIN: Self-INactivating
SNP: Single Nucleotide Polymorphism
SR1: StemRegenin-1
SRC: SCID-Repopulating Cell
SSA: Single-Strand Annealing
SSC: Side Scatter
SSTR: Single-Strand Template Repair
ssDNA: single-stranded DNA
ssODN: single-stranded OligoDeoxyNucleotide
shRNA: short RNA
ST-HSC: Short-Term HSC
SV40pA: polyadenylation signal derived from the Simian Virus 40
TALE(N): Transcription Activator-Like Effector Nuclease
TPO: ThromboPOietin
tracrRNA: trans-activating CRISPR RNA
TSS: Transcription Start Site
UT: UnTreated cells
UV: UltraViolet
WAS: Wiskott-Aldrich Syndrome
WPRE: Woodchuck hepatitis virus Post-transcriptional regulatory Element
WT: Wild Type
XLF: XRCC4-Like Factor
XRCC4: X-Ray Cross Complementing protein 4
ZFN: Zinc Finger Nuclease

CHAPTER I

INTRODUCTION

For thousands of years, humans took advantage of genetic crosses between species to improve their living conditions. In the mid-19th century, Darwin introduced the theory of evolution by natural selection, but it is only a century later that scientists could identify the source of genetic information and described the deoxyribonucleic acid (DNA) structure (Watson & Crick, 1953). After decades, human genetic and virology finally crossed roads opening new perspectives of therapies for inherited genetic diseases leading to the birth of the gene therapy field (Friedmann & Roblin, 1972; Blaese *et al*, 1995). Hematopoietic stem cells soon became a valuable target to treat blood disorders, such as adenosine deaminase deficiency (ADA-SCID), with the help of retroviruses as carriers of therapeutic gene (Abbott, 1992). However, in human history, new technologies always brought their part of unexpected events. This has also been the case for the "pioneering" gene therapy trials performed in pediatric SCID patients (bubble boy), some of whom developed hematological malignancies years after treatment, due to semi-random viral integration near some oncogene (Cavazzana-Calvo *et al*, 2004). Scientists thoroughly worked to developed safer approaches by exploring and exploiting virus biology, even if some limits still constrain its applicability (Dunbar *et al*, 2018).

Programmable molecules, such as Zinc Finger Nuclease (ZFN) and Transcription Activator-Like Effector Nuclease (TALEN) opened the door to targeted genome editing and became a precise and powerful technology to deal with the human genome. In early 2000s, the RNA-guided Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas discovery allowed the genome editing field to make a giant leap towards easier and more versatile tool, thus extending gene therapy applications for several hematological diseases (Jinek *et al*, 2012). Nowadays, scientists are harnessing this technology, especially for *in situ* HDR-mediated gene correction, but it still requires optimization to definitively pave the way for its clinical translation.

In my PhD thesis, I will provide state-of-the-art in genetic engineering of hematopoietic stem cells, starting from blood cells description and characterization, describing what gene therapy is and explaining how gene editing can be exploited to further extend applications of gene addition/correction for blood disorders. Moreover, I will present newly published data that hopefully will contribute to move the field toward clinical translation.

1. Hematopoietic Stem and Progenitor Cells (HSPCs)

Blood is a whole body regenerating liquid tissue and hematopoiesis is the biological process that guarantees life-long production of blood cells. This mechanism is involved in many physiological functions, such as coagulation, oxygen transport, immunity, and tissue remodeling (Boulais & Frenette, 2015). The bone marrow (BM) microenvironment is the primary site of human hematopoiesis which is a semi-solid tissue forming the medullary cavities of long and flat bones.

1.1. Hematopoiesis and hematopoietic hierarchy

At the beginning of the 20th century, for the first time the existence of a common progenitor for all blood cells has been theorized (Maximow, 1909). Later, scientists showed that mice exposed to lethal irradiation can be rescued by transplanting bone marrow cells leading to the first evidence of hematopoietic stem and progenitor cells (HSPCs) capable of functionally regenerating the hematopoietic system (Jacobson *et al*, 1951; Cole *et al*, 1955). In the following decades, further studies have defined a hierarchical tree-like model of hematopoiesis where all mature blood and immune cells derived from a restricted pool of multipotent HSCs capable of self-renewing and maintaining the stem cell state. Lineage-restricted progenitors are generated in a stepwise manner by subsequent binary branching decisions (Kondo *et al*, 1997; Akashi *et al*, 2000; Notta *et al*, 2011; Morrison *et al*, 1995).

At the top of the tree structure take place the long-term and short-term HSC (LT/ST-HSC), which progressively display diminished self-renewing potential and more differentiated phenotypes (Benveniste *et al*, 2010; Copley *et al*, 2012). Multipotent progenitor (MPP) is the immediate progeny which does not bear self-renewing capacity but robustly proliferate and differentiate in lineage committed progenitors (Seita & Weissman, 2010): i) common myeloid progenitor (CMP) differentiates in megakaryocyte-erythrocyte progenitor (MEP) ultimately giving rise to red blood cells and platelets, while granulocyte-macrophage progenitor cell (GMP) originates monocytes, granulocytes (neutrophils, basophils and eosinophils) and dendritic cells; ii) multipotent lymphoid progenitor (MLP) produces natural killer (NK) cells, T- and B- lymphocyte cells (**Figure 1**).

The recent advances in single-cell transcriptomic studies uncovered higher degree of complexity and heterogeneity within HSPC compartments. Indeed, individual HSC gradually acquires lineage biases along multiple directions. This differentiation process is defined as highly elastic and dynamic, where unilineage-restricted cells emerge from a "continuum of low-primed undifferentiated (CLOUD)-HSPCs (Velten *et al*, 2017; Laurenti & Göttgens, 2018).

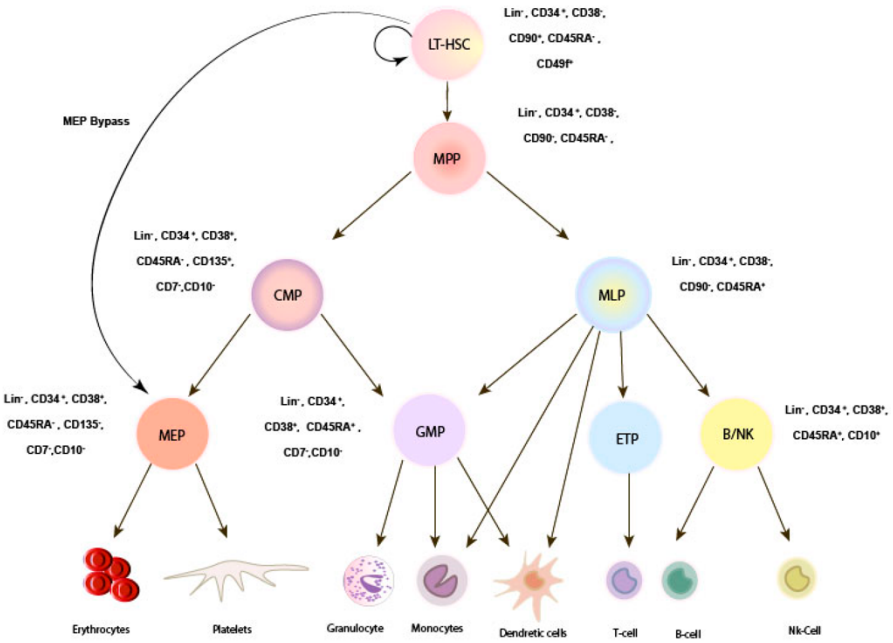

Figure 1: *Schematic representation of the revised model for human HSC hierarchy giving rise to the entire repertoire of hematopoietic cells.* In the classic model for the human HSC hierarchy LT-HSCs differentiates into MPP, CMP, MLP giving rise to all hematopoietic lineages. In the revised model, HSCs can differentiate directly into MEP bypassing CMP (Tajer *et al*, 2019).

Primitive HSCs are mainly characterized by quiescent state with low levels of mitochondrial activity, protein synthesis and glycolytic metabolism. The persistence in G_0 of cell cycle has been indicated as a mechanism preventing accumulation of mutations and preserving lifelong stemness reservoir. On some occasions, HSCs can transiently engage cell cycle activation to perform symmetric or asymmetric division, where a cell can divide to produce two progeny cells adopting either the same or different fates (**Figure 2**). Multiple studies in mouse indicate that the frequency of HSC division inversely correlates with their repopulating capacity (Wilson *et al*, 2008; Laurenti *et al*, 2015; Bernitz *et al*, 2016). Finally, the BM microenvironment also plays an essential role in the balance between self-renewing asymmetric division versus differentiation (Ho, 2005).

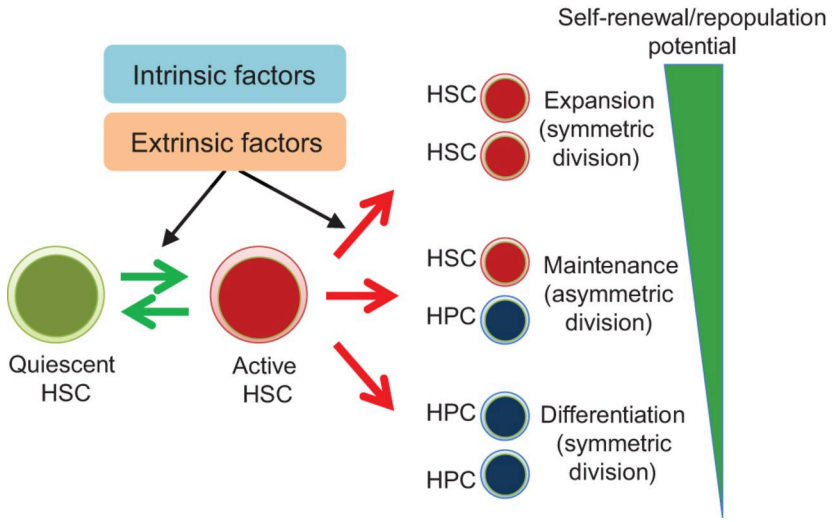

Figure 2: *Schematic of HS/PC divisions.* A quiescent HSC is activated by cellular -intrinsic or -extrinsic factors leading to symmetric or asymmetric cell divisions in relation to self-renewal and the repopulation potential of HSCs (Nakamura-Ishizu *et al*, 2014).

1.2. Phenotypic identification of HSPC subpopulations

In 1984, human $CD34^+$ cell surface glycoprotein promoting cell-to-cell adhesion has been proposed as suitable marker for enrichment of a heterogeneous population of hematopoietic multi- and oligo-potent progenitor cells (Civin *et al*, 1984; Berenson *et al*, 1988). Later, human $CD90^+$ cell surface glycoprotein implicated in cell-to-matrix interactions was also proposed to identify a subpopulation of more primitive HSCs capable of establishing multipotent long-term culture and guaranteeing long-term multilineage reconstitution *in vivo* (Weissman & Shizuru, 2008; Baum *et al*, 1992). Other surface markers were evidenced to further enrich for more primitive cells within the $CD34^+$ cell compartment, such as $CD133^+$ (transmembrane glycoprotein) (Yin *et al*, 1997), $CD49f^+$ (integrin α6) and EPCR/$CD201^+$ (endothelial protein receptor) (Notta *et al*, 2011; Fares *et al*, 2017). Inversely, primitive cells are associated with low or undetectable surface expression of $CD38^+$ (cyclic ADP ribose hydrolase) and $CD45RA^+$ (isoform of protein tyrosine phosphatase receptor type C) (Hao *et al*, 1996; Bhatia *et al*, 1997). Negative selection (Lin^-) from lineages markers can also be performed with a cocktail of antibodies recognizing surface antigens expressed by human hematopoietic mature cells, such as $CD3^+$, $CD14^+$, $CD16^+$, $CD19^+$, $CD20^+$, $CD56^+$. Importantly, expression of some of these surface cell markers can be altered during *ex vivo* manipulation of HSPCs due to culture conditions, treatment alterations or exposure to medium supplements (**Figure 1**).

Phenotypic identification of human HSPC subpopulations by cytofluorimetric is based on the use of fluorescent dye-conjugated antibodies which bind specific antigens on the cell membrane.

1.3. Homeostasis in the hematopoietic niche

HSPCs constitute a heterogeneous population coexisting in the same tissue. During homeostasis, the stem cell "niche" defined the BM micro-environment that plays a key role in the regulation of HSC quiescence, proliferation and differentiation. Mouse studies show that quiescent HSCs are located near bone surfaces or are associated with the sinusoidal endothelium (Kunisaki *et al*, 2013).

The stem cell niche is a complex structure composed by several cell types also called stromal cells that interact and regulate HSC homeostasis. Stromal cells secrete essential components for HSC maintenance such as CXC-chemokine ligand 12 (CXCL12) or stem cell factor (SCF). Endothelial cells, megakaryocytes, T cells, Schwann cells and adipocytes also participate to regulation of HSC homeostasis, long-term self-renewing and quiescence (**Figure 3**).

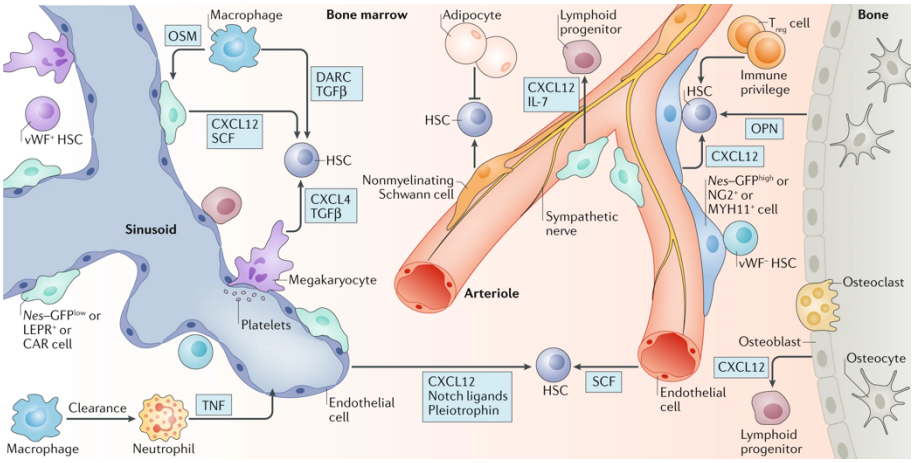

Figure 3: *Schematic representation of the adult bone marrow hematopoietic niche.* In homeostasis, various cell types and niche factors directly or indirectly regulate HSC activity. The stromal cells are key regulators of HSC maintenance by producing chemo-attractive factors as SCF or CXCL12. Moreover, HSC-derived such as macrophages, neutrophils, regulatory T cells and megakaryocytes can feedback and contribute to HSC maintenance or mobilization (Pinho & Frenette, 2019).

Chemo-attractive CXCL12 is recognized by CXCR4, a cell surface receptor expressed by HSPCs, which is involved for BM niche homing and mobilization. This is a dynamic process orchestrated by regulation of CXCL12/CXCR4 signaling leading to circulating HSPC capture into the niche, or inversely the release into the bloodstream (Ara *et al*, 2003). HSPC recirculation is important to maintain a stem cell pool ready-to-use in case of organ damages or infections (Ratajczak, 2018). Many studies have been performed to disentangle the complex BM niche network and to further improved clinical protocols for *ex vivo* HSC manipulation, nay, expansion.

1.4. HSPC collection and ex vivo manipulation

Novel therapies based on *ex vivo* HSPC engineering holds great potential for the treatment of several human blood disorders. To this aim, HSPCs can be harvested from umbilical cord blood (CB-CD34$^+$), bone marrow (BM-CD34$^+$) or peripheral blood (mPB-CD34$^+$) (Pelus & Broxmeyer, 2018). Peripheral blood leukapheresis is the most suitable method for clinical applications but it requires HSPC mobilization from the BM niche to the bloodstream. To this aim, the gold standard protocol refers to administration of granulocyte colony stimulating factor (G-CSF) (Publicover *et al*, 2013). Indeed, G-CSF induces granulocytes recruitment in BM leading to down-regulation of adhesion molecules (Korkmaz & Altuntas, 2017). More recently, G-CSF has been combined with plerixafor (or AMD3100), a small molecule, that antagonizes the CXCL12/CXCR4 bound (Matthys *et al*, 2001) resulting in higher HSPCs release into the bloodstream (Uy *et al*, 2008).

Culturing HSPCs is a challenge by considering the harness to reproduce physiological BM niche environment *in vitro*. To preserve human HSPCs properties, improvements in *ex vivo* manipulation have been made about medium supplementation with hematopoietic cytokines cocktail composed by human-derived FLT3L, SCF, TPO and IL-6/-3 (Sauvageau *et al*, 2004). Conversely, high cytokine concentration or prolonged stimulation with these cocktails promoted HSC differentiation (Uchida *et al*, 2003; Walasek *et al*, 2012). Recently, the screening of wide small molecule libraries was performed to identify novel compounds promoting HSPC maintenance, repopulation capacity or even expansion. StemRegenin 1 (SR1), an aryl hydrocarbon receptor (AhR) antagonist, increases the engraftment capacity of cultured CB-CD34$^+$ cells after transplantation in NSG mice (Boitano *et al*, 2010). Moreover, 16,16-dimethyl-prostaglandin E2 (dmPGE2) increases CXCR4 expression potentially favoring homing and protects HSPCs from apoptosis (Hoggatt *et al*, 2009). More recently, the pyrimidoindole derivative UM171 has been identified and remarkably enhances long-term repopulation capacity of cultured CB-CD34$^+$ (Fares *et al*, 2014). Moreover, successful expansion of long-term repopulating CB- and BM-CD34$^+$ have been made when embedded in a zwitterionic hydrogel mimicking the complex 3D structure of the BM niche, thus preventing excessive ROS production (Bai *et al*, 2019).

Finally, the overall toxicity of *ex vivo* HSPC engineering protocols, prompts the need for both *in vitro* and *in vivo* models to assess HSPC repopulation potential before moving forward human therapies.

1.5. Ex vivo HSPC functionality assessments

1.5.1. In vitro clonogenic assays

Clonogenic assays were developed to study the multilineage colony forming capacity of HSPCs (Pike & Robinson, 1970). In the colony forming cell (CFC) assay, a small number of human HSPCs is plated in semi-solid media in order to isolate colony forming unit cells (CFU-C). Culture supplementation with suitable factors triggers HSPC differentiation upon very limited round of divisions. The number of colonies is used as a surrogate measure of HSPCs retaining functional properties. Furthermore, it is possible to assess differentiation biases between myeloid and erythroid colonies that are distinguished by morphological criteria. Still, CFC assay readout is constrained by *ex vivo* culturing and might not reflect entirely the long-term repopulation capacity of HSCs pool in capability of self-renewing and homing to bone marrow niche after *in vivo* transplantation.

1.5.2. In vivo xenograft models

C.B-17 scid hematochimeric mouse model has been established first to study human hematopoiesis where human BM-derived hematopoietic cells were successfully transplanted (Kamel-Reid & Dick, 1988; Lapidot *et al*, 1992). However, human cell engraftment was low and transient, mainly due to the presence of functional host NK cells, macrophages and granulocytes. Later, backcrossing *C.B-17 scid* mice with nonobese diabetic (NOD) mice, which present deficient NK cell and macrophage functions, allowed higher levels of human HSPC engraftment (Greiner *et al*, 1995) but still this NOD/SCID mouse model suffered of low human B/T cells maturation and the high incidence of thymic lymphoma prevented long-term studies. Finally, NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ.NSG harboring a complete null mutation of the *IL2rg*^{null} gene prevents cytokine signaling through multiple receptors leading to a deficiency in functional murine B, T and NK cells. NSG constitutes the most immunodeficient mouse strain and began a gold standard as hematochimeric mouse model.

Xenotransplantation of human HSPCs requires sublethal irradiation to make space in the BM from recipient mice allowing robust engraftment of human CD34$^+$ hematopoietic stem cells (Ishikawa *et al*, 2005; Shultz *et al*, 2005). However, irradiation irremediably damages multiple organs and tissue, included the BM microenvironment. Recently, a new mouse strain called NBSGW has been obtained by crossing NSG mice with C57BL/6 ones carrying mutant *kit* (W41) and enables comparable levels of human cell to NSG mice, without the need for irradiation (McIntosh *et al*, 2015).

1.6. HSC-based therapies for hematological diseases

Knowledges in hematopoiesis and HSC biology enabled effective and life-saving treatments for a variety of blood diseases. Hematopoietic stem cell transplantation (HSCT) consists in administration of healthy HSC in patients presenting dysfunctional (deficiency syndromes, hemoglobinopathies) or depleted bone marrow (hematological malignancies) (Copelan, 2006). In 50's, the first successful syngeneic bone marrow transplantation has been done between twins to treat a patient with acute leukemia (Thomas *et al*, 1957). A decade later, a pediatric patient with severe combined immunodeficiency (SCID) syndrome successfully received allogeneic bone marrow transplantation from matched donor (Gatti *et al*, 1968). Allogenic HSCT broaden applicability of HSC-based therapies even still limited by donor availability. Indeed, genetic variability between individuals can be responsible for graft rejection in case of incomplete conditioning of the recipient, or graft-versus-host disease (GvHD) which is a potentially lethal immunological reaction of donor lymphocytes against host tissue.

The major histocompatibility complex (MHC) class I and class II proteins play a pivotal role in the adaptive immunity (Dausset *et al*, 1958). The Human Leukocyte Antigens (HLAs) encoded by genes located in MHC are responsible for immunological tissue compatibility by recognition of polymorphic fragments of foreign HLA molecules. HLA genotyping at A, B, C, DRB1 and DQB1 of both donor and recipient is crucial before performing HSCT. However, for many patients a perfectly matched related or unrelated donor is not always available. Thus, establishment of HSCT registries help to identify a potential HLA-matched unrelated donor (Nowak, 2008). Interestingly, HLA mismatches have been exploited to trigger the graft-versus-leukemia (GvL) response, between leukemic and donor HSCT cells, which accounts for reduced rates of relapse and contributes to malignancy eradication (Jenq & Van Den Brink, 2010).

Based on these considerations, allogenic HSCT has some limitations: i) necessity of a compatible donor; ii) occurrence of GvHD and associated life-threatening complications; iii) partial or unsuccessful HSC collection from matched donor; iv) genotoxic myeloablative or conditioning regimens are often needed to clear recipient cells and makes space for efficient donor stem cell engraftment (Bernardo & Aiuti, 2016). Recently, immunotoxin (CD45-saporin) has been develop to target specifically hematopoietic cells in the bone marrow avoiding neutropenia and anemia, spared bone marrow and thymic niches, and enabling rapid recovery of T and B cells with minimal overall toxicity (Palchaudhuri *et al*, 2016).

Finally, autologous transplantation of genetically modified HSPCs is an alternative option for patients affected by genetic blood diseases and missing HLA-matched donor. Autologous HSPCs transplantation is associated with lower risks of GvHD, graft rejection, or infection

during immunosuppression due to more rapid immune reconstitution. Overall, gene therapy by gene addition or site-specific correction is paving the way to safer and effective treatments of inherited blood diseases (Naldini, 2019).

2. *Ex vivo* engineering of autologous HSPCs

2.1. HSC gene therapy

The aim of gene therapy is to genetically modify the cellular genome to replace a defective gene or to provide a novel function in order to guarantee prolonged clinical benefit to patients (Naldini, 2015). In most cases, gene therapy exploits the knowledge on virus biology to generate, carry and transfer a functional copy of the disease-causing gene into patients' cells. *In vivo* delivery, either systemic or local, of the corrective transgene is required to target cells that are not isolated from the body, such as eye or muscle cells (Russell *et al*, 2017; Le Guiner *et al*, 2017). Instead, autologous HSPCs cells can be easily harvested from the patient (Pelus & Broxmeyer, 2018), cultured and engineered *ex vivo* before being ultimately re-infused back into the same subject. The gene-modified cells engraft in the bone marrow, where they self-renew potentially for the lifetime of the individual while giving rise to differentiating progeny along all hematopoietic lineages (**Figure 4**). The remarkable progresses *in vitro* manipulation of human primary cells (Boitano *et al*, 2010; Cieri *et al*, 2013) confines the manipulation to a defined cell subset, diminishing the risk of off-target effects and bystander toxicity spillover. Despite originally envisioned to treat inherited monogenic disorders (Friedmann & Roblin, 1972), *ex vivo* therapy strategies can also be applied to acquired diseases, such as genetic engineering of T cells for cancer immunotherapy (Sadelain *et al*, 2017).

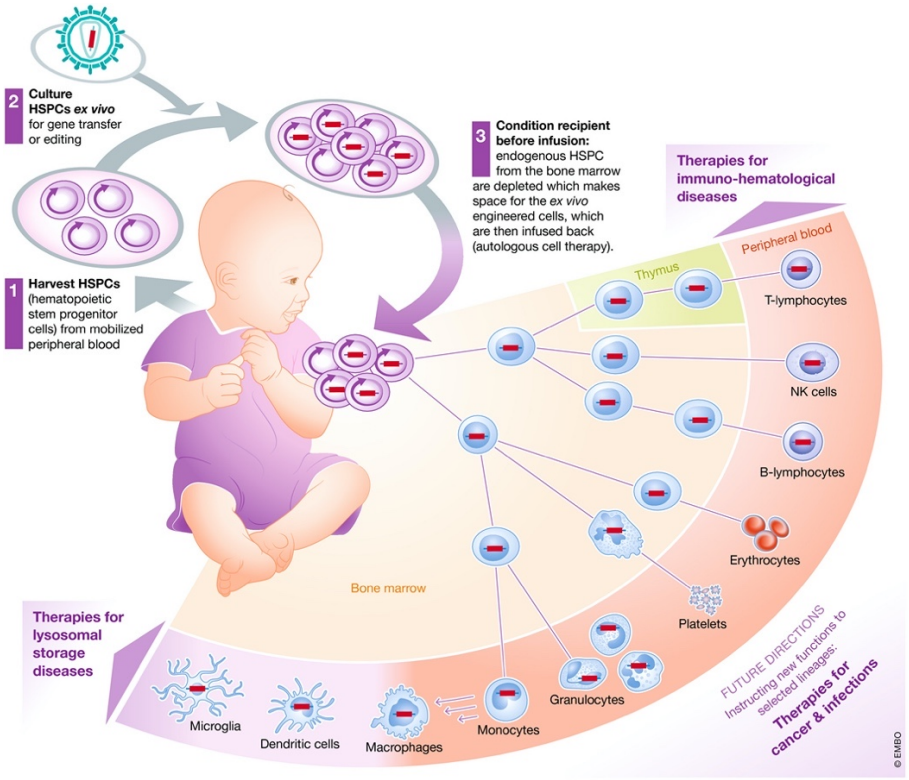

Figure 4: *Schematic of HSPCs gene engineering for blood diseases.* (1) HSPC are harvested from the patient and (2) ex vivo engineered before being (3) re-infused back to the patient (autologous cell therapy). Genetic engineering of hematopoiesis broaden applicability rather than replacing inherited defective genes, to cancer or chronic infections (Naldini, 2019).

Ex vivo gene addition exploits integrating retrovirus (RV) or lentivirus (LV) as viral vectors which results in permanent and semi-random integration(s) of one or more functional copies of the therapeutic transgene into the genome. In the last decades, gene therapy clinical trials have demonstrated therapeutic potential for the treatment of several blood diseases, such as adenosine deaminase deficiency (ADA-SCID), X-linked severe combined immunodeficiency (SCID-X1), chronic granulomatous disease (CGD), Wiskott-Aldrich Syndrome (WAS), β-thalassemia and hematological malignancies (Aiuti *et al*, 2002; Marktel *et al*, 2019; Thompson *et al*, 2018; Cavazzana-Calvo *et al*, 2000; Kohn *et al*, 2020; Ferrua *et al*, 2019). However, SCID-X1, CGD and WAS gene therapy trials raised safety issues concerning the potential malignant transformation of transduced cells (Cavazzana-Calvo *et al*, 2000; Braun *et al*, 2014; Stein *et al*, 2010). Indeed, viral-mediated gene therapy carries intrinsic genotoxic risks associated with semi-random insertional mutagenesis, as a consequence of integration close to or into proto-oncogenes, leading to their overexpression, or due to knock out of tumor suppressor genes (Cavazza *et al*, 2013). Furthermore, expression of the transgene itself may be unphysiological, as the endogenous regulation cannot be precisely recapitulated (Hacein-Bey-Abina *et al*, 2003; Woods *et al*, 2006). The outcomes differ depending on the transduced cell type, disease pathophysiology and

individual factors. For instance, RV integration near to proto-oncogenes caused leukemia in patients undergoing HSPC gene therapy for SCID-X1 (Hacein-Bey-Abina *et al*, 2003).

Improvements in vector design, such as self-inactivating LV, have greatly reduced the genotoxic risk related to insertional mutagenesis, supported by multiple years of cumulative follow-up. The development of such viral vectors substantially improved the safety profile of gene addition strategies, which have now reached advanced stages of clinical testing or even commercialization (Dunbar *et al*, 2018; Cicalese *et al*, 2016). Still, insertional mutagenesis occurs and thus genotoxicity remains a concern, particularly because knock-out of tumor suppressor genes cannot be excluded. Furthermore, constitutive or unregulated expression of the transgene is unsuitable for the treatment of a number of diseases that require granular control of gene expression. In this framework, precise modification of the human genome may extend the clinical applicability of gene therapy beyond conventional gene addition strategies, improving safety and efficacy.

2.2. HSC targeted genome editing

Targeted genome editing enables site-specific modification at an intended locus of the genome by introducing deletions, insertions, nucleotide substitutions or targeted integration of a therapeutic cassette. The development of engineered programmable nucleases able to deliver a site-specific DNA double-strand break (DSB) upon precise recognition of a nucleotide sequence has been a revolutionary milestone for the development of genome editing technologies. The genome editing toolbox includes several chimeric molecules composed by: i) an effector domain, which is often an endonuclease domain inducing a DSB in the proximity of the DNA binding site; ii) a protein- or RNA-based DNA binding structure, which dictates the specificity of the effector domain.

2.2.1 The genome editing toolbox

In 80's, crystallographic and biochemical studies uncovered the role of sequence-specific DNA binding proteins that regulate gene expression (Pabo & Sauer, 1983). DNA-binding domains (DBDs) confer to ZIF268 protein binding specificity (Pavletich & Pabo, 1991). The fusion of DBDs with a catalytic endonuclease domain can be exploited to target a specific DNA sequence in genome and to induce intentional lesion (Smith, 2000; Gersbach, 2014). The induction of DNA DSB triggers a complex cellular response, known as DNA damage response (DDR), which orchestrates the repair of the DNA DSB and promotes homologous recombination events (Bibikova *et al*, 2001). So far, engineered programmable nucleases, such as Zinc

Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and the RNA-guided Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas, are the most exploited platforms for targeted genome editing (Carroll, 2014). The criteria that define the choice of the nuclease is based on: i) portability to multiple genomic loci; ii) delivery to cells; iii) specificity (on-target activity); iv) minimal toxicity.

Zinc finger nucleases (ZFNs)

The most widely used ZFP class is the Cys2His2 (C2H2)-type, one of the most present in eukaryotic transcription factors. ZFNs are chimeric proteins typically composed by the FokI endonuclease domain and an array of three to six DBDs, thus recognizing a DNA sequence of 9 to 18 bp (Gaj *et al*, 2013). A pair of ZNFs is required to allow FokI to dimerize and to induce DNA DSB at the target site (Bitinaite *et al*, 1998; Mani *et al*, 2005). Moreover, ZFN monomers binds the DNA in a head-to-head configuration by associating with the positive and negative strands of DNA double helix (**Figure 5**). A minimal distance of 5-7 bp between the target sites of each ZFN monomer is required to maximize the efficiency of DNA cleavage (Isalan, 2012).

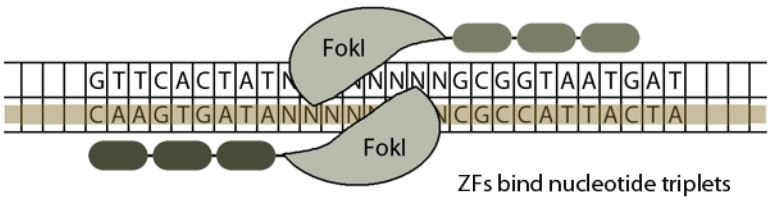

Figure 5: *Schematic of ZFN pairs binding targeted DNA sequence.* Each ZFN consists of the cleavage domain of FokI fused to a zinc-finger protein (ZFP) that has been customized to specifically recognize either a 'left' or 'right' half-site, which are separated by a spacer of either 5 or 6 bp. Simultaneous binding by both ZFNs enables dimerization of the FokI nuclease domain and DNA cleavage (copyright© Xenbase).

Different strategies were developed to speed up the design and optimized the structure to enhance ZFN binding specificity and nuclease domain efficiency (Cathomen & Keith Joung, 2008; Urnov *et al*, 2010). Although optimized ZFN have shown very promising results for therapeutic genome editing (Maier *et al*, 2013; Tebas *et al*, 2014), their design remain challenging, time-consuming and expensive.

Transcription activator-like effector nucleases (TALENs)

TALENs are composed by a tandem of TALE repeats (33-35 amino acids each) fused with a flexible linker to FokI endonuclease domain (Joung & Sander, 2013). Each TALE recognizes a specific nucleotide of the target sequence by non-covalent binding (Moscou & Bogdanove,

2009). Similarly to ZFNs, head-to-head dimerization of TALENs is required to promote FokI site-specific DNA DSB (**Figure 6**) (Joung & Sander, 2013).

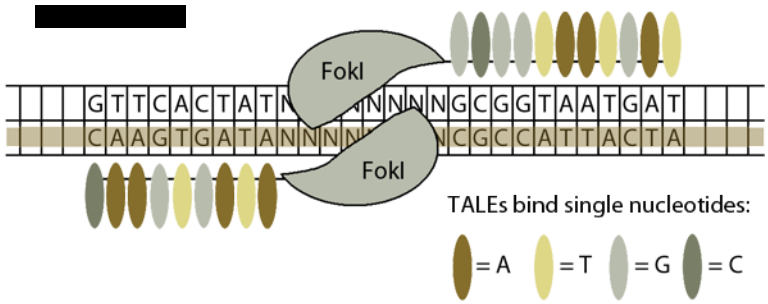

Figure 6: *Schematic of TALEN pairs binding targeted DNA sequence.* Each TALEN consists of the cleavage domain of FokI fused to TALE repeats that specifically bind each nucleotide of DNA target site. Cleavage by the FokI nuclease domains occurs in the 'spacer' sequence that lies between the two regions of the DNA bound by the two TALEN monomers (copyright© Xenbase).

Although TALEN design is easier than ZFNs, the length of each repeat, the repetitiveness within the DBDs and the high homology score among TALE repeats represent challenges for TALEN assembly and expression (Cermak *et al*, 2011; Morbitzer *et al*, 2011; Reyon *et al*, 2012).

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas nucleases

CRISPR/Cas are widespread systems in prokaryotes involved in adaptive immunity against phage or plasmid DNA (Sorek *et al*, 2008; van der Oost *et al*, 2009). During the last decade, CRISPR/Cas9 system derived from *Streptococcus pyogenes* has been adapted and optimized for the use as genome editing tools in eukaryotic cells and began the gold standard. Cas9 endonuclease is complexed to a guide RNA (gRNA) which is composed by two parts: i) a small invariable trans-activating (tra)crRNA molecule having 25 nucleotides complementary to the CRISPR repeats; ii) a commutable crRNA that dictates the specificity of the gRNA by complementary matching to the target DNA genomic sequence (Deltcheva *et al*, 2011). Further optimization of gRNA led to create a unique chimeric molecule know as single guide RNA (sgRNA) where crRNA and tracrRNA are fused by an RNA hairpin structure, that has been awarded in October 2020 by Nobel Prize in chemistry (Jinek *et al*, 2012). Importantly, the Cas9:gRNA ribonucleoprotein (RNP) complex activity is constrained by recognition of 5'-NGG-3' protospacer adjacent motif (PAM) sequence which must be present. Cas9 induces a DNA DSB at position -3 from the NGG PAM sequence and releases blunt DNA ends (**Figure 7**).

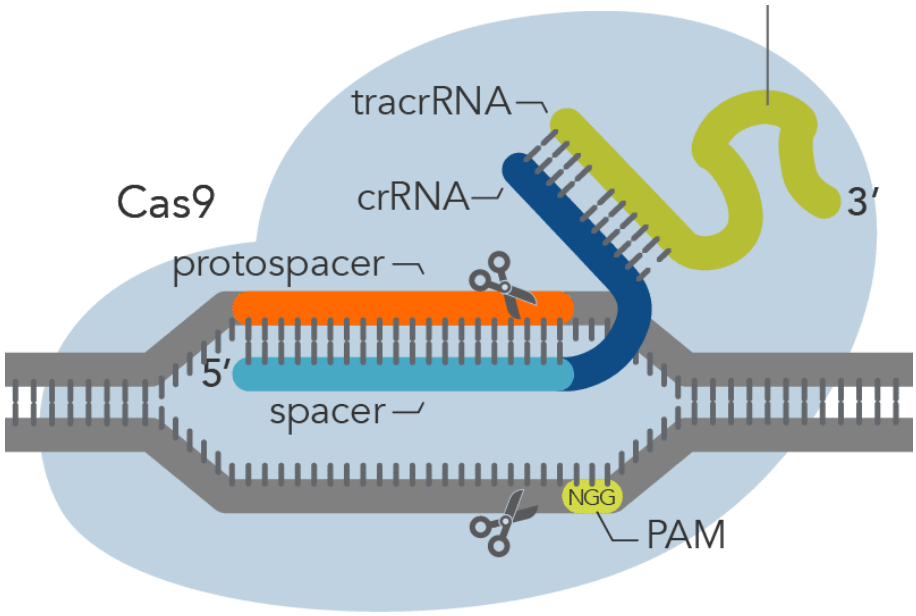

Figure 7: *Schematic of CRISPR/Cas9 binding to his targeted DNA sequence.* Hybridized crRNA:tracrRNA or sgRNA (not shown) form a complex with Cas9 nuclease to target specific genomic site. The crRNA recognizes protospacer 19 or 20 nt on the strand opposite from the PAM sequence and defines the active cleavage site (www.idtdna.com).

The distribution of PAM sequences in the human genome constrains the set of possible targets, and in turn the application of this platform. To expand the repertoire of targetable DNA sequences, other Cas9 homologues (e.g. Cas12a/Cpf1) (Zetsche *et al*, 2015) and Cas9 proteins requiring different PAM sequences have been identified in other bacteria species, such as *Staphylococcus aureus* (Ran *et al*, 2015), *Neisseria meningitis* (Lee *et al*, 2016), and *Streptococcus thermophilus* (Müller *et al*, 2016; Xu *et al*, 2015). Finally, Cas9 variants with relaxed PAM preferences (Cas-NG and xCas) (Hu *et al*, 2018; Nishimasu *et al*, 2018) or altered PAM profiles (SpCas9-VQR, VRQR and VRER) (Harrington *et al*, 2017; Kleinstiver *et al*, 2016) have been developed by directed evolution or structure-guided engineering and further expanded the range of editable targets. Recently, a SpCas9 variant (SpRY), requiring a 5'-NRN-3' PAM, has been generated to edit previously inaccessible genetic sites, significantly overcoming most PAM-related limitations (Walton *et al*, 2020).

In the last years, extensive engineering improved CRISPR/Cas9 specificity and efficiency by modifying gRNA and Cas9 protein architecture. Concerning gRNAs, 5'-truncated gRNAs (tru-gRNA) showed similar on-target activity than standard gRNAs but several-fold lower off-

target activity (Fu *et al*, 2014), likely reducing the interaction energy at the RNA–DNA heteroduplex level (Lim *et al*, 2016). The addition of two guanines at the 5' end of the gRNA reduced off-target activity, albeit also decreasing on-target editing in some cases (Cho *et al*, 2014). Instead, chemical modifications (2'-O-methyl-3'phosphorothiorate or 2'-O-methyl-3'thioPACE) of the three terminal nucleotides at the 5' and 3' ends improved the specificity profile and enhanced tolerability compared to unmodified gRNA in HSPCs (Hendel *et al*, 2015). Concerning Cas9 protein, novel variants (eSpCas9(1.1) and SpCas9-HF1) having higher cleavage fidelity due to the dampened interaction strength with the DNA (Slaymaker *et al*, 2016; Kleinstiver *et al*, 2016) have been identified by structure-guided mutagenesis. However, these variants showed lower on-target activity than wild-type SpCas9 in human HSPCs when delivered as RNP (DeWitt *et al*, 2016; Vakulskas *et al*, 2018). Recently, other highly specific SpCas9 variants, such as EvoCas9 (Casini *et al*, 2018), SniperCas9 (Lee *et al*, 2018) and HiFi-Cas9 (Vakulskas *et al*, 2018) were discovered by directed evolution approaches. The latter showed improved fidelity and high on-target editing over wild-type SpCas9 in human HSCs.

Finally, reducing time of Cas9 expression also improves nuclease specificity. The transient delivery of Cas9-gRNA preassembled ribonucleoprotein (RNP) complex showed lowered off-target activity compared to other delivery methods, likely due to faster Cas9 degradation (Dever *et al*, 2016).

2.2.2 Specificity of programmable nucleases

Programmable nucleases specificity is defined as the ratio between on-target and off-target activity, i.e. the DNA DSB frequency at the intended target site and at unintended genomic loci. Careful assessment of nuclease specificity is mandatory, particularly when aiming to clinical translation. In order to detect CRISPR/Cas9 off-targets, a panel of genome-wide assays *in silico* prediction algorithms as well as *in vitro* (e.g. CIRCLE-seq, DIGENOME-seq) and *in cellulo* assays (e.g. GUIDE-seq) have been developed (Kim *et al*, 2019).

CIRCLE-seq technique is based on the circularization of fragmented genomic DNA extracted from human cells. Circular DNA molecules are *in vitro* digested with Cas9. Hence, only circles containing an RNP cleavage site are linearized and will be ligated to adapters for paired-end high-throughput sequencing. Then, DNA regions upstream and downstream the DSB are sequenced (Tsai *et al*, 2017). DIGENOME-seq technique is based on specific pattern recognition of aligned sequencing reads. This technology requires Cas9 cleavage of linear genomic DNA fragments and exploits whole-genome sequencing. Potential off-target sites are identified

as genomic regions where reads constantly start from the same 5'-end. Despite the high sensitivity of this method, it requires extensive operator interaction and complex analyses (Kim *et al*, 2015).

Although aforementioned methods are useful to an initial screening of potential unintended cleavage sites, pre-identified putative off-target sequences must be validated in *in vivo* context. IDLV-trapping at the DNA DSB sites during repair can be mappable by Linear Amplification Mediated Polymerase Chain Reaction (LAM-PCR) and has been used as genome-wide approach to validate nuclease platforms (**Figure 8a**) (Osborn *et al*, 2013; Wang *et al*, 2015; Gabriel *et al*, 2011). Genome-wide unbiased identification of DSBs enabled by next generation sequencing (GUIDE-Seq) is a similar approach based on the trapping of double-stranded oligodeoxynucleotides (dsODNs) during repair of DNA DSBs and mapping with an unbiased amplification method and next generation sequencing (NGS) (**Figure 8b**) (Tsai *et al*, 2015). Unbiased genome-wide method without using any DNA bait has been developed by analyzing chromosomal translocation at DNA DSBs (**Figure 8c**) (Frock *et al*, 2015). More recently, discovery of *in situ* Cas off-targets and verification by sequencing (DISCOVER-seq) which is a chromatin immunoprecipitation (ChIP)-seq-based method has been published and allows efficient identification Cas9 off-targets by exploiting endogenous DNA repair machinery (Wienert *et al*, 2019).

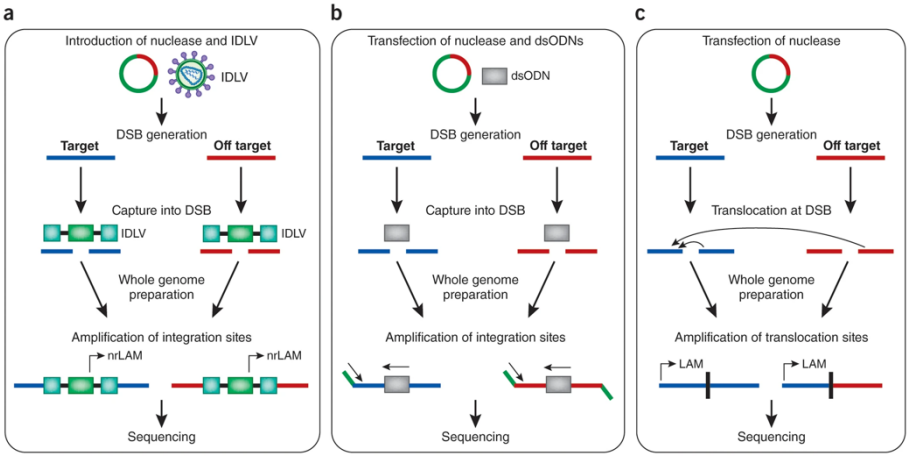

Figure 8: *unbiased genome-wide methods to assess nucleases specificity.* **a**, Wang *et al.* introduced a donor sequence together with an IDLV as a foreign DNA bait that is captured at nuclease cleavage sites. **b**, Tsai *et al.* used dsODNs as foreign DNA baits. **c**, Frock *et al.* exploited LAM-PCR to identify translocations of endogenous genome sequences (Gabriel e*t al*, 2015).

All these methods show considerable sensitivity and specificity issues and none of them alone allows to comprehensively and precisely identify nuclease off-target sites, even because no gold standard exists. The type of delivery, the cell types used for the off-target analysis and duration of Cas9 expression may influence the results of *in vivo* specificity assays (Cameron *et*

al, 2017). Although genome editing generally offers higher level of specificity than genetic engineering platforms based on semi-randomly integrating vectors, off-target activity is expected to be a major source of genotoxicity and its burden may vary depending on the nuclease platform and the targeted DNA sequence. Nuclease off-target activity may have no biological consequences, or instead be cytotoxic, knock-out tumor suppressor genes, induce off-target incorporation of the transgene or other chromosomal rearrangements. Moreover, unintended on-target events, such as excision or insertion of arbitrary DNA fragments, have been reported upon gene editing in non-hematopoietic cell types (Nelson *et al*, 2019; Kosicki *et al*, 2018; Hanlon *et al*, 2019).

In conclusion, *in silico* off-target prediction and multiple *in vitro* or *in vivo* assays should be performed to comprehensively investigate nuclease specificity. Indeed, different methods often return only partially overlapped lists of putative off-target sites. Importantly, cleavage activity at the candidate off-target sites in the cell type of interest must be confirmed by targeted deep-sequencing analysis.

2.2.3 DNA damage repair mechanisms upon DSB

Cells are continuously exposed to DNA damages at significant rates induced by endogenous and exogenous agents that may promote mutagenesis, carcinogenesis or ageing and generate a risk for genome stability (Lindahl, 1993; Hoeijmakers, 2009). Genome stability is of primary importance for the survival and proper functioning of all organisms. DNA damages induced by endogenous agents may result from spontaneous errors during DNA replication, bases deamination and the presence of reactive metabolites, e.g. reactive oxygen species (ROS). Exogenous damaging agents include environment, chemical and physical factors, such as ionizing and UV radiation, alkylating agents and viral infections (Chatterjee & Walker, 2017).

Double-stranded breaks in DNA are important threats to genome integrity because they can result in chromosomal aberrations that can lead to cell malfunctioning and cell death. The induction of one or multiple DNA DSB(s) by programmable nucleases triggers a complex cellular response, known as DNA damage response (DDR), which orchestrates the repair of the DNA DSB and the global cellular reaction to this genomic insult (**Figure 9**) (Turnell & Grand, 2012). DNA repair mainly occur by: i) the error-prone non-homologous end joining (NHEJ) pathway, which directly ligates the free DNA ends but may insert and/or delete some nucleotides (indels) at the DSB site, possibly leading to gene disruption; ii) the homology-directed repair (HDR) high-fidelity pathway, which exploits any DNA sequence with homologies for the target site as template for the repair, useful for targeted integration or gene correction. If reparation it is not

effective or the damage is too severe in HSPC, the DDR can induce cellular apoptosis, differentiation or senescence in a p53-dependent manner (**Figure 9**) (Schiroli *et al*, 2019).

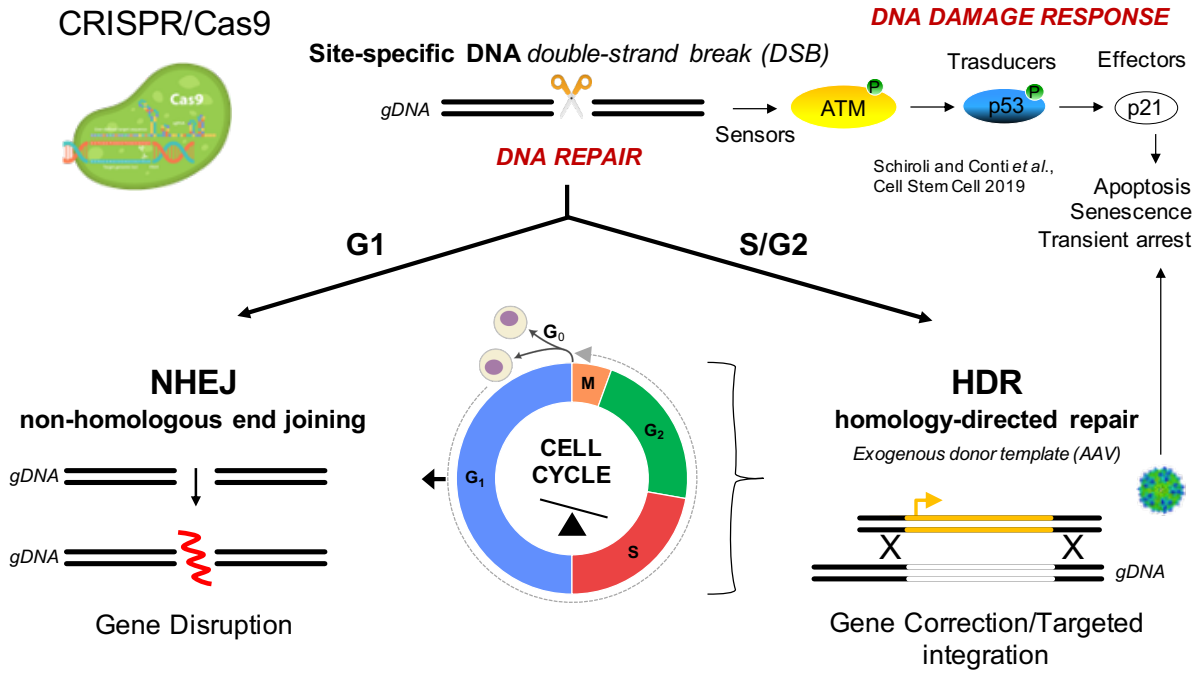

Figure 9: *Schematic of DSB repair pathways and DDR.* CRISPR/Cas9 nuclease platform is exploited to introduce a site-specific DNA break into the genome of HSC leading to DNA damage response which is driven by a robust activation of p53 pathway. Consequently, DNA repair may occur by NHEJ which can introduce indels at the repaired site or by Homology Directed Repair which can be exploited for targeted integration, but dependent on S and G2 cell cycle phases, when HDR machinery components are required for genome duplication. AAV viral vector can be used as exogenous HDR donor template because of his capability to carry a large coding cassette but concomitant exposure with Cas9, lead to cumulative p53 pathway activation and strongly impacts HSC biological functions.

The outcome of the DNA DSB repair is the result of a complex interplay between the editing tools and multiple factors, such as the cell cycle phase, the expression level of the DNA repair machinery, the chromatin context and the availability of the HDR template (Cannan & Pederson, 2016). The tumor suppressors 53BP1 and BRCA1 are two key players balancing NHEJ or HDR pathways. 53BP1 promotes the inhibition of the end-resection process and favor NHEJ during the G1 phase of the cell cycle (Bothmer *et al*, 2010), while BRCA1 favors HDR by counteracting 53BP1 activity in the S/G2 phases (Bunting *et al*, 2010). Other pathways, such as synthesis-dependent strand annealing (SDSA), single-strand template repair (SSTR), microhomology-mediated end joining (MMEJ) or single-strand annealing (SSA), can also be exploited for DNA DSB repair (**Figure 10**).

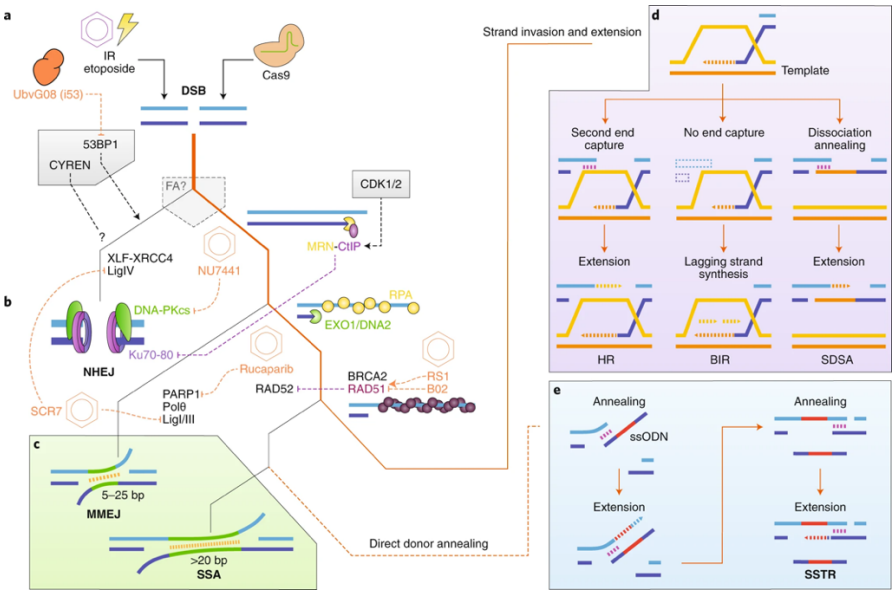

Figure 10: *Schematic of classical and alternative DSB repair pathways.* **a**, After the initial decision to enter NHEJ or other DSBR pathways is characterized by MRN-CtIP end resection, which is regulated by CDK1/2-dependent cell cycle activation. **b**, Persistent binding of the Ku complex (Ku70-80) to DSB ends and DNA-PKcs activation initiates NHEJ. Inhibition of NHEJ-factors has been attempted to increase HDR counterpart. **c**, Small homologies (5–25 bp) between resected ends enable microhomology-mediated end joining (MMEJ). Extended DSB end resection by long-distance exonucleases EXO1 or DNA2 is necessary for homology-directed repair (HDR). RAD52 is necessary for single-strand annealing (SSA), which requires homology between the resected DNA ends, like MMEJ. **d**, Templated HDR from a donor dsDNA requires RAD51 and can be used to integrate exogenous sequences in different ways (HR, BIR, SDSA). **e**, SSTR may mirror an SDSA-like process in which the donor anneals to the resected DSB end and is extended using the donor as a template (Yeh e*t al*, 2019).

Non-Homologous End Joining (NHEJ) Pathway

The NHEJ repair pathway is the most effective mechanism for DNA DSB repair with minimal processing in mammalian cells which is active independently from cell cycle activity (Burma *et al*, 2006). This is a robust, flexible, error-prone but predominant and fast pathway which stabilizes the DSB from genomic translocations (Soutoglou *et al*, 2007). The nuclear complex Ku70-Ku80 heterodimer recognizes and binds to the DNA DSB free ends, inducing recruitment of the DNA-dependent protein kinase catalytic subunit (DNA-PK), whose activity is crucial for NHEJ (Spagnolo *et al*, 2006; Davis *et al*, 2014). Ku and DNA-PKcs establish an extended filament and recruit other enzymes, such as Artemis, that cleave ssDNA-dsDNA to generate compatible blunt ends (Povirk *et al*, 2007; Chang *et al*, 2015). Furthermore, Ku recruits both the XRCC4-like factor and the X-ray repair cross-complementing protein 4 (XLF-XRCC4) which stabilizes the DNA ligase IV (LIG4) to seal the DSB (Mari *et al*, 2006). During processing, the DNA ends progressively become closer and ligation takes place, most of time

creating indels (**Figure 10b**) (Liu *et al*, 2019). In most cases, NHEJ is a precise repair mechanism, but often introduces small insertions and deletions at DSB, especially when exposed to prolong Cas9 assaults inducing multiple resections.

A number of small molecules or shRNA inhibiting NHEJ factors such as NU7441 or SCR7 have been studied to further enhance gene editing by homology-dependent DNA repair despite a marginal effect has been reported in HSPC (**Figure 10**) (Srivastava *et al*, 2012; Robert *et al*, 2015; Maruyama *et al*, 2015; Canny *et al*, 2017; Yao *et al*, 2017).

MMEJ and SSA are alternative repair pathways to the classical NHEJ sharing the initial ends resection step with homologous recombination, when respectively 5-25 bp or >200bp microhomology are present in the proximity of the DNA DSB (**Figure 10c**) (Truong *et al*, 2013).

Homology-Directed Repair (HDR) Pathway

HDR is a faithful pathway based on homologies recognition between a donor template (i.e. sister chromatid, vector genome, plasmid) and the genome locus, where occurring the DNA DSB. This is a natural molecular process responsible for chromosomal crossing-over and assuring genetic diversity. HDR machinery is required but constrains to S/G2 cell cycle phases, when genomic DNA is duplicating.

The process is initiated by MRN (MRE11, RAD51, NBS1) which binds DSB and activates ATM signaling response (Liu *et al*, 2014). MRE11 3'-5' endonuclease activity mediates "short-range" resection upon interaction with the C-terminal-binding protein interacting protein (CtIP). The end-resection process promotes the displacement of Ku70-Ku80 heterodimers and block NHEJ repair (Scully *et al*, 2019). Consequently, EXO1 and DNA2/BLM drive "long-range" resection through 5'-3' nuclease activity. The single strand is coated by the replication protein A (RPA) to unfold secondary structures and inhibit interactions with free ssDNA. Then, recombination mediators such as BRCA2 displace RPA protein and the recombinase RAD51 creates a nucleoprotein filament (San Filippo *et al*, 2008) that mediates strand invasion of the homologous DNA template (**Figure 10b**) (Chen *et al*, 2008). If a sufficient number of nucleotide pairs, the synapse is stabilized, and the non-paired strand form a displacement loop (D-loop). A DNA polymerase starts synthesis from the 3' free ends of the invading strand and the synapse is solved through crossover HR or non-crossover synthesis-dependent strand annealing (SDSA), the last being predominant. Rarely, failure of the displacement induces break-induced replication (BIR) error-prone repair mechanism, but such mutations have never been reported during genome editing (**Figure 10d**).

The single-strand template repair (SSTR) mirrors SDSA but is RAD51-independent and performs DSB repair using a single-strand donor annealing supplied in *trans*. SSTR mechanism is still debated, including whether the donor template is physically incorporated or dissociated from genome (**Figure 10e**) (Kan *et al*, 2017).

2.2.4 Delivery vehicles for ex vivo HDR editing

HDR of the nuclease-induced DNA DSB requires the presence of an exogenous DNA donor template flanked by homologous sequences to the target site. Several platforms have been used so far to deliver the HDR template in hematopoietic cells aiming to maximize HDR editing efficiency and minimize treatment toxicity. The transduction with viral vectors as integrase-defective LV (IDLV) or adeno-associated vectors serotype 6 (AAV6), as well as the electroporation of a single-stranded phosphorothioate-modified oligodeoxynucleotide (ssODN) have been largely preferred to deliver the HDR template in blood cells. Instead, adenoviral vectors and other non-viral vehicles (such as plasmids and double-stranded DNA templates) found limited applications in primary hematopoietic cells due to their poor efficiency and tolerability, albeit with some exceptions (Roth *et al*, 2018). Overall, these platforms offer a broad spectrum of cargo capacities and may be suitable for different editing strategies. Short ssODN are limited in length and may be applied for *in situ* gene correction of small disease-causing mutations (De Ravin *et al*, 2017; Romero *et al*, 2019; Pattabhi *et al*, 2019). Conversely, AAV6 and IDLV welcome larger payloads (approximately up to 4.7 and 8 kb, respectively) and may be useful for targeted integration of long therapeutic cassettes.

In human HSPCs, IDLV transduction combined with ZFNs led to 5-10% HDR editing in the bulk CD34+ population and 2-5% in the primitive CD34+CD133+CD90+ HSPC fraction, which entails cells with long-term engraftment capacity in immunodeficient mice (Genovese *et al*, 2014). Regardless of the nuclease platforms, AAV6 increased up to 5-fold the HDR editing efficiency in primitive HSPCs compared to the IDLV-based protocol (Dever *et al*, 2016; Pavel-Dinu *et al*, 2019; Schiroli *et al*, 2017; Wang *et al*, 2015; Kuo *et al*, 2018). The presence of intracellular restriction factors may challenge cell permissiveness to (ID)LV transduction (Petrillo *et al*, 2015), thus limiting availability of the HDR template into the nucleus. Accordingly, IDLV transduction in presence of cyclosporin H enhanced HDR efficiency up to 15-20% in the long-term progeny of human engrafting HSPCs by relieving interferon-induced transmembrane protein 3 (IFTM3)-mediated entry restriction (Petrillo *et al*, 2018). Nevertheless, molecular mechanisms for higher HDR efficiency with AAV6 still remain partially elusive.

Recruitment of HDR factors by AAV inverted terminal repeats (ITRs) (Hirsch, 2015) and engagement of alternative pathways exploiting single-stranded templates for DNA DSB repair may contribute to the enhancement of HDR editing (Hendrie & Russell, 2005). Finally, ssODN allows similar gene correction efficiencies compared to AAV6 in primitive HSPCs long term after xenotransplantation (Chen *et al*, 2015; De Ravin *et al*, 2017; Romero *et al*, 2019). Of note, ssODN likely engages DNA DSB repair mechanisms distinct from IDLV and possibly AAV6, preferring the Rad51-independent single-stranded template repair (SSTR) pathway rather than the conventional Rad51-dependent HDR (Yeh *et al*, 2019).

2.2.5 Strategies to improve HDR efficiency in HSC

The absolute and relative number of cells that need to be edited depends on the disease and on the therapeutic strategy. For instance, as SCID-X1 is lethal for developing lymphocytes, the strong *in vivo* selective advantage of few functional T cell progenitors over affected ones may compensate for relatively low editing efficiencies, and less than 10-15% of functional HSPCs are predicted to be sufficient to rescue the phenotype (Schiroli *et al*, 2017). Conversely, the minimal threshold of edited cell proportion must be substantially higher to benefit patients affected by other blood disorders, such as haemoglobinopathies (Abraham *et al*, 2017; Marktel *et al*, 2019). Despite initial proof-of-principle of gene editing in human HSPCs (Lombardo *et al*, 2007), many hurdles constrained efficient editing and in particular HDR-mediated targeted insertion or gene correction. This is particularly true in long-term repopulating HSPCs, which show: i) lower uptake of viral vectors; ii) lower permissiveness to HDR, likely due to their quiescent state and the low expression of the DNA repair machinery; iii) higher toxicity and sensitivity to prolonged and invasive *ex vivo* manipulation (Genovese *et al*, 2014).

Thus, depending on the setting, editing efficiency may constitute a challenge, and each step in the manipulation process, may introduce a bottleneck. Sourcing and culturing of the cells, delivery of the nucleases and the corrective template, limited nuclease activity and cell cycle constraints in engaging DNA repair pathways are significant barriers in hematopoietic cells. While NHEJ is active regardless of the cell cycle phase (Chang *et al*, 2017), HDR is exclusively engaged in the S/G_2, thus hampering HDR-mediated gene editing in slowly cycling and quiescent cells (Heyer *et al*, 2010). Therefore, several studies in the last years were focused on the optimization of the editing protocol and the development of novel tools and strategies to maximize editing efficiency by overcoming each of these technological and cellular barriers.

Optimizing HSC *ex vivo* procedure and genome editing reagents

Long-term repopulating HSCs display lower expression level of the DNA repair machinery and lower permissiveness to HDR than committed progenitors and activated T cells (Hustedt & Durocher, 2017; Beerman *et al*, 2014; Biechonski *et al*, 2018; Schiroli *et al*, 2019). Fine-tuning of culture conditions and editing timing has been pursued to promote HSPC cell cycle progression and activation to achieve sustained HDR editing, while preserving long-term persistence of edited cells. In fact, *ex vivo* culture of HSPCs for 48 or 72 hours before editing pushes repopulating cells to exit from quiescence and transit through S/G_2 phases, thus increasing HDR efficiency (Genovese *et al*, 2014; Zonari *et al*, 2017). However, prolonged culture times lead to cells differentiation and multipotency loss. Supplementation of the culture medium with stemness preserving compounds, such as Stem Regenin-1 (Boitano *et al*, 2010), UM171 (Fares *et al*, 2014) and $dmPGE_2$ (Hoggatt *et al*, 2009), helps to maintain the long-term multilineage repopulation capacity of human edited HSPCs transplanted in immunodeficient mouse models, partially overcoming the drawbacks of prolonged culture (Genovese *et al*, 2014).

Despite these substantial steps forward, HDR editing efficiency in HSPCs is still well below the current standards of gene addition therapies. Several strategies were proposed to enhance HDR efficiency in mammalian cells by transiently manipulating the DNA repair pathways or the cell cycle state (Liu *et al*, 2019). NHEJ inhibition by small molecules or proteins, tethering of HDR-promoting factors to Cas9 nuclease or S/G2 cell synchronization favored HDR engagement upon nuclease-induced DNA DSB in cell lines and pluripotent cells (Maruyama *et al*, 2015; Chu *et al*, 2015; Jayavaradhan *et al*, 2019; Charpentier *et al*, 2018; Gutschner *et al*, 2016). However, the efficacy of these approaches in long-term repopulating HSCs has been limited or so far unproven (Kuo *et al*, 2018). Recently, promoting cell cycle progression, either by maintaining low cell concentration during *ex vivo* manipulation (Charlesworth *et al*, 2018) or by providing cell-cycle modulators (Shin *et al*, 2020), has been reported as the most efficient strategy to enhance HDR editing in human HSPCs.

Hampering cellular toxicity and DNA damage response in HSC

As cells have continuously been subjected to multi-origin assaults by DNA damaging agents and pathogens, they evolved a panel of repair mechanisms to counteract genomic alterations and shield cellular integrity. Gene editing, which couples the delivery of exogenous molecules and the induction of the DNA DSB, may trigger complex cellular responses potentially leading to harmful outcomes (**Figure 11**). However, the consequences of gene editing on cell fitness,

as well as NHEJ/HDR proficiency, may vary among different cell types and strongly depend on cell biology and function. In addition, human primary cells, and particularly HSPCs, are generally more sensitive than cell lines to extensive manipulation (Urnov *et al*, 2005; Genovese *et al*, 2014), thus requiring substantial tailoring of the gene editing procedure to improve its tolerability.

As a first line of host defense, human immune cells (including HSPCs and T cells) exhibit pattern recognition receptors (PRRs), which sense pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and exogenous nucleic acids, and promote the release of type I interferons (IFNs) and other cytokines (Piras & Kajaste-Rudnitski, 2020; Rahman *et al*, 2009). Activation of PRRs in HSPCs and overexpression of IFN-stimulated genes (ISGs) have been reported to induce a variety of outcomes, such as exit from quiescence, differentiation and apoptosis (Passegué & Ernst, 2009; Essers *et al*, 2009; Liu *et al*, 2012). Nuclease-encoding mRNAs and *in vitro* transcribed 5'-triphosphate gRNAs may efficiently activate ISGs via PRRs, thus decreasing cell viability and the HSPC clonogenic potential (Mu *et al*, 2019). Dampening of these responses has been obtained by switching to high-pressure liquid chromatography (HPLC)-purified mRNAs incorporating base analogs or chemically synthetized gRNAs (Schiroli *et al*, 2017, 2019). Furthermore, electroporation of CRISPR/Cas9 machinery as ribonucleoprotein, rather than mRNA, is reported to be stealthier in human HSCs (Cromer *et al*, 2018). Of note, interferon induction may also affect concomitant viral transduction, thus constraining HDR template delivery (Petrillo *et al*, 2018).

Once delivered to the cells, the HDR template *per se* might be also sensed by innate cellular mechanisms. Hematopoietic cells poorly tolerate the transfer of naked dsDNA, which leads to substantial cell death and decreased proliferative potential (Hendel *et al*, 2015). Nevertheless, also secondary structures or nucleic acid hybrids present in AAV or in IDLV genomes may be recognized by the host and activate transient cellular responses (Piras & Kajaste-Rudnitski, 2020). Indeed, AAV and IDLV are not completely invisible to human HSPCs and robustly activate a p53-dependent DDR, regardless of the transgene (Piras *et al*, 2017). However, the molecular cascade leading to p53 activation has not been fully elucidated yet.

Recent works investigated the impact of gene editing on the fitness of human cells and highlighted p53-dependent nuclease toxicity in induced pluripotent stem cells (iPSCs) (Ihry *et al*, 2018) and cell lines (Haapaniemi *et al*, 2018; Enache *et al*, 2020), leading to apoptosis or cell cycle arrest. HSPCs are extremely sensitive to genotoxic insults and promptly activate DDR in response to DNA DSBs, leading to replicative arrest, apoptosis, differentiation or senescence (Milyavsky *et al*, 2010). Accordingly, nucleases inducing multiple DSBs transiently activate a

robust p53-dependent DDR, up to the establishment of pro-inflammatory programs, with a remarkable impact on HSPC clonogenic capacity (Schiroli *et al*, 2019). Conversely, one or few DNA DSB induce a low burden p53-dependent DDR, with a limited impact on HSPCs biology.

Another set of evidence indicate that HDR-mediated genome editing based on AAV6 or IDLV template cumulatively triggers p53 response and is weakly tolerated by HSPCs. Interestingly, transient p53 inhibition during editing procedure enhanced its tolerability and restored engraftment properties (Schiroli *et al*, 2019). Notably, the use of ssODN instead of viral vectors as HDR template does not cumulatively elicit p53 activation and is better tolerated by HSPCs, with no impact on their repopulation capacity (Pattabhi *et al*, 2019; Romero *et al*, 2019).

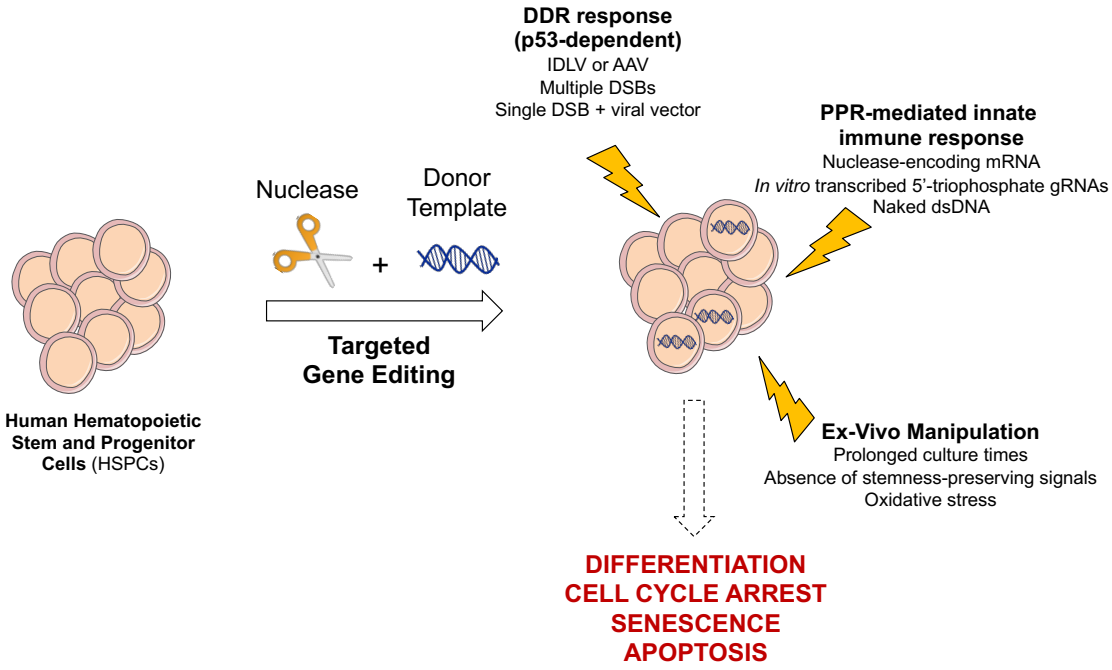

Figure 11: *Schematic of cellular responses triggered by targeted genome editing in HSC.* Ex vivo edited cells are subjected to different stimuli affecting the general cellular fitness and their repopulation potential in vivo, which are related directly to ex vivo culturing, or indirectly to editing reagents inducing DDR and PPR responses.

Overall, these findings support the contention that human HSPCs tolerate well NHEJ-mediated therapeutic genome editing, as also suggested by preliminary data from CRISPR/Cas clinical studies (Xu *et al*, 2019). On the contrary, future clinical testing is needed to provide indications about the feasibility of HDR editing in HSPCs.

3. HSC therapies for three paradigmatic blood disorders

The outstanding advantages and the current technological limitations of targeted genome editing are the main weights in the two sides of the scale when considering the opportunity of translating this intriguing therapeutic approach into clinics. However, their "weight" might remarkably change depending on the target disease. Therefore, the decision to move gene editing towards human testing necessarily requires a case-by-case assessment. In this scenario, the presence of competing treatments, either as standard of care or under clinical evaluation, and the costs of developing and commercializing advanced gene therapy medicinal products (Wilson & Carroll, 2019) might further restrict the space for the application of gene editing in blood disorders.

3.1 Human Immunodeficiency Virus (HIV)

The major cellular targets of HIV are human CD4+ T cells, which are infected by engaging the CD4 molecule as receptor and CCR5 and/or CXCR4 as coreceptors. Of note other cell types, such as macrophages (Ganor *et al*, 2019) and dendritic cells (Izquierdo-Useros *et al*, 2009; Manches *et al*, 2014) are also infected by the virus. If untreated, HIV leads to loss of CD4+ lymphocytes, acquired immunodeficiency, opportunistic infections, increased incidence of cancer and death.

The life expectancy of people living with HIV has been revolutionized thanks to antiretroviral therapy (ART) (Saag *et al*, 2018), trailing that of the general population by approximately 7 years, albeit with fewer comorbidity-free years (Marcus *et al*, 2020). ART must be continued lifelong, as viremia relapses upon treatment interruption. Latency has been mainly attributed to viral integration into the genome and persistence of HIV in long-lived CD4+ memory cells (Sengupta & Siliciano, 2018), but other cell types have been shown to contribute as well (Ganor *et al*, 2019).

Curing HIV requires clearing the virus and its reservoirs. Long-term remission has been reported in very few cases. The Berlin patient developed acute myeloid leukemia and subsequently received two allogenic HSCTs from a compatible donor, incidentally carrying the homozygous *CCR5*-Δ32 mutation, which impairs cell surface expression (Hutter *et al*, 2009), thus entering long-term HIV remission without alteration of normal hematopoiesis (Allers *et al*, 2011; Yukl *et al*, 2013). Similarly, the London patient underwent HSCT from a homozygous *CCR5*-Δ32 donor for Hodgkin's lymphoma and achieved ART-free remission (Gupta *et al*, 2020). Eradication is thought to have been achieved by the combination of reservoir clearance through conditioning, GvHD and the transplantation of homozygous *CCR5*-Δ32 cells in the

context of CCR5-tropic HIV infection (Sengupta & Siliciano, 2018; Gupta *et al*, 2019). Accordingly, CXCR4 tropic HIV may escape clearance, as observed in another patient that underwent allogenic HSCT from a homozygous *CCR5*-Δ32 donor (Kordelas *et al*, 2014). As individuals with biallelic mutations of *CCR5* are resistant to CCR5-tropic HIV, which predominates in the early course of the disease, and an HSCT bearing the same mutation can be curative, trials are investigating the potential of using *CCR5*-Δ32 donor cells for HSCT in HIV patients (NCT02140944).

However, as *CCR5*-Δ32 donors are scarce, gene disruption of *CCR5* is an attractive therapeutic strategy. Initially, *ex vivo* ZFN-mediated knockout of *CCR5* has been attempted in T cells of HIV patients, with efficiency below 30%. While no safety issues arose in the trial, little clinical benefit could be demonstrated (Tebas *et al*, 2014). Of note, since *CCR5* KO would not protect from HIV with CXCR4-tropism, some studies proposed CXCR4 gene knock-out alone or in combination with CCR5 KO in CD4+ T cells (Wilen *et al*, 2011; Didigu *et al*, 2014), the alternative being excluding patients with CXCR4-tropism from editing trials. Others are further extending the concept by weaponizing *CCR5*-KO T cells with a CD4-CAR (NCT03617198). Instead, *CCR5*-KO in HSPCs aims to exploit the propagation of the mutation in daughter cells, both myeloid and lymphoid, thus further restricting the pool of cells susceptible to HIV (Carter & Ehrlich, 2008; Manches *et al*, 2014). A trial with autologous ZFN *CCR5* KO HSPCs infused after busulfan conditioning is underway (NCT02500849).

Insights from Berlin and London patients however suggest that clearance of viral reservoirs requires a combination of intensive chemotherapy regimens, HSCT from HIV-negative donor and GvHD. These factors are incorporated in the premises of the NCT03164135 trial whereby *CCR5* KO is attempted on third party donors for allogenic HSCT, following development of hematological malignancies. However, only 5% of daughter T cells carried the *CCR5* disruption (Xu *et al*, 2019) and HIV was not eradicated.

Of note, while no safety concerns attributable to *CCR5* KO have emerged so far, *CCR2* shares a high degree of sequence similarity and may be a prominent off-target, with unknown consequences on hematopoietic cells (Mock *et al*, 2015). Beyond the disruption of CCR5 and CXCR4, the discovery of broadly neutralizing antibodies (bNAbs) has prompted the possibility of developing a completely different approach, which is still in its infancy. These Abs are rather peculiar in that they neutralize a wide range of HIV strains by targeting conserved epitopes. A "shock-and-kill" approach based on agonist Vesatolimod and bNAbs has indeed delivered with promising results in non-human primates (Borducchi *et al*, 2018). However, the generation of bNAbs by vaccination has so far proven challenging, as their emergence depends on a set of

improbable mutations (Saunders *et al*, 2019). For this reason, HDR-mediated editing of IgH locus in B cells, leading to silencing of native immunoglobulin genes and expression of HIV-bNAbs from the natural Ig loci, has been proposed as an alternative to bypass the convoluted evolution process of bNAbs development (Hartweger *et al*, 2019). It is yet too early to speculate whether bNAbs might constitute part of a potential cure or rather just complement to ART.

In summary, T-cell based therapies for HIV have been encouraging under the safety profile, but did not provide clinical benefit, likely due to suboptimal editing efficiency and their ineffectiveness in clearing the viral reservoir, which appears to be the main barrier to clear the virus. Arguably, candidates to allogenic HSCT due to other underlying diseases may be the ideal population to achieve the proof-of-concept of HIV clearance by gene editing. Novel approaches, such as HIV-CARs or bNAbs, hold the promise of overcoming the hurdles of chemotherapy, and suboptimal editing efficiency, but are presently in an embryonic development phase and their potential is still to be demonstrated.

3.2 Sickle Cell Disease (SCD)

SCD is the most common hemoglobinopathy. The homozygous Glu6Val missense mutation in the β-globin gene (*HBB*) results in the production of HbS, which has a strong tendency to polymerize when deoxygenated. This leads to sickling of red blood cells, causing hemolytic anemia and painful vaso-occlusive crises. SCD is a multisystemic disease whereby a cascade of events, i.e. inflammation, oxidative stress, hypercoagulability, platelet activation, results in and a wide range of chronic complications, culminating in reduced life expectancy and poor quality of life (Piel *et al*, 2017; Lubeck *et al*, 2019).

Early diagnosis, and prevention of complications, coupled with hydroxyurea or erythroexchange, are crucial to improve the clinical outcomes (Piel *et al*, 2017). HSCT from a matched sibling donor is a therapeutic option for SCD patients with high success rate (Arnold *et al*, 2016; Hsieh *et al*, 2014; Piel *et al*, 2017). However, this option may be offered to a minority of patients (fewer than 18%) because of the availability of suitable donors (Piel *et al*, 2017). Moreover, despite encouraging results in clinical trials and progressive improvement of transplant procedures, acute and chronic GvHD remain the main complications.

Thus, for the last two decades, significant work has been done to develop a safe and effective gene therapy strategy for SCD (Hoban *et al*, 2016). The most prominent strategies have revolved around using a LV carrying a functional (Chad *et al*, 2000; May *et al*, 2002) or a custom anti-sickling β-globin cassette (Pawliuk *et al*, 2001; Levasseur *et al*, 2003, 2004; Romero *et al*, 2013). Alternatively, the γ-globin gene has been used as LV cargo (Pestina *et al*, 2009;

Perumbeti *et al*, 2009; Moreau-Gaudry *et al*, 2001), as increased levels of HbF mitigate the SCD phenotype (Kraus *et al*, 1961; Akinsheye *et al*, 2011). Importantly, β-globin expression should be restricted to the erythroid compartment in order to avoid toxicities. Thus, the inclusion of the DNase-I hypersensitive sites (HS) within the locus control region (LCR) proved to be critical for sustained and erythroid specific expression of β-globin (Hoban *et al*, 2016). However, the inclusion of these long regulatory regions in the viral genome, strongly affected vector titer and transduction efficiency (Uchida *et al*, 2019; Hanawa *et al*, 2009). Accordingly, a LV carrying a custom β-globin gene harboring the anti-sickling β^{T87Q} mutation under the minimal *HBB* promoter and the HS elements, was first successfully tested in a single SCD patient (Ribeil *et al*, 2017), but this success could not be replicated in subsequent SCD patients possibly also due to low transduction efficiency (Kanter *et al*, 2016). Other GT clinical trials based on the anti-sickling approach are still in the recruitment phase (Lidonnici & Ferrari, 2018). Recent improvements in LV design and gene transfer protocols include reduced proviral length to improve titre and transduction efficiency (Morgan *et al*, 2019), novel insulator elements (Romero *et al*, 2015) and the use of transduction enhancers (Masiuk *et al*, 2019).

Beyond hurdles in reaching sustained β-globin expression, LV-based approaches share the common aforementioned drawbacks of semi-random integration and generation of anomalous transcripts, which might trigger oncogenesis. Instead, gene editing appears a very promising technology for the treatment of SCD, limiting the risk of genotoxic events to unwanted on- and off-target activity. Both functional correction with physiological expression of normal β-hemoglobin and reversion to endogenous HbF expression have been pursued. *HBB*-specific ZFNs combined with IDLV- or ssODN-based delivery of the corrective HDR template in patient-derived HSPCs resulted in high levels of gene editing and Hb tetramers production (Hoban *et al*, 2015). However, xenotransplantation of edited HSPCs in immunodeficient mice did not result in robust *HBB*-correction in the long-term graft. Similarly, another independent group showed correction levels up to 50% in patient-derived HSPCs by combining CRISPR/Cas9 and AAV6 *in vitro* (Dever *et al*, 2016). Yet only 5% of bulk *HBB*-edited cells engrafted at long term in murine xenograft models.

Restoration of the endogenous corrected Hb offers superior theoretical benefits than HbF reactivation, since the latter is expected to only partially alleviate the disease phenotype, at the expense of theoretically lower oxygen delivery capacity (Maurer *et al*, 1970). However, since HDR editing efficiency is limited in HSPCs, NHEJ approaches for SCD are closer to clinical development. In particular, NHEJ-mediated repair has been exploited to knock-out *BCL11A*, a transcriptional factor involved in HbF repression and fetal to adult Hb switching, thus resulting

in reactivation of HbF expression (Bauer *et al*, 2013; Wu *et al*, 2019). Promisingly, *BCL11A* locus disruption in HSPCs by both ZFN and CRISPR/Cas9 technologies reached phase I/II clinical trials (NCT03653247; NCT03745287; NCT04443907). As is the case for other applications, base editing approaches have been proposed to overcome the main limits of HSPCs gene editing. For instance, efficient and durable disruption of *BCL11A* erythroid enhancer by base-editing its core sequences has been reported (Zeng *et al*, 2020). However, improved efficiency and tolerability as well as careful and comprehensive off-target analysis are required before clinical implementation.

Tailoring gene therapy, and particularly gene editing, for SCD is an ambitious objective, as the disease is common, but not immediately life-threatening, and it is thus expected to meet very stringent safety requirements. Undoubtedly, a relative minority of patients with extensive transfusion alloimmunization or at high risk of complications, is in dire need of a novel therapeutic option. The need is even higher in resource-limited settings, where blood donation programs may not be optimal. Nevertheless, limited resources hamper the access to expensive therapies such as HSCT and gene therapy. In comparison to allogenic HSCT, gene therapy and gene editing approaches share the advantages of autologous transplantation, circumventing the need for matched healthy donors and avoiding the immunological complications. Still, autologous HSPCs harvest may be difficult due to the high risk of G-CSF-related complications (Fitzhugh *et al*, 2009; Salinas Cisneros & Thein, 2020). Over gene addition, gene editing has the theoretical rationale advantage of correcting the HbS mutation, in lieu of keeping it along with a novel β-globin cassette. As less than full graft editing is expected to suffice, both *HBB* correction and *BCL11A* disruption approaches are promising and may eventually succeed. Still, competition with novel more conventional drugs, such as Voxelotor (Vichinsky *et al*, 2019) and Crizanlizumab (Ataga *et al*, 2017), is expected to be fierce and may reshape the everyday care of sickle-cell patients, raising the bar for more invasive gene editing and gene therapy approaches.

3.3 X-linked Severe Combined Immunodeficiencies (SCID-X1)

SCID-X1 is a rare, life-threatening primary immunodeficiency caused by mutations of the *IL2RG* gene, which encodes for the common γ-chain (*γc*), a cytokine receptor subunit essential for development, survival, and function of T lymphocytes. Allogeneic HSCT is the standard treatment for SCID-X1 patients, who otherwise succumb to severe opportunistic infections that arise due to the paucity of lymphocytes (Noguchi *et al*, 1993; Cavazzana *et al*, 2016). HSCT

from a matched sibling donor restores T-cell immunity in more than 90% of the SCID-X1 patients with survival rates up to 97% in absence of conditioning. However, the use of a conditioning regimen is still under debate: while the lack of a functional host immune system does not require it to prevent rejection and graft derived lymphocytes have a strong selective advantage over the patient' cells, conditioning has been associated with a better T- and B- cell immune reconstitution (Buckley, 2011; Mazzolari *et al*, 2007). Whenever matched related donors are lacking, haploidentical or matched unrelated HSCT may be a valid alternative option, despite the lower survival rate (Haddad *et al*, 2018).

HSCT remains associated with significant toxicities, and no alternative curative treatments are available for patients not eligible for allogeneic transplant. Hence, autologous transplantation of *ex vivo* genetically modified HSPCs would be a gentler option with no risk of GvHD. Previous landmark studies of gene addition in HSPCs with semi-randomly integrating vector showed long-lasting correction and functional and polyclonal T cell reconstitution, thus demonstrating the potential of gene therapy for this disease. However, transactivation of oncogene due to insertional mutagenesis resulted in overt T cell leukemias (Cavazzana-Calvo *et al*, 2000; Hacein-Bey-Abina *et al*, 2003, 2008; Howe *et al*, 2008). Of note, the use of SIN vectors resulted in the persistence of functional T, B and NK cells without leukemic events, thus improving the safety profile of SCID-X1 gene addition therapies (Hacein-Bey-Abina *et al*, 2014; De Ravin *et al*, 2016). However, prolonged follow-ups are required to comprehensively assess the long-term safety of these approaches. For instance, unregulated expression of the *IL2RG* transgene has been proposed as an independent contributor to leukemogenesis in SCID-X1 patients (Woods *et al*, 2006; Ginn *et al*, 2010).

In this context, HDR-mediated therapeutic genome editing may be a valuable option for the treatment of SCID-X1, as it would in principle avoid insertional mutagenesis and/or unregulated *γc* expression and consequent leukemogenesis. Recently, independent groups have developed one-size-fits-all gene correction strategies to integrate the corrective partial cDNA in the *IL2RG* gene (Schiroli *et al*, 2017; Pavel-Dinu *et al*, 2019). Mice transplanted with *IL2RG*-edited HSPCs showed multilineage reconstitution of the hematopoietic system, development of functional edited T cells and editing efficiencies up to 10-20% in long-term human xenografts. Given the aforementioned selective advantage of the functional lymphoid progeny, the current HSPC gene editing efficiencies match the threshold required for clinical benefit, as predicted by mouse studies and gene therapy clinical trials (De Ravin *et al*, 2016; Schiroli *et al*, 2017).

In summary, gene therapy may position itself ahead of HSCT, as long as it is presumed to be safer. Milder conditioning, faster engraftment and the absence of GvHD must be balanced

by the hurdles of cell collection, which may be cumbersome in very young patients, and the delays associated with the manufacturing process, which instead are not a prerogative of haploidentical donors, who are immediately available. Nevertheless, gene therapy does not preclude the opportunity of performing a subsequent allogenic mismatched HSCT and may thus be the preferred first choice when both are available. Indeed, gene therapy can provide sustained clinical benefit for SCID-X1 patients and appears to be an equal, if not superior, alternative to haploidentical HSCT so far (Touzot *et al*, 2015). In this context, the competitive advantage of gene editing over gene addition resides in the presumed safer genomic and *γc* expression profile.

SCOPE OF THE THESIS

HSC targeted genome editing is an attractive therapeutic option for the treatment of several hematological diseases by *in situ* correction of the disease-causing mutation and restoration of physiological gene function. Engineered nucleases such as CRISPR/Cas enable efficient disruption of a targeted locus by delivering site-specific DSBs, but most gene correction strategies require homology-directed repair (HDR) of the DSB through an exogenous template. However, HDR efficiency remains constrained in long-term repopulating HSCs. Whereas a limited proportion of edited HSPCs may result in safe and effective treatment of diseases characterized by selective advantage of the functional progeny, this might be insufficient in other conditions, where a large proportion of co-infused non-edited cells would compete with the edited ones. Furthermore, the biological consequences of the DNA DSB and its repair processes on the preservation of crucial biological properties of human HSC remain to be fully ascertained, given the challenges of stringently assessing long-term hematopoietic output, multi-lineage potential and self-renewal capacity, as well as on clonal composition in xenotransplantation models. Indeed, reconstitution of the hematopoietic system by a limited number of HSPC clones might expose the patient to infections by delaying immune reconstitution, increase the risk of graft failure or even drive myelodysplastic/leukemogenic events.

My PhD project was conducted within the context of a broader study undertaken in our laboratory, where we addressed a major barrier to successful targeted gene editing of human HSC. In this study, we investigated the biological bases underlying constrained gene editing in human long-term repopulating HSC and we showed that: i) a robust p53-mediated response to the combination of DNA DSBs and viral template delivery induces an unanticipated substantial loss of engrafting HSC clones; ii) low expression of HDR machinery limits HDR proficiency together with delayed cell cycle progression in the most primitive HSCs. We then overcome these barriers by transient expression of proteins counteracting the p53 response and forcing cell cycle progression and upregulation of HDR machinery. These changes allowed establishing a novel enhanced gene editing protocol that reaches high and stable proportions of HDR edited HSC in long-term human xenografts (up to 50%). Importantly, we validated these improvements by performing *in vivo* clonal tracking of edited HSCs and showed polyclonal hematopoietic reconstitution with fully preserved multilineage and long-term self-renewal over serial transplantation in mouse model. Therefore, high yield and proportion of HDR-edited HSPCs with preserved multi-lineage long-term repopulation potential allow to broaden applicability of HSPC gene editing and strengthen the rationale for first-in-man clinical trials.

REFERENCES

Abarrategi A, Foster K, Hamilton A, Mian SA, Passaro D, Gribben J, Mufti G & Bonnet D (2017) Versatile humanized niche model enables study of normal and malignant human hematopoiesis. *J Clin Invest*

Abbott A (1992) Italians first to use stem cells. *Nature* doi:10.1038/356465a0

Abraham A, Hsieh M, Eapen M, Fitzhugh C, Carreras J, Keesler D, Guilcher G, Kamani N, Walters MC, Boelens JJ, *et al* (2017) Relationship between Mixed Donor–Recipient Chimerism and Disease Recurrence after Hematopoietic Cell Transplantation for Sickle Cell Disease. *Biol Blood Marrow Transplant*

Adikusuma F, Piltz S, Corbett MA, Turvey M, McColl SR, Helbig KJ, Beard MR, Hughes J, Pomerantz RT & Thomas PQ (2018) Large deletions induced by Cas9 cleavage. *Nature*

Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, Morecki S, Andolfi G, Tabucchi A, Carlucci F, *et al* (2002) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science (80-)*

Akashi K, Traver D, Miyamoto T & Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*

Akinsheye I, Alsultan A, Solovieff N, Ngo D, Baldwin CT, Sebastiani P, Chui DHK & Steinberg MH (2011) Fetal hemoglobin in sickle cell anemia. *Blood* doi:10.1182/blood-2011-03-325258

Aleem E, Kiyokawa H & Kaldis P (2005) Cdc2-cyclin E complexes regulate the G1/S phase transition. *Nat Cell Biol*

Allers K, Hütter G, Hofmann J, Loddenkemper C, Rieger K, Thiel E & Schneider T (2011) Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. *Blood*

Ara T, Tokoyoda K, Sugiyama T, Egawa T, Kawabata K & Nagasawa T (2003) Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. *Immunity*

Arnold SD, Bhatia M, Horan J & Krishnamurti L (2016) Haematopoietic stem cell transplantation for sickle cell disease – current practice and new approaches. *Br J Haematol* doi:10.1111/bjh.14167

Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, Guthrie TH, Knight-Madden J, Alvarez OA, Gordeuk VR, *et al* (2017) Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med*

Bai T, Li J, Sinclair A, Imren S, Merriam F, Sun F, O'Kelly MB, Nourigat C, Jain P, Delrow JJ, *et al* (2019) Expansion of primitive human hematopoietic stem cells by culture in a zwitterionic hydrogel. *Nat Med*

Bates D, Mächler M, Bolker BM & Walker SC (2015) Fitting linear mixed-effects models using lme4. *J Stat Softw*

Bauer DE, Kamran SC, Lessard S, Xu J, Fujiwara Y, Lin C, Shao Z, Canver MC, Smith EC, Pinello L, *et al* (2013) An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. *Science (80-)*

Baum CM, Weissman IL, Tsukamoto AS, Buckle AM & Peault B (1992) Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A* 89: 2804–2808

Beerman I, Seita J, Inlay MA, Weissman IL & Rossi DJ (2014) Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. *Cell Stem Cell*

Beerman I, Seita J, Inlay MA, Weissman IL & Rossi DJ (2014) Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. *Cell Stem Cell*

Benveniste P, Frelin C, Janmohamed S, Barbara M, Herrington R, Hyam D & Iscove NN (2010) Intermediate-Term Hematopoietic Stem Cells with Extended but Time-Limited Reconstitution Potential. *Cell Stem Cell* 6: 48–58

Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD & Bernstein ID (1988) Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J Clin Invest* 81: 951–955

van den Berg J, Manjón AG, Kielbassa K, Feringa FM, Freire R & Medema RH (2018) A limited number of double-strand DNA breaks is sufficient to delay cell cycle progression. *Nucleic Acids Res*

Bernardo ME & Aiuti A (2016) The Role of Conditioning in Hematopoietic Stem-Cell Gene Therapy. *Hum Gene Ther*

Bernitz JM, Kim HS, MacArthur B, Sieburg H & Moore K (2016) Hematopoietic Stem Cells Count and Remember Self-Renewal Divisions. *Cell* 167: 1296-1309.e10

Bhatia M, Wang JCY, Kapp U, Bonnet D & Dick JE (1997) Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A* 94: 5320–5325

Bibikova M, Carroll D, Segal DJ, Trautman JK, Smith J, Kim Y-G & Chandrasegaran S (2001) Stimulation of Homologous Recombination through Targeted Cleavage by Chimeric Nucleases. *Mol Cell Biol*

Biechonski S, Olender L, Zipin-Roitman A, Yassin M, Aqaqe N, Marcu-Malina V, Rall-Scharpf M, Trottier M, Meyn MS, Wiesmüller L, *et al* (2018) Attenuated DNA damage responses and increased apoptosis characterize human hematopoietic stem cells exposed to irradiation. *Sci Rep* 8

Bitinaite J, Wah DA, Aggarwal AK & Schildkraut I (1998) Foki dimerization is required for dna cleavage. *Proc Natl Acad Sci U S A* 95: 10570–10575

Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, *et al* (1995) T lymphocyte-directed gene therapy for ADA-SCID: Initial trial results after 4 years. *Science (80-)*

Boitano AE, Wang J, Romeo R, Bouchez LC, Parker AE, Sutton SE, Walker JR, Flaveny CA, Perdew GH, Denison MS, *et al* (2010) Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science (80-)*

Borducchi EN, Liu J, Nkolola JP, Cadena AM, Yu WH, Fischinger S, Broge T, Abbink P, Mercado NB, Chandrashekar A, *et al* (2018) Antibody and TLR7 agonist delay viral rebound in SHIV-infected monkeys. *Nature*

Bothmer A, Robbiani DF, Feldhahn N, Gazumyan A, Nussenzweig A & Nussenzweig MC (2010) 53BP1 regulates DNA resection and the choice between classical and alternative end joining during class switch recombination. *J Exp Med* 207: 855–865

Boulais PE & Frenette PS (2015) Making sense of hematopoietic stem cell niches. *Blood*

Braun CJ, Boztug K, Paruzynski A, Witzel M, Schwarzer A, Rothe M, Modlich U, Beier R, Göhring G, Steinemann D, *et al* (2014) Gene therapy for Wiskott-Aldrich syndrome-long-term efficacy and genotoxicity. *Sci Transl Med*

Buckley RH (2011) Transplantation of hematopoietic stem cells in human severe combined immunodeficiency: Longterm outcomes. *Immunol Res* doi:10.1007/s12026-010-8191-9

Bunting SF, Callén E, Wong N, Chen HT, Polato F, Gunn A, Bothmer A, Feldhahn N, Fernandez-Capetillo O, Cao L, *et al* (2010) 53BP1 inhibits homologous recombination in brca1-deficient cells by blocking resection of DNA breaks. *Cell* 141: 243–254

Burma S, Chen BPC & Chen DJ (2006) Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. *DNA Repair (Amst)* 5: 1042–1048 doi:10.1016/j.dnarep.2006.05.026

Cameron P, Fuller CK, Donohoue PD, Jones BN, Thompson MS, Carter MM, Gradia S, Vidal

B, Garner E, Slorach EM, *et al* (2017) Mapping the genomic landscape of CRISPR-Cas9 cleavage. *Nat Methods* 14: 600–606

Cannan WJ & Pederson DS (2016) Mechanisms and Consequences of Double-Strand DNA Break Formation in Chromatin. *J Cell Physiol* doi:10.1002/jcp.25048

Canny MD, Moatti N, Wan LCK, Fradet-Turcotte A, Krasner D, Mateos-Gomez PA, Zimmermann M, Orthwein A, Juang Y-C, Zhang W, *et al* (2017) Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR–Cas9 genome-editing efficiency. *Nat Biotechnol*

Carroll D (2014) Genome Engineering with Targetable Nucleases. *Annu Rev Biochem*

Carter CA & Ehrlich LS (2008) Cell biology of HIV-1 infection of macrophages. *Annu Rev Microbiol* doi:10.1146/annurev.micro.62.081307.162758

Casini A, Olivieri M, Petris G, Montagna C, Reginato G, Maule G, Lorenzin F, Prandi D, Romanel A, Demichelis F, *et al* (2018) A highly specific SpCas9 variant is identified by in vivo screening in yeast. *Nat Biotechnol*

Cathomen T & Keith Joung J (2008) Zinc-finger nucleases: The next generation emerges. *Mol Ther* 16: 1200–1207 doi:10.1038/mt.2008.114

Cavazza A, Moiani A & Mavilio F (2013) Mechanisms of retroviral integration and mutagenesis. *Hum Gene Ther* doi:10.1089/hum.2012.203

Cavazzana-Calvo M, Hacein-Bey S, De Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, *et al* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science (80-)*

Cavazzana-Calvo M, Thrasher A & Mavillo F (2004) The future of gene therapy. *Nature* doi:10.1038/427779a

Cavazzana M, Six E, Lagresle-Peyrou C, André-Schmutz I & Hacein-Bey-Abina S (2016) Gene Therapy for X-Linked Severe Combined Immunodeficiency: Where Do We Stand? *Hum Gene Ther*

Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia N V., Bogdanove AJ & Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res* 39

Chad M, Rivella S, Callegari J, Heller G, Gaensler KML, Luzzatto L & Sadelain M (2000) Therapeutic haemoglobin synthesis in β-thalassaemic mice expressing lentivirus-encoded human β-globin. *Nature*

Chang HHY, Pannunzio NR, Adachi N & Lieber MR (2017) Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nat Rev Mol Cell Biol* doi:10.1038/nrm.2017.48

Chang HHY, Watanabe G & Lieber MR (2015) Unifying the DNA end-processing roles of the artemis nuclease: Ku-dependentartemis resection atbluntdnaends. *J Biol Chem*

Charlesworth CT, Camarena J, Cromer MK, Vaidyanathan S, Bak RO, Carte JM, Potter J, Dever DP & Porteus MH (2018) Priming Human Repopulating Hematopoietic Stem and Progenitor Cells for Cas9/sgRNA Gene Targeting. *Mol Ther - Nucleic Acids*

Charpentier M, Khedher AHY, Menoret S, Brion A, Lamribet K, Dardillac E, Boix C, Perrouault L, Tesson L, Geny S, *et al* (2018) CtIP fusion to Cas9 enhances transgene integration by homology-dependent repair. *Nat Commun*

Chatterjee N & Walker GC (2017) Mechanisms of DNA damage, repair, and mutagenesis. *Environ Mol Mutagen* 58: 235–263 doi:10.1002/em.22087

Chen F, Pruett-Miller SM & Davis GD (2015) Gene editing using ssODNs with engineered endonucleases. *Methods Mol Biol*

Chen Z, Yang H & Pavletich NP (2008) Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. *Nature* 453: 489–494

Cheng H, Zheng Z & Cheng T (2020) New paradigms on hematopoietic stem cell

differentiation. *Protein Cell* 11: 34–44 doi:10.1007/s13238-019-0633-0
Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S & Kim JS (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res*
Chu VT, Weber T, Wefers B, Wurst W, Sander S, Rajewsky K & Kühn R (2015) Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat Biotechnol* 33: 543–548
Cicalese MP, Ferrua F, Castagnaro L, Pajno R, Barzaghi F, Giannelli S, Dionisio F, Brigida I, Bonopane M, Casiraghi M, *et al* (2016) Update on the safety and efficacy of retroviral gene therapy for immunodeficiency due to adenosine deaminase deficiency. *Blood*
Cieri N, Camisa B, Cocchiarella F, Forcato M, Oliveira G, Provasi E, Bondanza A, Bordignon C, Peccatori J, Ciceri F, *et al* (2013) IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. *Blood*
Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF & Shaper JH (1984) Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol* 133: 157–65
Clement K, Rees H, Canver MC, Gehrke JM, Farouni R, Hsu JY, Cole MA, Liu DR, Joung JK, Bauer DE, *et al* (2019) CRISPResso2 provides accurate and rapid genome editing sequence analysis. *Nat Biotechnol* doi:10.1038/s41587-019-0032-3
Cole LJ, Habermeyer JG & Bond VP (1955) Recovery from acute radiation injury in mice following administration of rat bone marrow. *J Natl Cancer Inst*
Conti A & Di Micco R (2018) p53 activation: A checkpoint for precision genome editing? *Genome Med* doi:10.1186/s13073-018-0578-6
Copelan EA (2006) Hematopoietic stem-cell transplantation. *N Engl J Med* 354: 1813–1826 doi:10.1056/NEJMra052638
Copley MR, Beer PA & Eaves CJ (2012) Hematopoietic stem cell heterogeneity takes center stage. *Cell Stem Cell* 10: 690–697 doi:10.1016/j.stem.2012.05.006
Del Core Luca, Montini Eugenio, Di Serio Clelia CA (2018) Dealing with Data Evolution and Data Integration: An approach using Rarefaction. In *49th Scientific meeting of the Italian Statistical Society*
Cromer MK, Vaidyanathan S, Ryan DE, Curry B, Lucas AB, Camarena J, Kaushik M, Hay SR, Martin RM, Steinfeld I, *et al* (2018) Global Transcriptional Response to CRISPR/Cas9-AAV6-Based Genome Editing in CD34+ Hematopoietic Stem and Progenitor Cells. *Mol Ther*
Cullot G, Boutin J, Toutain J, Prat F, Pennamen P, Rooryck C, Teichmann M, Rousseau E, Lamrissi-Garcia I, Guyonnet-Duperat V, *et al* (2019) CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. *Nat Commun*
Dausset J, Colombani J & Evelin J (1958) Présence de l'antigène Rh (D) dans les leucocytes et les plaquettes humaines. *Vox Sang*
Davis AJ, Chen BPC & Chen DJ (2014) DNA-PK: A dynamic enzyme in a versatile DSB repair pathway. *DNA Repair (Amst)*
Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J & Charpentier E (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471: 602–607
Dever DP, Bak RO, Reinisch A, Camarena J, Washington G, Nicolas CE, Pavel-Dinu M, Saxena N, Wilkens AB, Mantri S, *et al* (2016) CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. *Nature* 539: 384–389
DeWitt MA, Magis W, Bray NL, Wang T, Berman JR, Urbinati F, Heo SJ, Mitros T, Muñoz DP, Boffelli D, *et al* (2016) Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med*
Didigu CA, Wilen CB, Wang J, Duong J, Secreto AJ, Danet-Desnoyers GA, Riley JL, Gregory PD, June CH, Holmes MC, *et al* (2014) Simultaneous zinc-finger nuclease editing of the HIV

coreceptors ccr5 and cxcr4 protects CD4+ T cells from HIV-1 infection. *Blood*
Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K & Sadelain M (2018) Gene therapy comes of age. *Science (80-)* doi:10.1126/science.aan4672
Enache OM, Rendo V, Abdusamad M, Lam D, Davison D, Pal S, Currimjee N, Hess J, Pantel S, Nag A, *et al* (2020) Cas9 activates the p53 pathway and selects for p53-inactivating mutations. *Nat Genet*
Essers MAG, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA & Trumpp A (2009) IFNα activates dormant haematopoietic stem cells in vivo. *Nature*
Fares I, Chagraoui J, Gareau Y, Gingras S, Ruel R, Mayotte N, Csaszar E, Knapp DJHFHF, Miller P, Ngom M, *et al* (2014) Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science (80-)* 345: 1509–1512
Fares I, Chagraoui J, Lehnertz B, MacRae T, Mayotte N, Tomellini E, Aubert L, Roux PP & Sauvageau G (2017) EPCR expression marks UM171-expanded CD34 + cord blood stem cells. *Blood*: blood-2016-11-750729
Ferrari S, Jacob A, Beretta S, Unali G, Albano L, Vavassori V, Cittaro D, Lazarevic D, Brombin C, Cugnata F, *et al* (2020) Efficient gene editing of human long-term hematopoietic stem cells validated by clonal tracking. *Nat Biotechnol*
Ferrua F, Cicalese MP, Galimberti S, Giannelli S, Dionisio F, Barzaghi F, Migliavacca M, Bernardo ME, Calbi V, Assanelli AA, *et al* (2019) Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study. *Lancet Haematol*
Fitzhugh CD, Hsieh MM, Bolan CD, Saenz C & Tisdale JF (2009) Granulocyte colony-stimulating factor (G-CSF) administration in individuals with sickle cell disease: Time for a moratorium? *Cytotherapy*
Frese KK, Lee SS, Thomas DL, Latorre IJ, Weiss RS, Glaunsinger BA & Javier RT (2003) Selective PDZ protein-dependent stimulation of phosphatidylinositol 3-kinase by the adenovirus E4-ORF1 oncoprotein. *Oncogene*
Friedmann T & Roblin R (1972) Gene therapy for human genetic disease? *Science (80-)*
Frock RL, Hu J, Meyers RM, Ho YJ, Kii E & Alt FW (2015) Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol*
Fu Y, Sander JD, Reyon D, Cascio VM & Joung JK (2014) Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol*
Gabriel R, Lombardo A, Arens A, Miller JC, Genovese P, Kaeppel C, Nowrouzi A, Bartholomae CC, Wang J, Friedman G, *et al* (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat Biotechnol*
Gaj T, Gersbach CA & Barbas CF (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 31: 397–405 doi:10.1016/j.tibtech.2013.04.004
Ganor Y, Real F, Sennepin A, Dutertre CA, Prevedel L, Xu L, Tudor D, Charmeteau B, Couedel-Courteille A, Marion S, *et al* (2019) HIV-1 reservoirs in urethral macrophages of patients under suppressive antiretroviral therapy. *Nat Microbiol*
Gatti RA, Meuwissen HJ, Allen HD, Hong R & Good RA (1968) Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet*
Genovese P, Schiroli G, Escobar G, Di Tomaso T, Firrito C, Calabria A, Moi D, Mazzieri R, Bonini C, Holmes MC, *et al* (2014) Targeted genome editing in human repopulating haematopoietic stem cells. *Nature* 510: 235–240
Gersbach CA (2014) Genome engineering: The next genomic revolution. *Nat Methods* 11: 1009–1011
Ginn SL, Liao SH, Dane AP, Hu M, Hyman J, Finnie JW, Zheng M, Cavazzana-Calvo M, Alexander SI, Thrasher AJ, *et al* (2010) Lymphomagenesis in SCID-X1 mice following lentivirus-mediated phenotype correction independent of insertional mutagenesis and γc

overexpression. *Mol Ther*

Greiner DL, Shultz LD, Yates J, Appel MC, Perdrizet G, Hesselton RM, Schweitzer I, Beamer WG, Shultz KL, Pelsue SC, *et al* (1995) Improved engraftment of human spleen cells in NOD/LtSz-scid/scid mice as compared with C.B-17-scid/scid mice. *Am J Pathol* 146: 888–902

Le Guiner C, Servais L, Montus M, Larcher T, Fraysse B, Moullec S, Allais M, François V, Dutilleul M, Malerba A, *et al* (2017) Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. *Nat Commun*

Gupta RK, Abdul-Jawad S, McCoy LE, Mok HP, Peppa D, Salgado M, Martinez-Picado J, Nijhuis M, Wensing AMJ, Lee H, *et al* (2019) HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature*

Gupta RK, Peppa D, Hill AL, Gálvez C, Salgado M, Pace M, McCoy LE, Griffith SA, Thornhill J, Alrubayyi A, *et al* (2020) Evidence for HIV-1 cure after CCR5Δ32/Δ32 allogeneic haemopoietic stem-cell transplantation 30 months post analytical treatment interruption: a case report. *Lancet HIV*

Gutschner T, Haemmerle M, Genovese G, Draetta GF & Chin L (2016) Post-translational Regulation of Cas9 during G1 Enhances Homology-Directed Repair. *Cell Rep*

Gwiazda KS, Grier AE, Sahni J, Burleigh SM, Martin U, Yang JG, Popp NA, Krutein MC, Khan IF, Jacoby K, *et al* (2016) High Efficiency CRISPR/Cas9-mediated Gene Editing in Primary Human T-cells Using Mutant Adenoviral E4orf6/E1b55k 'Helper' Proteins. *Mol Ther* 24: 1–11

Haapaniemi E, Botla S, Persson J, Schmierer B & Taipale J (2018) CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. *Nat Med*

Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier E, Caccavelli L, Delabesse E, Beldjord K, *et al* (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, *et al* (2003) LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. *Science (80-)*

Hacein-Bey-Abina S, Pai SY, Gaspar HB, Armant M, Berry CC, Blanche S, Bleesing J, Blondeau J, De Boer H, Buckland KF, *et al* (2014) A modified γ-retrovirus vector for X-linked severe combined immunodeficiency. *N Engl J Med*

Haddad E, Logan BR, Griffith LM, Buckley RH, Parrott RE, Prockop SE, Small TN, Chaisson J, Dvorak CC, Murnane M, *et al* (2018) SCID genotype and 6-month posttransplant CD4 count predict survival and immune recovery. *Blood*

Hanawa H, Yamamoto M, Zhao H, Shimada T & Persons DA (2009) Optimized lentiviral vector design improves titer and transgene expression of vectors containing the chicken β-globin locus HS4 insulator element. *Mol Ther*

Hanlon KS, Kleinstiver BP, Garcia SP, Zaborowski MP, Volak A, Spirig SE, Muller A, Sousa AA, Tsai SQ, Bengtsson NE, *et al* (2019) High levels of AAV vector integration into CRISPR-induced DNA breaks. *Nat Commun*

Hao QL, Thiemann FT, Petersen D, Smogorzewska EM & Crooks GM (1996) Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood* 88: 3306–3313

Harrington LB, Paez-Espino D, Staahl BT, Chen JS, Ma E, Kyrpides NC & Doudna JA (2017) A thermostable Cas9 with increased lifetime in human plasma. *Nat Commun*

Hartweger H, McGuire AT, Horning M, Taylor JJ, Dosenovic P, Yost D, Gazumyan A, Seaman MS, Stamatatos L, Jankovic M, *et al* (2019) HIV-specific humoral immune responses by CRISPR/Cas9-edited B cells. *J Exp Med*

Hendel A, Bak RO, Clark JT, Kennedy AB, Ryan DE, Roy S, Steinfeld I, Lunstad BD, Kaiser

RJ, Wilkens AB, *et al* (2015) Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat Biotechnol*

Hendrie PC & Russell DW (2005) Gene targeting with viral vectors. *Mol Ther* doi:10.1016/j.ymthe.2005.04.006

Heyer WD, Ehmsen KT & Liu J (2010) Regulation of homologous recombination in eukaryotes. *Annu Rev Genet* doi:10.1146/annurev-genet-051710-150955

Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP & Butler KS (2020) Gene editing and CRISPR in the clinic: Current and future perspectives. *Biosci Rep* doi:10.1042/BSR20200127

Hirsch ML (2015) Adeno-associated virus inverted terminal repeats stimulate gene editing. *Gene Ther*

Ho AD (2005) Kinetics and symmetry of divisions of hematopoietic stem cells. *Exp Hematol*

Hoban MD, Cost GJ, Mendel MC, Romero Z, Kaufman ML, Joglekar A V., Ho M, Lumaquin D, Gray D, Lill GR, *et al* (2015) Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. *Blood*

Hoban MD, Cost GJ, Mendel MC, Romero Z, Kaufman ML, Joglekar A V., Ho M, Lumaquin D, Gray D, Lill GR, *et al* (2015) Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. *Blood*

Hoeijmakers JHJ (2009) DNA damage, aging, and cancer

Hoggatt J, Singh P, Sampath J & Pelus LM (2009) Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood* 113: 5444–5455

Hoggatt J, Singh P, Sampath J & Pelus LM (2009) Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood*

Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempski H, Brugman MH, Pike-Overzet K, Chatters SJ, De Ridder D, *et al* (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest*

Hsieh MM, Fitzhugh CD, Weitzel RP, Link ME, Coles WA, Zhao X, Rodgers GP, Powell JD & Tisdale JF (2014) Nonmyeloablative HLA-matched sibling allogeneic hematopoietic stem cell transplantation for severe sickle cell phenotype. *JAMA - J Am Med Assoc*

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, *et al* (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol*

Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z, *et al* (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature*

Huang MM & Hearing P (1989) The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev* 3: 1699–1710

Hustedt N & Durocher D (2017) The control of DNA repair by the cell cycle. *Nat Cell Biol* doi:10.1038/ncb3452

Hutter G, Nowak D, Mossner M, Ganepola S, Müß A, Allers K, Schneider T, Hofmann J, Külcherer C, Blau O, *et al* (2009) Long-term control of HIV by CCR5 delta32/delta32 stem-cell transplantation. *N Engl J Med*

Ihry RJ, Worringer KA, Salick MR, Frias E, Ho D, Theriault K, Kommineni S, Chen J, Sondey M, Ye C, *et al* (2018) P53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat Med*

Isalan M (2012) Zinc-finger nucleases: How to play two good hands. *Nat Methods* doi:10.1038/nmeth.1805

Ishikawa F, Yasukawa M, Lyons B, Yoshida S, Miyamoto T, Yoshimoto G, Watanabe T, Akashi K, Shultz LD & Harada M (2005) Development of functional human blood and

immune systems in NOD/SCID/IL2 receptor γ chainnull mice. *Blood*
Izquierdo-Useros N, Naranjo-Gómez M, Archer J, Hatch SC, Erkizia I, Blanco J, Borràs FE, Puertas MC, Connor JH, Fernádez-Figueras MT, *et al* (2009) Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway. *Blood*
Jacobson LO, Simmons EL, Marks EK & Eldredge JH (1951) Recovery from radiation injury. *Science (80)*
Javier RT & Rice AP (2011) Emerging Theme: Cellular PDZ Proteins as Common Targets of Pathogenic Viruses. *J Virol*
Jayavaradhan R, Pillis DM, Goodman M, Zhang F, Zhang Y, Andreassen PR & Malik P (2019) CRISPR-Cas9 fusion to dominant-negative 53BP1 enhances HDR and inhibits NHEJ specifically at Cas9 target sites. *Nat Commun*
Jenq RR & Van Den Brink MRM (2010) Allogeneic haematopoietic stem cell transplantation: Individualized stem cell and immune therapy of cancer. *Nat Rev Cancer* 10: 213–221 doi:10.1038/nrc2804
Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA & Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science (80)*
Joung JK & Sander JD (2013) TALENs: A widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol* 14: 49–55 doi:10.1038/nrm3486
Kamel-Reid S & Dick JE (1988) Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science (80-)* 242: 1706–1709
Kan Y, Ruis B, Takasugi T & Hendrickson EA (2017) Mechanisms of precise genome editing using oligonucleotide donors. *Genome Res*
Kanter J, Walters MC, Hsieh MM, Krishnamurti L, Kwiatkowski J, Kamble RT, von Kalle C, Kuypers FA, Cavazzana M, Leboulch P, *et al* (2016) Interim Results from a Phase 1/2 Clinical Study of Lentiglobin Gene Therapy for Severe Sickle Cell Disease. *Blood*
Karikó K, Muramatsu H, Ludwig J & Weissman D (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res*
Kim D, Bae S, Park J, Kim E, Kim S, Yu HR, Hwang J, Kim J Il & Kim JS (2015) Digenome-seq: Genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods* 12: 237–243
Kim D, Luk K, Wolfe SA & Kim JS (2019) Evaluating and enhancing target specificity of gene-editing nucleases and deaminases. *Annu Rev Biochem*
Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z & Joung JK (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*
Kobayashi H, Morikawa T, Okinaga A, Hamano F, Hashidate-Yoshida T, Watanuki S, Hishikawa D, Shindou H, Arai F, Kabe Y, *et al* (2019) Environmental Optimization Enables Maintenance of Quiescent Hematopoietic Stem Cells Ex Vivo. *Cell Rep*
Kohn DB, Booth C, Kang EM, Pai SY, Shaw KL, Santilli G, Armant M, Buckland KF, Choi U, De Ravin SS, *et al* (2020) Lentiviral gene therapy for X-linked chronic granulomatous disease. *Nat Med* doi:10.1038/s41591-019-0735-5
Komori H, Enomoto M, Nakamura M, Iwanaga R & Ohtani K (2005) Distinct E2F-mediated transcriptional program regulates p14ARF gene expression. *EMBO J*
Kondo M, Weissman IL & Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*
Kordelas L, Verheyen J & Esser S (2014) Shift of HIV tropism in stem-cell transplantation with CCR5 delta32 mutation. *N Engl J Med* doi:10.1056/NEJMc1405805
Korkmaz S & Altuntas F (2017) What is the role of biosimilar G-CSF agents in hematopoietic

stem cell mobilization at present? *Transfus Apher Sci* 56: 795–799
Kosicki M, Tomberg K & Bradley A (2018) Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol*
Kraus AP, Koch B & Burckett L (1961) Two families showing interaction of haemoglobin c or thalassaemia with high foetal haemoglobin in adults. *Br Med J*
Kumar S & Geiger H (2017) HSC Niche Biology and HSC Expansion Ex Vivo. *Trends Mol Med* doi:10.1016/j.molmed.2017.07.003
Kunisaki Y, Bruns I, Scheiermann C, Ahmed J, Pinho S, Zhang D, Mizoguchi T, Wei Q, Lucas D, Ito K, *et al* (2013) Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* 502: 637–643
Kuo CY, Long JD, Campo-Fernandez B, de Oliveira S, Cooper AR, Romero Z, Hoban MD, Joglekar A V., Lill GR, Kaufman ML, *et al* (2018) Site-Specific Gene Editing of Human Hematopoietic Stem Cells for X-Linked Hyper-IgM Syndrome. *Cell Rep*
Kuo CYCY, Long JDJD, Campo-Fernandez B, de Oliveira S, Cooper AR, Romero Z, Hoban MDMD, Joglekar AVA V., Lill GRGR, Kaufman MLML, *et al* (2018) Site-Specific Gene Editing of Human Hematopoietic Stem Cells for X-Linked Hyper-IgM Syndrome. *Cell Rep* 23: 2606–2616
Lapidot T, Pflumio F, Doedens M, Murdoch B, Williams DE & Dick JE (1992) Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID Mice. *Science (80-)* 255: 1137–1141
Lassmann T (2015) TagDust2: A generic method to extract reads from sequencing data. *BMC Bioinformatics*
Laurenti E, Frelin C, Xie S, Ferrari R, Dunant CF, Zandi S, Neumann A, Plumb I, Doulatov S, Chen J, *et al* (2015) CDK6 levels regulate quiescence exit in human hematopoietic stem cells. *Cell Stem Cell*
Laurenti E & Göttgens B (2018) From haematopoietic stem cells to complex differentiation landscapes. *Nature* 553: 418–426 doi:10.1038/nature25022
Lee CM, Cradick TJ & Bao G (2016) The neisseria meningitidis CRISPR-Cas9 system enables specific genome editing in mammalian cells. *Mol Ther*
Lee JK, Jeong E, Lee J, Jung M, Shin E, Kim Y hoon, Lee K, Jung I, Kim D, Kim S, *et al* (2018) Directed evolution of CRISPR-Cas9 to increase its specificity. *Nat Commun*
Levasseur DN, Ryan TM, Pawlik KM & Townes TM (2003) Correction of a mouse model of sickle cell disease: Lentiviral/ antisickling β-globin gene transduction of unmobilized, purified hematopoietic stem cells. *Blood*
Levasseur DN, Ryan TM, Reilly MP, McCune SL, Asakura T & Townes TM (2004) A recombinant human hemoglobin with anti-sickling properties greater than fetal hemoglobin. *J Biol Chem*
Lidonnici MR & Ferrari G (2018) Gene therapy and gene editing strategies for hemoglobinopathies. *Blood Cells, Mol Dis* doi:10.1016/j.bcmd.2017.12.001
Lim Y, Bak SY, Sung K, Jeong E, Lee SH, Kim JS, Bae S & Kim SK (2016) Structural roles of guide RNAs in the nuclease activity of Cas9 endonuclease. *Nat Commun*
Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature* 362: 709–715 doi:10.1038/362709a0
Liu C, Srihari S, Cao KAL, Chenevix-Trench G, Simpson PT, Ragan MA & Khanna KK (2014) A fine-scale dissection of the DNA double-strand break repair machinery and its implications for breast cancer therapy. *Nucleic Acids Res* 42: 6106–6127 doi:10.1093/nar/gku284
Liu J, Guo YM, Hirokawa M, Iwamoto K, Ubukawa K, Michishita Y, Fujishima N, Tagawa H, Takahashi N, Xiao W, *et al* (2012) A synthetic double-stranded RNA, poly I: C, induces a rapid apoptosis of human CD34+ cells. *Exp Hematol*
Liu M, Rehman S, Tang X, Gu K, Fan Q, Chen D & Ma W (2019) Methodologies for improving

HDR efficiency. *Front Genet* doi:10.3389/fgene.2018.00691
Lombardo A, Cesana D, Genovese P, Di Stefano B, Provasi E, Colombo DF, Neri M, Magnani Z, Cantore A, Lo Riso P, *et al* (2011) Site-specific integration and tailoring of cassette design for sustainable gene transfer. *Nat Methods* 8: 861–869
Lombardo A, Genovese P, Beausejour CM, Colleoni S, Lee YL, Kim KA, Ando D, Urnov FD, Galli C, Gregory PD, *et al* (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol*
Lubeck D, Agodoa I, Bhakta N, Danese M, Pappu K, Howard R, Gleeson M, Halperin M & Lanzkron S (2019) Estimated Life Expectancy and Income of Patients With Sickle Cell Disease Compared With Those Without Sickle Cell Disease. *JAMA Netw open*
Maier DA, Brennan AL, Jiang S, Binder-Scholl GK, Lee G, Plesa G, Zheng Z, Cotte J, Carpenito C, Wood T, *et al* (2013) Efficient clinical scale gene modification via zinc finger nuclease-targeted disruption of the HIV co-receptor CCR5. *Hum Gene Ther* 24: 245–258
Manches O, Frleta D & Bhardwaj N (2014) Dendritic cells in progression and pathology of HIV infection. *Trends Immunol* doi:10.1016/j.it.2013.10.003
Mani M, Smith J, Kandavelou K, Berg JM & Chandrasegaran S (2005) Binding of two zinc finger nuclease monomers to two specific sites is required for effective double-strand DNA cleavage. *Biochem Biophys Res Commun* 334: 1191–1197
Marcus JL, Leyden WA, Alexeeff SE, Anderson AN, Hechter RC, Hu H, Lam JO, Towner WJ, Yuan Q, Horberg MA, *et al* (2020) Comparison of Overall and Comorbidity-Free Life Expectancy Between Insured Adults With and Without HIV Infection, 2000-2016. *JAMA Netw open*
Mari PO, Florea BI, Persengiev SP, Verkaik NS, Brüggenwirth HT, Modesti M, Giglia-Mari G, Bezstarosti K, Demmers JAA, Luider TM, *et al* (2006) Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. *Proc Natl Acad Sci U S A*
Marktel S, Scaramuzza S, Cicalese MP, Giglio F, Galimberti S, Lidonnici MR, Calbi V, Assanelli A, Bernardo ME, Rossi C, *et al* (2019) Intrabone hematopoietic stem cell gene therapy for adult and pediatric patients affected by transfusion-dependent ß-thalassemia. *Nat Med*
Martins CP, Brown-Swigart L & Evan GI (2006) Modeling the Therapeutic Efficacy of p53 Restoration in Tumors. *Cell*
Maruyama T, Dougan SK, Truttmann M, Bilate AM, Ingram JR & Ploegh HL (2015) Inhibition of non-homologous end joining increases the efficiency of CRISPR/Cas9-mediated precise [TM: inserted] genome editing. *Nature*
Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR & Ploegh HL (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol*
Masiuk KE, Zhang R, Osborne K, Hollis RP, Campo-Fernandez B & Kohn DB (2019) PGE2 and Poloxamer Synperonic F108 Enhance Transduction of Human HSPCs with a β-Globin Lentiviral Vector. *Mol Ther - Methods Clin Dev*
Matthys P, Hatse S, Vermeire K, Wuyts A, Bridger G, Henson GW, De Clercq E, Billiau A & Schols D (2001) AMD3100, a Potent and Specific Antagonist of the Stromal Cell-Derived Factor-1 Chemokine Receptor CXCR4, Inhibits Autoimmune Joint Inflammation in IFN- Receptor-Deficient Mice. *J Immunol*
Maurer HS, Behrman RE & Honig GR (1970) Dependence of the oxygen affinity of blood on the presence of foetal or adult haemoglobin. *Nature*
Maximow AA (1909) The lymphocyte as a stem cell, common to different blood elements in embryonic development and during the post-fetal life of mammals. *Folia Haematol*
May C, Rivella S, Chadburn A & Sadelain M (2002) Successful treatment of murine β-thalassemia intermedia by transfer of the human β-globin gene. *Blood*

Mazzolari E, Forino C, Guerci S, Imberti L, Lanfranchi A, Porta F & Notarangelo LD (2007) Long-term immune reconstitution and clinical outcome after stem cell transplantation for severe T-cell immunodeficiency. *J Allergy Clin Immunol*

McIntosh BE, Brown ME, Duffin BM, Maufort JP, Vereide DT, Slukvin II & Thomson JA (2015) Nonirradiated NOD,B6.SCID Il2rγ-/- kitW41/W41 (NBSGW) mice support multilineage engraftment of human hematopoietic cells. *Stem Cell Reports* 4: 171–180

Menendez D, Nguyen T-AA, Snipe J & Resnick MA (2017) The cytidine deaminase APOBEC3 family is subject to transcriptional regulation by p53. *Mol Cancer Res*

Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, Schurra C, Garré M, Giovanni Nuciforo P, Bensimon A, *et al* (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature*

Milyavsky M, Gan OI, Trottier M, Komosa M, Tabach O, Notta F, Lechman E, Hermans KG, Eppert K, Konovalova Z, *et al* (2010) A Distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal. *Cell Stem Cell* 7: 186–197

Mjelle R, Hegre SA, Aas PA, Slupphaug G, Drabløs F, Sætrom P & Krokan HE (2015) Cell cycle regulation of human DNA repair and chromatin remodeling genes. *DNA Repair (Amst)*

Mock U, MacHowicz R, Hauber I, Horn S, Abramowski P, Berdien B, Hauber J & Fehse B (2015) mRNA transfection of a novel TAL effector nuclease (TALEN) facilitates efficient knockout of HIV co-receptor CCR5. *Nucleic Acids Res*

Morbitzer R, Elsaesser J, Hausner J & Lahaye T (2011) Assembly of custom TALE-type DNA binding domains by modular cloning. *Nucleic Acids Res* 39: 5790–5799

Moreau-Gaudry F, Xia P, Jiang G, Perelman NP, Bauer G, Ellis J, Surinya KH, Mavilio F, Shen CK & Malik P (2001) High-level erythroid-specific gene expression in primary human and murine hematopoietic cells with self-inactivating lentiviral vectors. *Blood*

Morgan RA, Unti MJ, Aleshe B, Brown D, Osborne KS, Koziol C, Smith OB, O'Brien R, Tam C, Miyahira E, *et al* (2019) Improved Titer and Gene Transfer by Lentiviral Vectors Using Novel, Small β-Globin Locus Control Region Elements. *Mol Ther*

Morrison SJ, Uchida N & Weissman IL (1995) The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol* doi:10.1146/annurev.cb.11.110195.000343

Moscou MJ & Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. *Science (80-)* 326: 1501

Mu W, Tang N, Cheng C, Sun W, Wei X & Wang H (2019) In vitro transcribed sgRNA causes cell death by inducing interferon release. *Protein Cell* doi:10.1007/s13238-018-0605-9

Müller M, Lee CM, Gasiunas G, Davis TH, Cradick TJ, Siksnys V, Bao G, Cathomen T & Mussolino C (2016) Streptococcus thermophilus CRISPR-Cas9 systems enable specific editing of the human genome. *Mol Ther*

Nakamura-Ishizu A, Takizawa H & Suda T (2014) The analysis, roles and regulation of quiescence in hematopoietic stem cells. *Dev* 141: 4656–4666

Naldini L (2015) Gene therapy returns to centre stage. *Nature* doi:10.1038/nature15818

Naldini L (2019) Genetic engineering of hematopoiesis: current stage of clinical translation and future perspectives. *EMBO Mol Med*

Nelson CE, Wu Y, Gemberling MP, Oliver ML, Waller MA, Bohning JD, Robinson-Hamm JN, Bulaklak K, Castellanos Rivera RM, Collier JH, *et al* (2019) Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. *Nat Med* 25: 427–432

Nishimasu H, Shi X, Ishiguro S, Gao L, Hirano S, Okazaki S, Noda T, Abudayyeh OO, Gootenberg JS, Mori H, *et al* (2018) Engineered CRISPR-Cas9 nuclease with expanded targeting space. *Science (80-)*

Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBride OW & Leonard WJ (1993) Interleukin-2 receptor γ chain mutation results in X-linked severe

combined immunodeficiency in humans. *Cell*
Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I & Dick JE (2011) Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science (80)*
Nowak J (2008) Role of HLA in hematopoietic SCT. *Bone Marrow Transplant* doi:10.1038/bmt.2008.288
Obert S, O'Connor RJ, Schmid S & Hearing P (1994) The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. *Mol Cell Biol* 14: 1333–46
van der Oost J, Jore MM, Westra ER, Lundgren M & Brouns SJJ (2009) CRISPR-based adaptive and heritable immunity in prokaryotes. *Trends Biochem Sci* 34: 401–407 doi:10.1016/j.tibs.2009.05.002
Osborn MJ, Starker CG, McElroy AN, Webber BR, Riddle MJ, Xia L, Defeo AP, Gabriel R, Schmidt M, Von Kalle C, *et al* (2013) TALEN-based gene correction for epidermolysis bullosa. *Mol Ther* 21: 1151–1159
Pabo CO & Sauer RT (1983) Protein-DNA recognition. *Annu Rev Biochem*
Palchaudhuri R, Saez B, Hoggatt J, Schajnovitz A, Sykes DB, Tate TA, Czechowicz A, Kfoury Y, Ruchika FNU, Rossi DJ, *et al* (2016) Non-genotoxic conditioning for hematopoietic stem cell transplantation using a hematopoietic-cell-specific internalizing immunotoxin. *Nat Biotechnol* 34: 738–745
Passegué E & Ernst P (2009) IFN-α wakes up sleeping hematopoietic stem cells. *Nat Med* doi:10.1038/nm0609-612
Pattabhi S, Lotti SN, Berger MP, Singh S, Lux CT, Jacoby K, Lee C, Negre O, Scharenberg AM & Rawlings DJ (2019) In Vivo Outcome of Homology-Directed Repair at the HBB Gene in HSC Using Alternative Donor Template Delivery Methods. *Mol Ther - Nucleic Acids*
Pavel-Dinu M, Wiebking V, Dejene BT, Srifa W, Mantri S, Nicolas CE, Lee C, Bao G, Kildebeck EJ, Punjya N, *et al* (2019) Gene correction for SCID-X1 in long-term hematopoietic stem cells. *Nat Commun*
Pavletich NP & Pabo CO (1991) Zinc finger-DNA recognition: Crystal structure of a Zif268-DNA complex at 2.1 Å. *Science (80-)* 252: 809–817
Pawliuk R, Westerman KA, Fabry ME, Payen E, Tighe R, Bouhassira EE, Acharya SA, Ellis J, London IM, Eaves CJ, *et al* (2001) Correction of sickle cell disease in transgenic mouse models by gene therapy. *Science (80-)*
Pelus LM & Broxmeyer HE (2018) Peripheral Blood Stem Cell Mobilization: a Look Ahead. *Curr Stem Cell Reports* doi:10.1007/s40778-018-0141-9
Perumbeti A, Higashimoto T, Urbinati F, Franco R, Meiselman HJ, Witte D & Malik P (2009) A novel human gamma-globin gene vector for genetic correction of sickle cell anemia in a humanized sickle mouse model: Critical determinants for successful correction. *Blood*
Pestina TI, Hargrove PW, Jay D, Gray JT, Boyd KM & Persons DA (2009) Correction of murine sickle cell disease using γ-globin lentiviral vectors to mediate high-level expression of fetal hemoglobin. *Mol Ther*
Petrillo C, Cesana D, Piras F, Bartolaccini S, Naldini L, Montini E & Kajaste-Rudnitski A (2015) Cyclosporin A and rapamycin relieve distinct lentiviral restriction blocks in hematopoietic stem and progenitor cells. *Mol Ther*
Petrillo C, Thorne LG, Unali G, Schiroli G, Giordano AMS, Piras F, Cuccovillo I, Petit SJ, Ahsan F, Noursadeghi M, *et al* (2018) Cyclosporine H Overcomes Innate Immune Restrictions to Improve Lentiviral Transduction and Gene Editing In Human Hematopoietic Stem Cells. *Cell Stem Cell*
Piel FB, Steinberg MH & Rees DC (2017) Sickle cell disease. *N Engl J Med* doi:10.1056/NEJMra1510865
Pike BL & Robinson WA (1970) Human bone marrow colony growth in agar-gel. *J Cell*

Physiol
Pinheiro J, Bates D, DebRoy S, Sarkar D & Team the RC (2007) The nlme Package: Linear and Nonlinear Mixed Effects Models. *R-project*
Pinho S & Frenette PS (2019) Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol* 20: 303–320 doi:10.1038/s41580-019-0103-9
Piras F & Kajaste-Rudnitski A (2020) Antiviral immunity and nucleic acid sensing in haematopoietic stem cell gene engineering. *Gene Ther* doi:10.1038/s41434-020-0175-3
Piras F, Riba M, Petrillo C, Lazarevic D, Cuccovillo I, Bartolaccini S, Stupka E, Gentner B, Cittaro D, Naldini L, *et al* (2017) Lentiviral vectors escape innate sensing but trigger p53 in human hematopoietic stem and progenitor cells. *EMBO Mol Med*
Povirk LF, Zhou T, Zhou R, Cowan MJ & Yannone SM (2007) Processing of 3′-phosphoglycolate-terminated DNA double strand breaks by artemis nuclease. *J Biol Chem*
Provasi E, Genovese P, Lombardo A, Magnani Z, Liu PQ, Reik A, Chu V, Paschon DE, Zhang L, Kuball J, *et al* (2012) Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nat Med*
Publicover A, Richardson DS, Davies A, Hill KS, Hurlock C, Hutchins D, Jenner MW, Johnson PW, Lamb J, Launders H, *et al* (2013) Use of a biosimilar granulocyte colony-stimulating factor for peripheral blood stem cell mobilization: An analysis of mobilization and engraftment. *Br J Haematol* 162: 107–111
Querido E, Blanchette P, Yan Q, Kamura T, Morrison M, Boivin D, Kaelin WG, Conaway RC, Conaway JW & Branton PE (2001) Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev* 15: 3104–3117
Radhakrishnan SK, Feliciano CS, Najmabadi F, Haegebarth A, Kandel ES, Tyner AL & Gartel AL (2004) Constitutive expression of E2F-1 leads to p21-dependent cell cycle arrest in S phase of the cell cycle. *Oncogene*
Rahman AH, Taylor DK & Turka LA (2009) The contribution of direct TLR signaling to T cell responses. *Immunol Res* doi:10.1007/s12026-009-8113-x
Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, *et al* (2015) In vivo genome editing using Staphylococcus aureus Cas9. *Nature*
Ratajczak MZ (2018) Circulating Stem Cells in Physiology and Pathology - Recent Studies Published in Stem Cell Reviews and Reports. *Stem Cell Rev Reports* doi:10.1007/s12015-018-9842-9
De Ravin SS, Li L, Wu X, Choi U, Allen C, Koontz S, Lee J, Theobald-Whiting N, Chu J, Garofalo M, *et al* (2017) CRISPR-Cas9 gene repair of hematopoietic stem cells from patients with X-linked chronic granulomatous disease. *Sci Transl Med*
De Ravin SS, Reik A, Liu P-Q, Li L, Wu X, Su L, Raley C, Theobald N, Choi U, Song AH, *et al* (2016) Targeted gene addition in human CD34(+) hematopoietic cells for correction of X-linked chronic granulomatous disease. *Nat Biotechnol* 34: 1–8
De Ravin SS, Wu X, Moir S, Anaya-O'Brien S, Kwatemaa N, Littel P, Theobald N, Choi U, Su L, Marquesen M, *et al* (2016) Lentiviral hematopoietic stem cell gene therapy for X-linked severe combined immunodeficiency. *Sci Transl Med*
Reyon D, Tsai SQ, Khgayter C, Foden JA, Sander JD & Joung JK (2012) FLASH assembly of TALENs for high-throughput genome editing. *Nat Biotechnol* 30: 460–465
Ribeil JA, Hacein-Bey-Abina S, Payen E, Magnani A, Semeraro M, Magrin E, Caccavelli L, Neven B, Bourget P, El Nemer W, *et al* (2017) Gene therapy in a patient with sickle cell disease. *N Engl J Med*
Robert F, Barbeau M, Éthier S, Dostie J & Pelletier J (2015) Pharmacological inhibition of DNA-PK stimulates Cas9-mediated genome editing. *Genome Med*
Romero Z, Campo-Fernandez B, Wherley J, Kaufman ML, Urbinati F, Cooper AR, Hoban MD,

Baldwin KM, Lumaquin D, Wang X, *et al* (2015) The human ankyrin 1 promoter insulator sustains gene expression in a β-globin lentiviral vector in hematopoietic stem cells. *Mol Ther - Methods Clin Dev*

Romero Z, Lomova A, Said S, Miggelbrink A, Kuo CY, Campo-Fernandez B, Hoban MD, Masiuk KE, Clark DN, Long J, *et al* (2019) Editing the Sickle Cell Disease Mutation in Human Hematopoietic Stem Cells: Comparison of Endonucleases and Homologous Donor Templates. *Mol Ther*

Romero Z, Urbinati F, Geiger S, Cooper AR, Wherley J, Kaufman ML, Hollis RP, De Assin RR, Senadheera S, Sahagian A, *et al* (2013) β-globin gene transfer to human bone marrow for sickle cell disease. *J Clin Invest*

Roth TL, Puig-Saus C, Yu R, Shifrut E, Carnevale J, Li PJ, Hiatt J, Saco J, Krystofinski P, Li H, *et al* (2018) Reprogramming human T cell function and specificity with non-viral genome targeting. *Nature*

Russell S, Bennett J, Wellman JA, Chung DC, Yu ZF, Tillman A, Wittes J, Pappas J, Elci O, McCague S, *et al* (2017) Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *Lancet*

Saag MS, Benson CA, Gandhi RT, Hoy JF, Landovitz RJ, Mugavero MJ, Sax PE, Smith DM, Thompson MA, Buchbinder SP, *et al* (2018) Antiretroviral drugs for treatment and prevention of HIV infection in adults: 2018 recommendations of the international antiviral society-USA panel. In *JAMA - Journal of the American Medical Association*

Sadelain M, Rivière I & Riddell S (2017) Therapeutic T cell engineering. *Nature* doi:10.1038/nature22395

Sakofsky CJ, Saini N, Klimczak LJ, Chan K, Malc EP, Mieczkowski PA, Burkholder AB, Fargo D & Gordenin DA (2019) Repair of multiple simultaneous double-strand breaks causes bursts of genome-wide clustered hypermutation. *PLoS Biol*

Salinas Cisneros G & Thein SL (2020) Recent Advances in the Treatment of Sickle Cell Disease. *Front Physiol* doi:10.3389/fphys.2020.00435

San Filippo J, Sung P & Klein H (2008) Mechanism of Eukaryotic Homologous Recombination. *Annu Rev Biochem* 77: 229–257

Saunders KO, Wiehe K, Tian M, Acharya P, Bradley T, Munir Alam S, Go EP, Scearce R, Sutherland L, Henderson R, *et al* (2019) Targeted selection of HIV-specific antibody mutations by engineering B cell maturation. *Science (80-)*

Sauvageau G, Iscove NN & Humphries RK (2004) In vitro and in vivo expansion of hematopoietic stem cells. *Oncogene* 23: 7223–7232 doi:10.1038/sj.onc.1207942

Schaley J, O'Connor RJ, Taylor LJ, Bar-Sagi D & Hearing P (2000) Induction of the Cellular E2F-1 Promoter by the Adenovirus E4-6/7 Protein. *J Virol*

Schaley JE, Polonskaia M & Hearing P (2005) The Adenovirus E4-6/7 Protein Directs Nuclear Localization of E2F-4 via an Arginine-Rich Motif. *J Virol*

Schiroli G, Conti A, Ferrari S, della Volpe L, Jacob A, Albano L, Beretta S, Calabria A, Vavassori V, Gasparini P, *et al* (2019) Precise Gene Editing Preserves Hematopoietic Stem Cell Function following Transient p53-Mediated DNA Damage Response. *Cell Stem Cell*

Schiroli G, Ferrari S, Conway A, Jacob A, Capo V, Albano L, Plati T, Castiello MC, Sanvito F, Gennery AR, *et al* (2017) Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1. *Sci Transl Med*

Scully R, Panday A, Elango R & Willis NA (2019) DNA double-strand break repair-pathway choice in somatic mammalian cells. *Nat Rev Mol Cell Biol* 20: 698–714

Seandel M, Butler JM, Kobayashi H, Hooper AT, White IA, Zhang F, Vertes EL, Kobayashi M, Zhang Y, Shmelkov S V., *et al* (2008) Generation of a functional and durable vascular niche by the adenoviral E4ORF1 gene. *Proc Natl Acad Sci* 105: 19288–19293

Seita J & Weissman IL (2010) Hematopoietic stem cell: Self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* 2: 640–653 doi:10.1002/wsbm.86

Sengupta S & Siliciano RF (2018) Targeting the Latent Reservoir for HIV-1. *Immunity* doi:10.1016/j.immuni.2018.04.030

Shin JJ, Schröder MS, Caiado F, Wyman SK, Bray NL, Bordi M, Dewitt MA, Vu JT, Kim WT, Hockemeyer D, *et al* (2020) Controlled Cycling and Quiescence Enables Efficient HDR in Engraftment-Enriched Adult Hematopoietic Stem and Progenitor Cells. *Cell Rep*

Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, *et al* (2005) Human Lymphoid and Myeloid Cell Development in NOD/LtSz-scid IL2R γ null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells . *J Immunol*

Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX & Zhang F (2016) Rationally engineered Cas9 nucleases with improved specificity. *Science (80-)*

Smith J (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucleic Acids Res* 28: 3361–3369

Sorek R, Kunin V & Hugenholtz P (2008) CRISPR - A widespread system that provides acquired resistance against phages in bacteria and archaea. *Nat Rev Microbiol* 6: 181–186

Soutoglou E, Dorn JF, Sengupta K, Jasin M, Nussenzweig A, Ried T, Danuser G & Misteli T (2007) Positional stability of single double-strand breaks in mammalian cells. *Nat Cell Biol*

Spagnolo L, Rivera-Calzada A, Pearl LH & Llorca O (2006) Three-Dimensional Structure of the Human DNA-PKcs/Ku70/Ku80 Complex Assembled on DNA and Its Implications for DNA DSB Repair. *Mol Cell*

Srivastava M, Nambiar M, Sharma S, Karki SS, Goldsmith G, Hegde M, Kumar S, Pandey M, Singh RK, Ray P, *et al* (2012) An inhibitor of nonhomologous end-joining abrogates double-strand break repair and impedes cancer progression. *Cell* 151: 1474–1487

Stanelle J, Stiewe T, Theseling CC, Peter M & Pützer BM (2002) Gene expression changes in response to E2F1 activation. *Nucleic Acids Res*

Steensma DP (2018) Myelodysplastic syndromes current treatment algorithm 2018. *Blood Cancer J*

Stein S, Ott MG, Schultze-Strasser S, Jauch A, Burwinkel B, Kinner A, Schmidt M, Krämer A, Schwäble J, Glimm H, *et al* (2010) Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med*

Täuber B & Dobner T (2001) Adenovirus early E4 genes in viral oncogenesis. *Oncogene* 20: 7847–7854 doi:10.1038/sj/onc/1204914

Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G, Spratt SK, Surosky RT, Giedlin MA, Nichol G, *et al* (2014) Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med* 370: 901–910

Thomas ED, Lochte HL, Lu WC & Ferrebee JW (1957) Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med*

Thompson AA, Walters MC, Kwiatkowski J, Rasko JEJ, Ribeil JA, Hongeng S, Magrin E, Schiller GJ, Payen E, Semeraro M, *et al* (2018) Gene therapy in patients with transfusion-dependent β-thalassemia. *N Engl J Med*

Touzot F, Moshous D, Creidy R, Neven B, Frange P, Cros G, Caccavelli L, Blondeau J, Magnani A, Luby JM, *et al* (2015) Faster T-cell development following gene therapy compared with haploidentical HSCT in the treatment of SCID-X1. *Blood*

Truong LN, Li Y, Shi LZ, Hwang PYH, He J, Wang H, Razavian N, Berns MW & Wu X (2013) Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *Proc Natl Acad Sci USA*

Tsai SQ, Nguyen NT, Malagon-Lopez J, Topkar V V., Aryee MJ & Joung JK (2017) CIRCLE-

seq: a highly sensitive in vitro screen for genome-wide CRISPR–Cas9 nuclease off-targets. *Nat Methods* 14

Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar V V., Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, *et al* (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol*

Turnell AS & Grand RJ (2012) DNA viruses and the cellular DNA-damage response. *J Gen Virol* doi:10.1099/vir.0.044412-0

Uchida N, Dykstra B, Lyons KJ, Leung FYK & Eaves CJ (2003) Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. *Exp Hematol* 31: 1338–1347

Uchida N, Hsieh MM, Raines L, Haro-Mora JJ, Demirci S, Bonifacino AC, Krouse AE, Metzger ME, Donahue RE & Tisdale JF (2019) Development of a forward-oriented therapeutic lentiviral vector for hemoglobin disorders. *Nat Commun*

Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD & Holmes MC (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*

Urnov FD, Rebar EJ, Holmes MC, Zhang HS & Gregory PD (2010) Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 11: 636–646 doi:10.1038/nrg2842

Uy GL, Rettig MP & Cashen AF (2008) Plerixafor, a CXCR4 antagonist for the mobilization of hematopoietic stem cells. *Expert Opin Biol Ther*

Vakulskas CA, Dever DP, Rettig GR, Turk R, Jacobi AM, Collingwood MA, Bode NM, McNeill MS, Yan S, Camarena J, *et al* (2018) A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. *Nat Med*

Velten L, Haas SF, Raffel S, Blaszkiewicz S, Islam S, Hennig BP, Hirche C, Lutz C, Buss EC, Nowak D, *et al* (2017) Human haematopoietic stem cell lineage commitment is a continuous process. *Nat Cell Biol* 19: 271–281

Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A, Hassab H, Achebe MM, Alkindi S, Brown RC, *et al* (2019) A Phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med*

Wagenblast E, Azkanaz M, Smith SA, Shakib L, McLeod JL, Krivdova G, Araújo J, Shultz LD, Gan OI, Dick JE, *et al* (2019) Functional profiling of single CRISPR/Cas9-edited human long-term hematopoietic stem cells. *Nat Commun*

Walasek MA, van Os R & de Haan G (2012) Hematopoietic stem cell expansion: Challenges and opportunities. *Ann N Y Acad Sci* 1266: 138–150

Walton RT, Christie KA, Whittaker MN & Kleinstiver BP (2020) Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. *Science (80-)*

Wang CX & Cannon PM (2016) The clinical applications of genome editing in HIV. *Blood* doi:10.1182/blood-2016-01-678144

Wang J, Exline CM, DeClercq JJ, Llewellyn GN, Hayward SB, Li PW-LL, Shivak DA, Surosky RT, Gregory PD, Holmes MC, *et al* (2015) Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors. *Nat Biotechnol* 33: 1256–1263

Wang JCY, Doedens M & Dick JE (1997) Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. *Blood*

Wang X, Wang Y, Wu X, Wang J, Wang Y, Qiu Z, Chang T, Huang H, Lin RJ & Yee JK (2015) Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENs using integrase-defective lentiviral vectors. *Nat Biotechnol* 33: 175–179

Watson JD & Crick FHC (1953) Molecular structure of nucleic acids: A structure for

deoxyribose nucleic acid. *Nature*

Weissman IL & Shizuru JA (2008) The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood* 112: 3543–3553

Wienert B, Wyman SK, Richardson CD, Yeh CD, Akcakaya P, Porritt MJ, Morlock M, Vu JT, Kazane KR, Watry HL, *et al* (2019) Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. *Science (80-)*

Wilen CB, Wang J, Tilton JC, Miller JC, Kim KA, Rebar EJ, Sherrill-Mix SA, Patro SC, Secreto AJ, Jordan APO, *et al* (2011) Engineering HIV-resistant human CD4+ T cells with CXCR4-specific zinc-finger nucleases. *PLoS Pathog*

Wilson A, Laurenti E, Oser G, van der Wath RC, Blanco-Bose W, Jaworski M, Offner S, Dunant CF, Eshkind L, Bockamp E, *et al* (2008) Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell*

Wilson RC & Carroll D (2019) The Daunting Economics of Therapeutic Genome Editing. *CrisJ*

Woods NB, Bottero V, Schmidt M, Von Kalle C & Verma IM (2006) Gene therapy: Therapeutic gene causing lymphoma. *Nature*

Wu Y, Zeng J, Roscoe BP, Liu P, Yao Q, Lazzarotto CR, Clement K, Cole MA, Luk K, Baricordi C, *et al* (2019) Highly efficient therapeutic gene editing of human hematopoietic stem cells. *Nat Med*

Xie SZ, Garcia-Prat L, Voisin V, Ferrari R, Gan OI, Wagenblast E, Kaufmann KB, Zeng AGX, Takayanagi S ichiro, Patel I, *et al* (2019) Sphingolipid Modulation Activates Proteostasis Programs to Govern Human Hematopoietic Stem Cell Self-Renewal. *Cell Stem Cell*

Xu K, Ren C, Liu Z, Zhang T, Zhang T, Li D, Wang L, Yan Q, Guo L, Shen J, *et al* (2015) Efficient genome engineering in eukaryotes using Cas9 from Streptococcus thermophilus. *Cell Mol Life Sci*

Xu L, Wang J, Liu Y, Xie L, Su B, Mou D, Wang L, Liu T, Wang X, Zhang B, *et al* (2019) CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia. *N Engl J Med*

Yao X, Wang X, Hu X, Liu Z, Liu J, Zhou H, Shen X, Wei Y, Huang Z, Ying W, *et al* (2017) Homology-mediated end joining-based targeted integration using CRISPR/Cas9. *Nat Publ Gr*: 1–14

Yeh CD, Richardson CD & Corn JE (2019) Advances in genome editing through control of DNA repair pathways. *Nat Cell Biol* 21: 1468–1478

Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J & Buck DW (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*

Yukl SA, Boritz E, Busch M, Bentsen C, Chun TW, Douek D, Eisele E, Haase A, Ho YC, Hütter G, *et al* (2013) Challenges in Detecting HIV Persistence during Potentially Curative Interventions: A Study of the Berlin Patient. *PLoS Pathog*

Zeng J, Wu Y, Ren C, Bonanno J, Shen AH, Shea D, Gehrke JM, Clement K, Luk K, Yao Q, *et al* (2020) Therapeutic base editing of human hematopoietic stem cells. *Nat Med*

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, *et al* (2015) Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. *Cell*

Zhao H, Dahlö M, Isaksson A, Syvänen AC & Pettersson U (2012) The transcriptome of the adenovirus infected cell. *Virology*

Zonari E, Desantis G, Petrillo C, Boccalatte FE, Lidonnici MR, Kajaste-Rudnitski A, Aiuti A, Ferrari G, Naldini L & Gentner B (2017) Efficient Ex Vivo Engineering and Expansion of Highly Purified Human Hematopoietic Stem and Progenitor Cell Populations for Gene Therapy. *Stem Cell Reports* 8: 977–990

CHAPTER II

Specific contribution of the candidate

In 2014, a paper from our laboratory by Genovese *et al.* published in *Nature* journal showed the feasibility to reach HDR-mediated gene editing in human HSC by targeting the *IL2RG* locus in the context of SCID-X1 immunodeficiency. In 2017, another paper from our laboratory by Schiroli *et al.* published in *Science Translational Medicine* journal defined a threshold of 10% of targeted gene correction for SCID-X1 to reach a therapeutic benefit in mouse model, and also highlighted the selective advantage of these corrected cells in the context of SCID-X1 disease. Importantly, the use of AAV6 as HDR donor template instead of IDLV increased 5-fold the HDR editing efficiency in human long-term engrafting HSCs after xenotransplantation in immunodeficient NSG mice. In 2019, another paper by Schiroli *et al.* published in *Cell Stem Cell* journal shed light on genome editing toxicity in HSC where a robust p53 activation led to human graft shrinking after xenotransplantation in NSG mice. Importantly, the transient delivery of mRNA coding for a p53 inhibitor (e.g. GSE56) hampered p53-mediated toxicity and led to 6-fold higher human graft size in mouse. Despite these technological advances, HSC biology and cell cycle status still constrained HDR gene editing efficiency and required further improvements to expand the number of therapeutic targets, which cannot benefit of a selective advantage. Discovering a strategy to overcome this remaining barrier thus became the major goal of my Ph.D. project.

We reasoned that some proteins derived from wild-type Adenovirus, a natural co-helper of AAV virus during infection and previously reported to promote cell cycle progression of infected cells may enhance HDR in HSC. For this purpose, we screened a large panel of adenoviral proteins derived from several serotypes variants by transient expression in HSC by mRNA delivery during electroporation together with the Cas9 nuclease. As main readouts, we evaluated cell growth, HDR editing efficiency, p53/p21 toxicity, and clonogenic assays. We found that the adenovirus 5 protein E4orf6/7, which operates the major cell cycle controller E2F, induced a 2-fold increase in HDR editing efficiency in the most primitive HSC compartment (Figure 2 and Supplementary 2). To further investigate the underlying mechanism of this effect, we performed RNA bulk sequencing and targeted gene expression analyses and showed increased engagement of cells transiently expressing Ad5-E4orf6/7 in S/G2 phases with concomitant upregulation of all major components of the HDR machinery, thus explaining the increased efficiency of targeted transgene insertion. Interestingly, synergic effect was observed

when combining E4orf6/7 with the p53 inhibitor GSE56, partially counteracting a p53/p21 negative feedback loop response triggered by Ad5-E4orf6/7 (Figure 3 and Supplementary 3). We then validated the activity of Ad5-E4orf6/7 in primitive HSC by transplanting the edited cells in NSG immunodeficient mouse model (Figure 5 and Supplementary 5). Combined E4orf6/7 expression and p53 inhibition during gene editing enhanced up to 50% the HDR efficiency within the long-term human graft, well surpassing the levels reported until now in the literature. Such outcome was reproducible across several HSPC donors and sources, genomic loci and is conceivably portable to most types of editing platforms.

The PhD work of mine described above was assembled in a paper describing the enhanced HSC gene editing protocol together with a detailed clonal characterization (BAR-seq) of the reconstituted hematopoiesis in xenotransplanted mice performed by my colleague and candidate Ph.D. Samuele Ferrari. After peer review the study was published as original research article in *Nature Biotechnology* on June 29th, 2020 (10.1038/s41587-020-0551-y) with myself and Samuele Ferrari co-first authors. A patent describing the enhanced editing protocol was also filed with myself listed as co-inventor (WO2020002380A1).

All experimental results presented in the Figures of the thesis were obtained by me, except for:

Figure 1: Experiments with small drugs which were performed by Dr. Samuele Ferrari (SR-TIGET, San Raffaele University, Milan).

Figure 3: RNA bulk sequencing and gene expression analyses which were performed 50/50 by Dr. Samuele Ferrari and myself.

Figure 5: BAR-seq and Indels-based clonal tracking which were performed by Dr. Samuele Ferrari (SR-TIGET, San Raffaele University, Milan).

Other results/works mentioned in the thesis were obtained through a joint effort as follows:

- RNA-Seq and indel-based clonal tracking bioinformatic analysis were performed in collaboration with Dr. Stefano Beretta;
- BAR-Seq bioinformatic pipeline was developed in collaboration with Dr. Stefano Beretta, Dr. Ivan Merelli and Dr. Davide Cittaro (SR-Tiget Bioinformatic Core and Center for Omics Sciences, IRCCS San Raffaele Scientific Institute, Milan);
- Sequencing was performed by Dr Dejan Lazarevic (Center for Omics Sciences, IRCCS San Raffaele Scientific Institute, Milan).
- Statistics were performed and analyzed in collaboration with Dr. Chiara Brombin and Dr. Federica Cugnata (CUSSB - University Center for Statistics in the Biomedical Sciences, Vita-Salute San Raffaele University).

Efficient gene editing of human long-term hematopoietic stem cells validated by clonal tracking

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ABSTRACT

Targeted gene editing in hematopoietic stem cells (HSCs) is a promising treatment for several diseases. However, the limited efficiency of homology-directed repair (HDR) in HSCs and the unknown impact of the procedure on clonal composition and dynamics upon transplantation have hampered clinical translation. Here, we apply a barcoding strategy to clonal tracking of edited cells (BAR-Seq) and show that editing activates p53, which significantly shrinks the HSC clonal repertoire in hematochimeric mice, although engrafted edited clones preserved multilineage and self-renewing capacity. Transient p53 inhibition restored polyclonal graft composition. We increased HDR efficiency by forcing cell cycle progression and upregulating components of the HDR machinery through transient expression of the Adenovirus 5 E4orf6/7 protein, which recruits the cell cycle controller E2F on its target genes. Combined E4orf6/7 expression and p53 inhibition resulted in HDR editing efficiencies of up to 50% in the long-term human graft, without perturbing repopulation and self-renewal of edited HSCs. This enhanced protocol should broaden applicability of HSC gene editing and pave its way to clinical translation.

INTRODUCTION

The therapeutic potential of HSC gene therapy has been shown in several clinical trials for inherited diseases and may be advanced by targeted genome editing, which allows *in situ* correction of mutant alleles, restoring function and physiological expression control (Naldini, 2019). Programmable nucleases, such as CRISPR/Cas, enable gene editing by introducing site-specific DNA double strand breaks (DSBs) into the genome (Carroll, 2014). DSB repair may occur by non-homologous end-joining (NHEJ), which often introduces small insertion/deletion (indels) at the repaired site, or by the high-fidelity homology-directed repair (HDR), which is exploited for gene correction or targeted integration using an exogenous DNA template. HDR predominantly occurs in S/G2 phases. Unfortunately, HSCs are poorly permissive to HDR, likely due to quiescence and limited template uptake (Genovese *et al*, 2014). Despite recent improvements achieved by culturing hematopoietic stem/progenitor cells (HSPC) with StemRegenin-1 (SR1) and UM171 (Genovese *et al*, 2014; Schiroli *et al*, 2017; Boitano *et al*, 2010; Fares *et al*, 2014), and using adeno-associated vector serotype 6 (AAV6) for template delivery (De Ravin *et al*, 2016; Pavel-Dinu *et al*, 2019; Wang *et al*, 2015; Dever *et al*, 2016; Kuo *et al*, 2018; Schiroli *et al*, 2017), HDR remains constrained in long-term repopulating HSCs (LT-HSCs) and thus limits applicability of gene correction. Whereas a limited proportion of edited HSCs may successfully treat diseases characterized by selective advantage of the functional progeny (Schiroli *et al*, 2017), this might be insufficient in most other conditions, where a large proportion of non-corrected cells competes with the edited ones for engraftment and limits functional hematopoietic reconstitution. Several strategies have been attempted to enhance HDR(Yeh *et al*, 2019) but the efficacy of these approaches in HSPCs is limited. Some helper adenoviral (Ad) proteins which, during AAV infection, promote viral genome processing and modulate host cell responses, have been shown to increase AAV template expression (Gwiazda *et al*, 2016; Chu *et al*, 2015) but not HDR in LT-HSCs (Kuo *et al*, 2018).

We recently showed that transient activation of the p53 pathway occurs in HSPCs even after a single DSB, leading to reversible proliferation delay (Schiroli *et al*, 2019). Concomitant exposure to AAV6 led to cumulative and robust p53 activation, causing proliferation arrest and strongly impacting hematopoietic reconstitution upon xenotransplantation in immunodeficient mice. Inhibition of this p53 response by transient expression of a dominant-negative p53 mutant protein (GSE56) during editing increased hematopoietic repopulation by treated cells. It remains unknown whether such outcome was due to altered growth properties or improved preservation of LT-HSCs during editing.

Little information is available on the clonogenic output and multilineage repopulation capacity of individual HSPCs after editing. A low yield of edited HSCs may delay hematopoietic recovery, exposing patients to high risk of infection, and result in oligoclonal hematopoiesis, which may impair graft resilience and potentially increase the risk of leukemia and myelodysplastic syndrome (Steensma, 2018).

Here, we developed an enhanced gene editing protocol yielding high proportions of edited LT-HSCs by overcoming two major biological barriers, robust p53 response and constrained HDR. Clonal tracking of edited HSPCs proved polyclonal reconstitution and preserved self-renewal and multi-potency of individual edited HSCs, giving confidence to future clinical translation.

RESULTS

Barcoded template enables clonal tracking of edited HSPCs and shows reconstitution by few dominant clones with preserved multilineage potential

We selected the Adeno-Associated Virus Site 1 (*AAVS1*) as paradigmatic safe harbor for targeted transgene insertion(Lombardo *et al*, 2011). We embedded a 22-bp degenerated heritable "barcode" sequence (BAR) in the repair template downstream of a GFP reporter cassette (**Fig. 1a**) and generated a plasmid library and an AAV6 pool of high and comparable complexity ($7.5x10^5$ and $5.9x10^5$ unique BARs, respectively) and nearly homogeneous representation of degenerated consensus sequences (**Fig. 1b**).

We then edited *AAVS1* in human cord blood (CB) HSPCs by electroporating CRISPR/*Sp*Cas9 ribonucleoprotein (RNP) with a highly specific chemically modified guide RNA (gRNA) (Schiroli *et al*, 2019) and found similar editing efficiency of the barcoded library compared to non-barcoded AAV6, as assessed by GFP^+ cells percentage in the treated cells outgrowth (**Extended Data Fig. 1a**). Deep sequencing of on-target BARs in HSPCs revealed highly diverse repertoire of similar magnitude as the edited cells (54,865 and 27,477 unique BARs retrieved from ~200,000 cells edited to 65% efficiency), with only one or two slightly overrepresented (< 0.25%) BAR/sample (**Extended Data Fig. 1b**).

The impact of prolonging culture time on graft clonality and the stem-preserving activity of SR1 and UM171 in the context of gene editing have never been evaluated. We treated the same starting number of HSPCs for *AAVS1* editing in presence or absence of SR1/UM171 and transplanted the total outgrowth in NSG mice one day (corresponding to 4^{th} day of culture; "+4 days") or one week after editing ("+10 days") (**Extended Data Fig. 1c**). Analyses of treated cells showed more phenotypically primitive progenitors ($CD34^+CD133^+CD90^+$, hereafter named "$CD90^+$") in presence of SR1/UM171 at both times, with comparable editing efficiencies between treatments. $CD90^+$ cells decreased with time in all cultures and became nearly absent without SR1/UM171 (**Extended Data Fig. 1d, e**). Concordantly, "+4 days"-transplanted mice showed higher human cell engraftment in peripheral blood (PB) and hematopoietic organs compared to the "+10 days" groups. SR1/UM171 increased human engraftment in both comparisons (**Fig. 1c** and **Extended Data Fig. 1f**). Despite similar and high percentage of GFP^+ cells among all groups in PB at 4 weeks after transplant, "+10 days"-transplanted mice showed decreased GFP marking at later times (**Fig. 1d** and **Extended Data Fig. 1g**).

Mice showing detectable (>0.1%) engraftment of human GFP^+ cells in PB at 18 weeks were selected for clonal analyses, which included all mice of the "+4 days" and only 6/10 and 3/10

mice of the "+10 days" groups with or without SR1/UM171, respectively. Sequencing of on-target BARs ("BAR-Seq") from PB mononuclear cells (PBMCs) of "+4 days"-transplanted mice at different times after transplant revealed from ~60 to ~700 unique BARs/mouse, which segregated in two populations with log-difference in abundance. Upon ranking from the most to the least abundant BAR within each sample, we applied a saturation-based approach and defined "dominant" the small set of BARs accounting for >90% of total abundance and "rare" the remaining ones (**Fig. 1e** and **Extended Data Fig. 1h, i**). We then focused our clonal dynamics analyses on dominant BARs, which robustly contribute to hematopoiesis.

Longitudinal analysis within PB of "+4 days"-transplanted mice showed progressive shrinking, up to disappearance, of some dominant clones between 8 and 12-18 weeks, as well as emergence of new dominant BARs at 12 and 18 weeks (**Fig. 1f**). The fraction of BARs shared between different time points in each mouse was higher in the 8 vs. 12 weeks comparison respect to the 8 or 12 vs. 18 weeks ones (**Fig. 1g**), independently from *ex vivo* culture conditions (**Extended Data Fig. 1j**) and suggested distinct early and steady-state reconstitution phases driven by different clones. The number of dominant BARs was significantly lower in the "+10 days" transplanted groups, suggesting loss of engrafting clones and/or expansion of a limited subset in prolonged culture. Despite a trend for higher number of dominant BARs short term after transplant with HSPCs cultured in presence of SR1/UM171, the edited long-term graft was mostly composed by 6-7 dominant clones per mouse (**Fig. 1h**).

Contribution to different lineages was similar among treatments, with myeloid and T cells showing highest and lowest clonality, respectively (**Fig. 1f, i** and **Extended Data Fig. 1j, k**). Most BARs retrieved from $CD34^+CD38^-$ HSPCs sorted from bone marrow (BM) of engrafted mice were shared with ≥2 differentiated hematopoietic lineages, confirming at clonal level the multipotent long-term repopulation capacity of individual HDR-edited HSPCs *in vivo* (**Fig. 1f, j** and **Extended Data Fig. 1j**).

Inter-mice BAR sharing was rare but detectable within the same experimental group. The high complexity of the library makes it unlikely that the same BAR integrates in different LT-HSC and collisions during sequencing can be ruled out because we focused our analysis only on abundant clones. Thus, detection of the same BAR among dominant clones of two mice suggests duplication during *ex vivo* culture of HDR-edited HSPCs which maintain repopulation potential in xenografts (**Fig. 1f** and **Extended Data Fig. 1j**). There was no linear correlation between the number of HDR-edited dominant clones and the percentage of GFP^+ cells within the human graft. The fewer were the engrafting clones (and, consequently, the smaller the human graft) the higher the variability in GFP marking, as expected from limited sampling of the

input population; the more were the engrafting clones (and the larger the size of the human graft) the less the variability in GFP marking and the closer its level to that of the input population (**Extended Data Fig. 1l**).

Overall, these results show oligoclonal composition of the human graft reconstituted by edited HSPCs which have maintained multi-lineage output and short or long-term self-renewing potential. Addition of SR1/UM171 improved the early phase of reconstitution by increasing the number of short-term contributing clones and the overall extent of repopulation after prolonged *ex vivo* culture.

P53 activation constrains the number but not output of repopulating edited HSPCs

Co-electroporation of mRNA for the dominant-negative p53 mutant GSE56 with the editing reagents substantially increased (>5 fold) the number of dominant BARs contributing to xenograft hematopoiesis (**Fig. 1k**), providing a mechanistic explanation for the reported increase in the human graft (Schiroli *et al*, 2019). This finding held true also when we expanded the analysis to encompass a larger proportion of BAR reads (**Extended Data Fig. 1m**). The size of the human edited graft (measured as percentage of human $hCD45^{+}GFP^{+}$ cells within the total live PBMCs) in mice transplanted with a non-saturating dose of edited cells significantly correlated at early and late times of reconstitution with the number of unique dominant BARs identified, indicating that neither p53 activation induced by gene editing nor its alleviation by GSE56 altered the average clonal output of individual repopulating human HSPCs. Moreover, the average clonal output of individual progenitors was lower at early than late times post-transplant, in line with progressive exhaustion of short-term progenitors (**Fig. 1l** and **Extended Data Fig. 1n**).

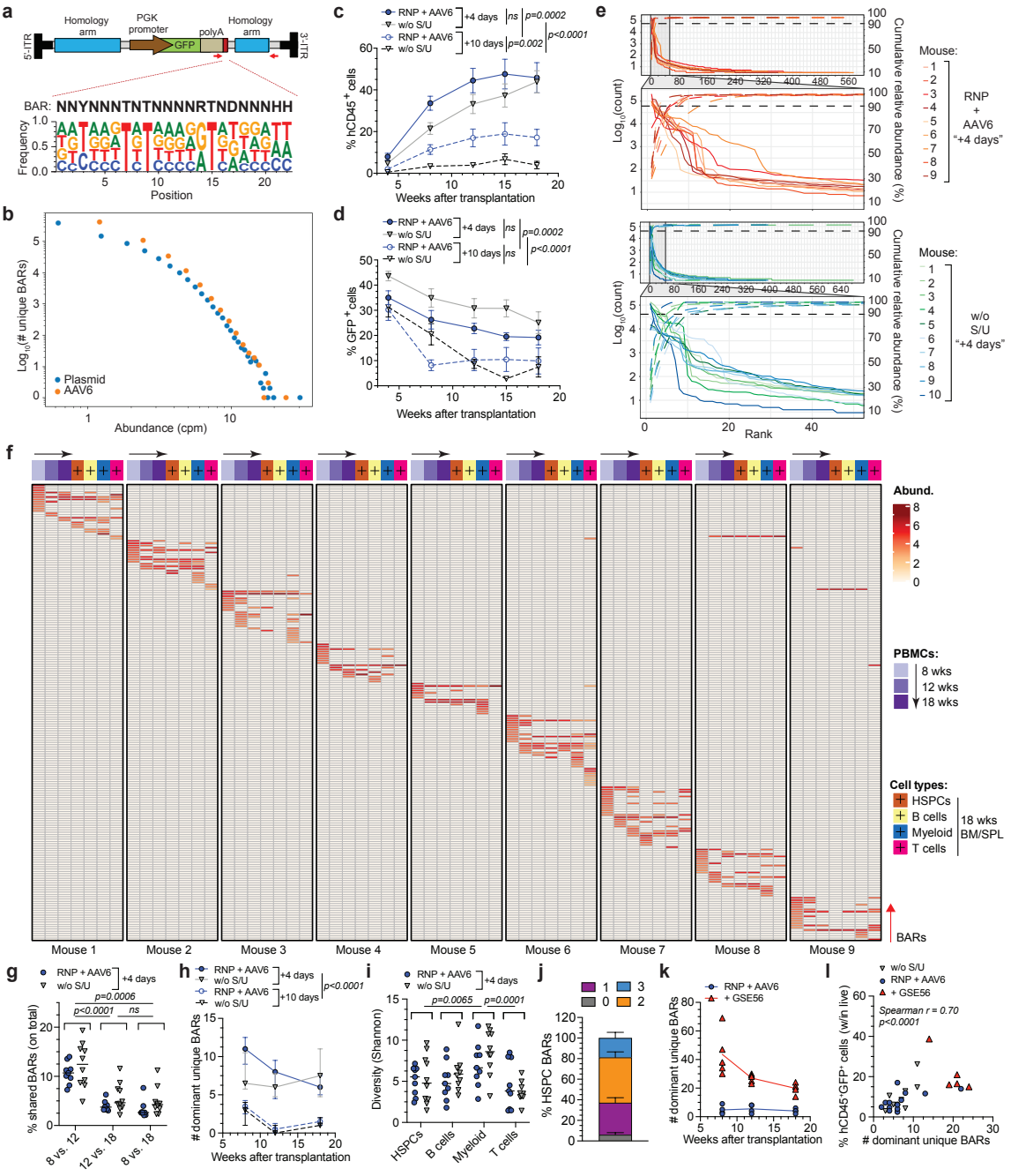


Figure 1. BAR-Seq enables clonal tracking of human HDR-edited HSPCs. a, Top: schematic of the barcoded AAV6 library for *AAVS1* editing and BAR consensus sequence downstream of the GFP reporter. Arrows indicate primer binding sites for plasmid/AAV sequencing. Bottom: logo plot showing the nucleotide frequency in the BAR sequence. **b**, Number of unique BARs and their abundances (counts per million, cpm) in plasmid and AAV6 libraries. **c-d**, Percentage of circulating human CD45$^+$ (hCD45$^+$) cells (**c**) and GFP$^+$ cells within human graft (**d**) in mice transplanted one day ("+4 days") or one week ("+10 days") after editing of HSPCs cultured in presence ("RNP + AAV6") or absence ("w/o S/U") of SR1/UM171 (n = 9, 10, 10,

10). Mean ± SEM. Linear Mixed Effects models (LME) followed by post-hoc analysis. Statistics are shown for the last timepoint. **e**, Abundance of ranked BARs in 18-weeks PBMCs from "+4 days" mice. Solid and dashed lines show absolute and cumulative relative abundance (saturation curves) of ranked BARs, respectively. Magnification of most abundant BARs is shown. **f**, Heatmap showing the abundance (red-scaled palette) of dominant unique BARs (rows) in "RNP + AAV6 (+4 days)" mice (separated columns) in PBMCs at indicated times after transplant and sorted hCD45$^+$ cell types. **g**, Percentage of BARs shared between PBMCs harvested at indicated timepoints ("+4 days" mice; n = 9, 10). Median. Friedman test with Dunn's multiple comparisons. **h**, Longitudinal PBMC analysis showing the number of dominant unique BARs in analyzed mice from Fig. 1c (n = 9, 10, 6, 3). Median with interquartile range (IQR). Generalized linear Mixed Effects models (GLMER) for count data. **i**, Clonal diversity within sorted hCD45$^+$ cell types ("+4 days" mice; n = 9, 10). Median. Friedman test with Dunn's multiple comparisons. **j**, Percentage of dominant unique HSPC BARs shared with none, 1, 2 or 3 sorted hCD45$^+$ cell lineages ("+4 days" mice; n = 19). Mean ± SEM. **k**, Longitudinal PBMC analysis showing the number of dominant unique BARs in mice transplanted with HSPCs edited in absence or presence of GSE56 and retrieved when including in the analysis >90% of total BAR reads (n = 4, 5). Median. **l**, Correlation between the percentage of hCD45$^+$GFP$^+$ cells and the number of dominant unique BARs in 18-weeks PBMCs of mice from Fig. 1h, k (n = 28). Spearman correlation coefficient was calculated. For Fig. 1g, h, i, j experimental groups were unified for statistical analysis. All statistical tests are two-tailed. n indicate independent animals.

Adenoviral protein E4orf6/7 improves editing efficiency of human HSPCs

We screened a panel of Ad proteins known to function as helpers in Ad-AAV co-infection (Zhao *et al*, 2012; Täuber & Dobner, 2001). We focused on E4orf1 and E4orf6/7, which interact with cellular components involved in survival (Seandel *et al*, 2008; Frese *et al*, 2003; Javier & Rice, 2011) and cell cycle (Huang & Hearing, 1989). Since some viral gene properties differ among Ad serotypes, we screened four serotype variants (**Extended Data Fig. 2a, b**). We also tested serotype 5 E1B55K and E4orf6 proteins, previously described to increase AAV DNA second strand synthesis (Chu *et al*, 2015; Gwiazda *et al*, 2016; Kuo *et al*, 2018). Ad proteins were expressed by HPLC-purified mRNAs (Karikó *et al*, 2011) co-delivered with *AAVS1*-editing reagents (**Fig. 2a**). All E4orf6/7 variants and Ad9-E4orf1 increased HDR in HSPCs, including the CD90$^+$ fraction (**Extended Data Fig. 2c, d**). As previously described in primary T cells (Gwiazda *et al*, 2016), the combination of E1B55K and E4orf6 increased the percentage of

GFP$^+$ HSPCs but molecular analysis did not show any HDR improvement, suggesting that increased GFP expression was mainly derived from the AAV6 donor whose double-stranded DNA synthesis was promoted (**Extended Data Fig. 2e**). As expected from previous reports (Pattabhi *et al*, 2019; Romero *et al*, 2019; Schiroli *et al*, 2019), HSPC editing decreased the proportion of colony-forming cells from the cumulative impact of several steps of the treatment (**Extended Data Fig. 2f**). E4orf1 variants increased cell proliferation counteracting the editing induced delay, while Ad4- and Ad5-E4orf6/7 did not influence cell growth. Ad3- and Ad23-E4orf6/7 as well as the combination of Ad5-E1B55K and Ad5-E4orf6 strongly decreased cell growth (**Extended Data Fig. 2g, h**). In agreement, the *in vitro* colony-forming potential of treated HSPCs was increased by E4orf1 proteins, while it was strongly reduced by the combination of E1B55K and E4orf6 or E4orf6/7 proteins, except for the Ad5-E4orf6/7 variant, which resulted similar to the standard condition (**Extended Data Fig. 2i**). Overall, this screening prompted further investigation of Ad5-E4orf6/7 and Ad9-E4orf1 (**Supplementary Fig. 1**) as enhancers of HDR editing.

We then evaluated the effect of Ad5-E4orf6/7 together with GSE56, transiently co-expressed by separate mRNAs or single RNA encoding a fusion protein with P2A self-cleaving peptide. Across multiple independent experiments, these combinations increased HDR by an average 50% in CD90$^+$ HSPCs as compared to the standard protocol and elected the fusion construct for further studies to lower overall mRNA input (**Fig. 2b, c**). Cell growth was higher when adding GSE56, as previously reported (Schiroli *et al*, 2019), although less markedly when combined with Ad5-E4orf6/7 (**Fig. 2d**). Ad5-E4orf6/7 treatment, with or without GSE56, decreased the fraction of CD90$^+$ cells measured in culture (**Fig. 2e**), an effect apparently due to lower CD90 expression on cell surface (**Extended Data Fig. 2j**). Toxicity was mild for all treatments, doubling the fraction of apoptotic/necrotic cells detected in untreated samples up to an average 15% in bulk and CD90$^+$ cells (**Extended Data Fig. 2k** and **Fig. 2f**). Colony-forming potential was similarly reduced for all editing treatments as compared to untreated cells, with a trend towards more colonies for GSE56 treated cells and less for the GSE56/Ad5-E4orf6/7 combination, without detectable difference in erythroid to myeloid ratio (**Fig. 2g**). We also reproduced the increase in HDR editing by Ad5-E4orf6/7 in human mobilized peripheral blood (mPB) HSPCs, reaching up to 1.5-fold within phenotypically primitive cells (**Fig. 2h** and **Extended Data Fig. 2l, m**). On the contrary, we did not observe higher efficiency of targeted integration in T cells (**Extended Data Fig. 2n**).

We then asked whether the effect of Ad5-E4orf6/7 on HDR editing was specific for the AAV donor template. Integrase defective lentiviral vectors (IDLV), although generally less effective

than AAV6 as donor template for HSPCs, might be useful to increase cargo capacity and lower predicted immunogenicity. We thus edited *AAVS1* with a suitably matching IDLV donor in presence or absence of GSE56, Ad5-E4orf6/7 and cyclosporin H, which increases IDLV transduction (Petrillo *et al*, 2018). Ad5-E4orf6/7 boosted HDR up to 1.5-fold in primitive CB HSPCs, reaching up to 35% HDR, with similar effects on culture composition as reported above for the AAV template (**Figure 2i** and **Extended Data Fig. 2o, p**).

We then assessed whether our AAV-based protocol was portable to other genomic sites by measuring HDR editing in *IL2RG* and *CD40LG*, whose defective mutation cause severe primary immunodeficiencies amenable to HSPC gene therapy. We found a 1.4/1.5-fold increase in HDR by Ad5-E4orf6/7 with or without GSE56 in $CD90^+$ cells as compared to standard condition, reaching up to an average of 50% and 35% GFP^+ cells upon *IL2RG* or *CD40LG* editing, respectively (**Fig. 2j**).

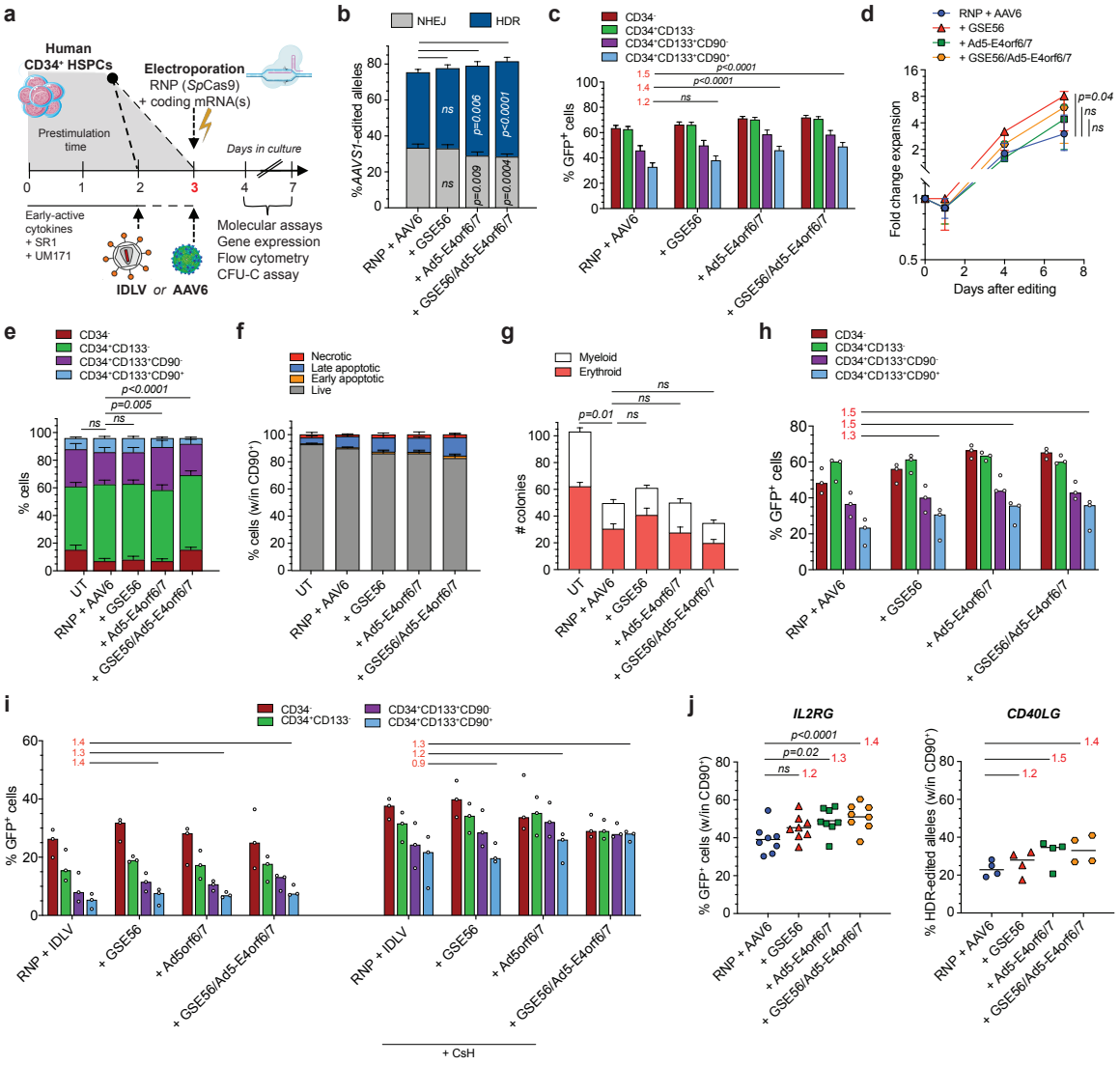


Figure 2. Combined transient expression of Ad5-E4orf6/7 and GSE56 improves editing efficiency in human HSPCs. a, Experimental workflow. **b-c**, Percentage of HDR/NHEJ-edited alleles in bulk CB HSPCs (**b**) and GFP$^+$ cells within subpopulations (**c**) 96 h after *AAVS1* editing with standard protocol ("RNP + AAV6"), in presence of GSE56, Ad5-E4orf6/7 or their combination (35 HSPC donors; n = 15). Mean ± SEM. **d**, Fold change expansion of live HSPCs after indicated treatments (9 HSPC donors; n = 5). Median ± IQR. Statistical analysis performed at the last timepoint. **e**, Culture composition 96 h after editing in experiments from Fig. 2b (n = 15). Mean ± SEM. **f**, Percentage of live, early/late apoptotic and necrotic CD90$^+$ cells 24 h after editing in the indicated conditions (7 HSPC donors; n = 3). Mean ± SEM. **g**, Number of colonies in the indicated conditions (19 HSPC donors; n = 10). Mean ± SEM. **h**, Percentage of GFP$^+$ cells within subpopulations 96 h after *AAVS1* editing of mPB HSPCs with the indicated treatments (5 HSPC donors; n = 3). Median. **i**, Percentage of GFP$^+$ cells within subpopulations 96

h after IDLV-based *AAVS1* editing of CB HSPCs with indicated treatments (3 HSPC donors; n = 3). Median. **j**, Percentage of GFP^+ cells (left) and HDR-edited alleles (right) in $CD90^+$ cells 96 h after *IL2RG* or *CD40LG* editing, respectively (*IL2RG*: 12 HSPC donors; n = 8. *CD40LG*: 4 HSPC donors; n = 4). Median. For all panels with statistical analyses: Friedman test with two-tailed Dunn's multiple comparisons against "RNP + AAV6". For panel 2c, h-j: red numbers represent the fold increases of the center values for the percentage of GFP^+ cells over "RNP + AAV6" within $CD90^+$ compartment. n indicate independent experiments.

Ad5-E4orf6/7 activates the E2F transcriptional pathway upregulating HDR machinery and forcing progression to S/G2 cell cycle phases

We investigated the cellular response triggered by Ad5-E4orf6/7, which has been reported to directly interact with the master cell cycle regulator E2F, leading to its binding and transcriptional activation of the Ad E2 promoter (Obert *et al*, 1994; Huang & Hearing, 1989). We measured expression of cell cycle-related genes upon *AAVS1* editing in presence or absence of GSE56 and/or Ad5-E4orf6/7, both in CB and mPB HSPCs. As reported(Schiroli *et al*, 2019), GSE56 dampened the editing-induced activation of DNA damage response (DDR) through p53 target genes, such as *CDKN1A* (p21), *RPS27L*, *PHLDA3* and *APOBEC3H*(Menendez *et al*, 2017). Ad5-E4orf6/7 transiently activated *CDK2*, which promotes S/G2progression(Aleem *et al*, 2005), but also upregulated the E2F target genes *CDKN1A* (p21) and *CDKN2A* ($p14^{ARF}$), which foster cell cycle arrest(Radhakrishnan *et al*, 2004; Komori *et al*, 2005). Ad5-E4orf6/7 downregulated *APOBEC3H*, *RPS27L*, *PHLDA3* and *CDKN2A* ($p16^{INK4a}$), an effect further increased by combination with GSE56. No differences were found in these transcriptional responses across HSPC sources (**Fig. 3a** and **Extended Data Fig. 3a, b**). p21 responses to the different treatments showed similar patterns when using IDLV instead of AAV template (**Extended Data Fig. 3c**). Whereas editing-induced p53 activation was also dampened by the combination of E1B55K and E4orf6 (**Extended Data Fig. 3d**) as expected from its p53 degradation activity(Querido *et al*, 2001), this treatment decreased cell growth and clonogenicity (**Extended Data Fig. 2g, i** above), showing that pleiotropic proteins interfering with DDR may have drastically different outcomes.

To further investigate these transcriptional changes, we performed whole transcriptomic analysis on CB HSPCs 12 hours after *AAVS1* editing, when the transient response peaked. We tested editing in presence or absence of GSE56, Ad5-E4orf6/7 or their combination. We iden-

tified a large subset of significant differentially expressed genes (DEGs) modulated by electroporation *per se* which was further expanded when performing editing, with p53 targets (*APOBEC3H*, *EDA2R*, *CDKN1A* and *MIR34AHG*) mostly upregulated (**Extended Data Fig. 3e**). The number of DEGs upon GSE56 addition compared to standard editing protocol was relatively limited, while addition of Ad5-E4orf6/7 modulated expression of a higher number of genes (including *CDK2*, *CDKN1A* and *CDKN2A*). Combination of GSE56 and Ad5-E4orf6/7 broadened the number of DEGs (**Fig. 3b**).

Gene Set Enrichment Analysis (GSEA) highlighted significant positive normalized enrichment scores (NES) for p53 pathway and inflammatory/TNFα dependent responses, and negative NES for cell cycle related categories (E2F pathway, G2M checkpoint, c-myc targets) when comparing mock electroporated with untreated cells, suggesting proliferation slowdown. These responses were further exacerbated in cells undergoing standard editing (**Fig. 3c**). GSEA between cells edited in presence or absence of GSE56 identified negative NES for the p53 pathway and positive for cell cycle-related categories, indicating dampening but not full DDR abrogation. Ad5-E4orf6/7 addition scored the E2F pathway and G2M checkpoints as top ranking positive categories (**Fig. 3c, d**). Several genes encoding for HDR machinery (Mjelle *et al*, 2015; Yeh *et al*, 2019) (*EXO1*, *DNA2*, *RBBP8*, *RPA4*, *RAD50*, *NBN*, *BRCA1/2*, *RPA1*, *RAD51C*, *RAD51AP1*, *BARD1*, *POLD3*, *PCNA*) were upregulated and the HDR pathway emerged from a more granular GSEA (**Extended Data Fig. 3f**). Surprisingly, the allograft rejection category scored with a negative NES in presence of Ad5-E4orf6/7, indicating downregulation of immune response related genes, such as *HLAs*, *CCL5/CCR5*, *IL1B*, *IRF7*, *CD28, CD4* and *THY1* (in agreement with the decrease of CD90 surface protein expression in Ad5-E4orf6/7 treated cells) (**Extended Data Fig. 3g** and **Fig. 3d**). The combination of GSE56 and Ad5-E4orf6/7 further enriched cell cycle-related categories (**Fig. 3d**).

Unsupervised clustering of E2F targets highlighted four subsets of genes showing similar expression dynamics across treatments (**Fig. 3e** and **Supplementary Table 1**). While editing downregulated genes within all subsets compared to controls, GSE56 partially rescued expression for genes of the first and second cluster, which enriched for genes promoting HDR (**Extended Data Fig. 3h**) and master regulators of cell cycle (*CHEK1*, *CHEK2*), respectively. Expression of the genes in the first cluster was upregulated by Ad5-E4orf6/7 and even more by its combination with GSE56 (**Fig. 3e** and **Extended Data Fig. 3i, top**). The third cluster enriched for CDK inhibitor genes (*CDKN1A*, *CDKN2A*, *CDKN2C*), which are involved in cell cycle arrest and were selectively upregulated by Ad5-E4orf6/7, except for *CDKN1A*, which was al-

ready increased by standard editing and further upregulated by Ad5-E4orf6/7, suggesting induction of a feedback loop to limit E2F-driven cell cycle progression (**Fig. 3e** and **Extended Data Fig. 3i, bottom**).

Expression analysis on a panel of cell cycle related genes (**Supplementary Table 2**) in sorted $CD90^+$ cells edited in presence or absence of Ad5-E4orf6/7 showed concordance with RNA-Seq data, suggesting that the transcriptional response described in bulk cultures similarly occurs in primitive HSPCs (**Fig. 3f**).

To investigate the impact of editing enhancers on cell cycle progression, we performed cell cycle analysis of edited HSPCs. Ad5-E4orf6/7 addition almost doubled the fraction of cells in S/G2 at 12-24 hours after editing, both in bulk and $CD90^+$ cells (**Fig. 3g, top**). Combination of GSE56 and Ad5-E4orf6/7 showed even more pronounced S/G2 phases transit. As expected from transient expression of the enhancers, their effects on cell cycle extinguished 96 hours after editing (**Fig. 3g, bottom**).

We then considered the potential genotoxic risk of forcing S/G2 transition and DNA replication in newly activated HSPCs. By exploiting an *IL2RG*-targeting nuclease which also detectably cleaves one off-target site (Schiroli *et al*, 2019), we measured the frequency of chromosomal translocations between on- and off-targets in presence of Ad5-E4orf6/7 and observed similar low levels in the *in vitro* outgrowth of all treated cells and none in their *in vivo* outgrowth upon transplantation in NSG mice (**Fig. 3h**).

Overall, these data suggest that transient overexpression of GSE56 and Ad5-E4orf6/7 triggers E2F-dependent cell cycle progression and upregulation of the HDR machinery, while dampening the editing-induced p53 response (**Fig. 3i**).

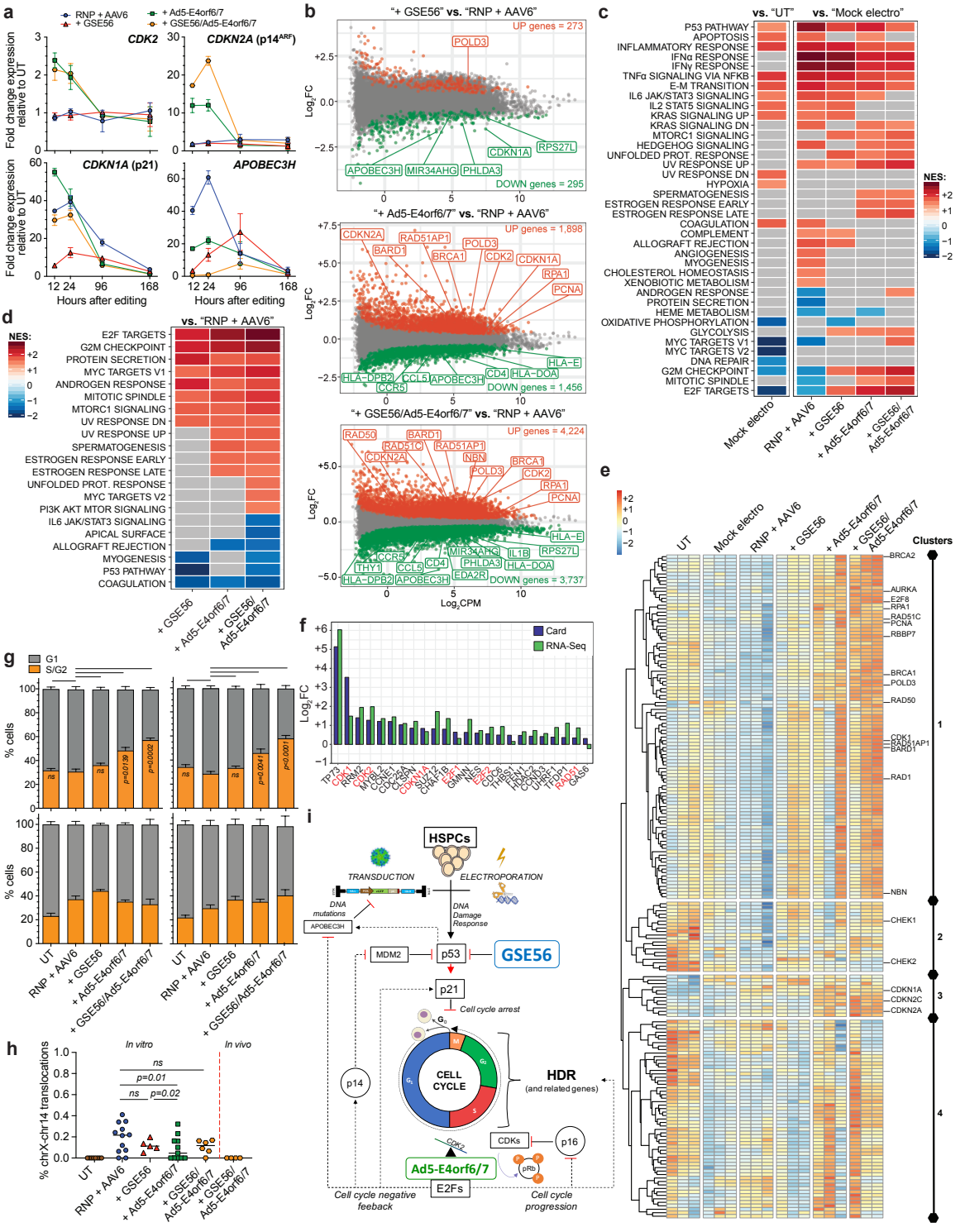


Figure 3. Ad5-E4orf6/7 forces cell cycle progression and upregulates HDR machinery via E2F pathway. a, Fold change expression over time of *CDK2*, *CDKN2A* (p14[arf]), *CDKN1A* (p21), *APOBEC3H* relative to untreated cells 'UT' (7 HSPC donors; n = 3). Median. **b**, MA plots showing significant down- (green) and up- (red) regulated genes after *AAVS1* editing in presence of GSE56 (top), Ad5-E4orf6/7 (middle) and their combination (bottom) against standard protocol (n = 3). **c-d**, Heatmaps showing NES from GSEA of indicated comparisons against

the Hallmark gene set (Molecular Signatures Database). DEGs were ranked by log_2FC expression. **e**, Heatmap showing normalized read counts for E2F target genes (Hallmark gene set) across samples. Full gene list is available in **Supplementary Table 1**. **f**, Log_2FC expression values for top 25 up-regulated cell cycle related genes in sorted $CD90^+$ HSPCs edited in presence or absence of Ad5-E4orf6/7 (blue) (n = 1). Comparison with RNA-Seq log_2FC expression values (green) is shown. **g**, Percentage of bulk (left) and $CD90^+$ (right) HSPCs in G1 or S/G2 phases 12-24 h (top; n = 6) and 96 h (bottom; n = 3) after indicated treatments. Mean ± SEM. Friedman test with Dunn's multiple comparisons against "RNP + AAV6" for 12-24 h. **h)** Percentage of *IL2RG* alleles harboring chromosome X-14 translocation 3 days after indicated treatments from Fig. 2j and in splenocytes of mice from Figure 4j (n = 8, 12, 5, 11, 6, 4). Median. LME followed by post-hoc analysis. **i)** Schematic summarizing the molecular mechanisms engaged upon enhanced editing. All statistical tests are two-tailed. n indicate independent experiments, except for Fig. 3b where n indicates independent samples.

Ad5-E4orf6/7 and GSE56 enhance gene editing in human LT-HSCs

To investigate the repopulation potential of edited HSPCs, we transplanted matched saturating or limiting cell doses into NSG mice (**Extended Data Fig. 4a**). At saturating cell doses, we observed similar human engraftment across treatments, which reached a plateau of 60% circulating cells (**Fig. 4a**). At limiting cell doses, we confirmed that GSE56 addition allowed 3-fold higher engraftment than the standard protocol, while its combination with Ad5-E4orf6/7 reduced this increase. Instead, addition of the Ad protein alone showed engraftment comparable to standard treatment (**Fig. 4b**). Similar patterns of engraftment were found long term after reconstitution in the hematopoietic organs (**Extended Data Fig. 4b**). Combination of GSE56 and Ad5-E4orf6/7 enabled higher and stable percentages of GFP^+ cells across four independent experiments compared to standard protocol, reaching a mean of 50% of the total graft (**Fig. 4c**). BM analysis showed multi-lineage long-term reconstitution with all treatments with higher proportion of progenitors when using editing enhancers (**Extended Data Fig. 4c**). The percentage of GFP^+ cells within the human graft, sorted progenitors and individual lineages were consistent with the levels observed in the blood, with the combination of GSE56/Ad5-E4orf6/7 outperforming other treatments (**Fig. 4d, e**). By comparing the percentage of GFP^+ cells to the fraction of HDR-edited alleles, we found that GSE56 tended to increase the fraction of biallelic HDR-editing (**Extended Data Fig. 4d**). To further investigate long-term repopulation capacity of

edited HSPCs, we performed secondary transplant (from one primary transplantation experiment) by purifying and pooling human CD34$^+$ cells from BM of primary recipients (**Extended Data Fig. 4a**). Results confirmed higher and stable fraction of HDR-edited cells when using the GSE56/Ad5-E4orf6/7 combination (**Fig. 4f, g**). This combination outperformed other treatments even when editing mPB-derived HSPCs, with an average 35% GFP$^+$ cells in long-term human PBMCs (**Fig. 4h, i**), or when targeting *IL2RG* in CB HSPCs (**Figure 4j** and **Extended Data Fig. 4e**).

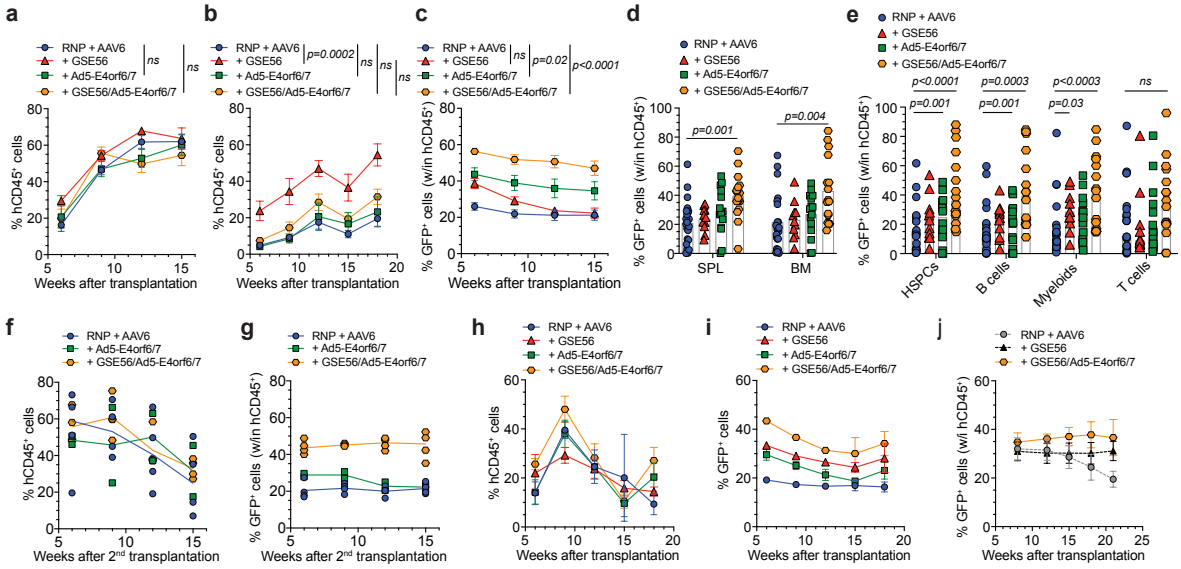


Figure 4. Editing enhancers enable high proportion of HDR-edited HSPCs and stable reconstitution in xenograft model. a-b, Percentage of circulating hCD45$^+$ cells in mice transplanted with the outgrown progeny of starting-matched saturating (**a**) (n = 11, 4, 7, 9) or limiting (**b**) (n = 12, 7, 8, 7) doses of CB HSPCs edited in *AAVS1* with indicated treatments. Each panel is a pool of two independent experiments. Mean ± SEM. Statistics are shown for the last timepoint. **c**, Percentage of GFP$^+$ cells within human graft in mice from Fig. 4a, b (n = 23, 11, 15, 16). Mean ± SEM. Statistics are shown for the last timepoint. **d-e**, Percentage of GFP$^+$ cells within human graft in hematopoietic organs (**d**) and lineages (**e**) of mice from Fig. 4a, b (n = 23, 11, 15, 16). Mean ± SEM. **f-g**, Percentage of circulating hCD45$^+$ cells (**f**) and GFP$^+$ cells within human graft (**g**) in secondary recipients transplanted with human BM-derived CD34$^+$ cells harvested from mice of one experiment in Fig. 4a (n = 4, 2, 4). Median. **h-i**, Percentage of circulating hCD45$^+$ (**h**) and GFP$^+$ cells within human graft (**i**) in mice transplanted with the outgrown progeny of starting-matched saturating doses of mPB HSPCs edited in *AAVS1* with

indicated treatments (n = 3, 5, 5, 5). Mean ± SEM. **j**, Percentage of GFP^+ cells within human graft in mice transplanted with CB HSPCs edited in *IL2RG* with editing enhancers (n = 4). Comparison with previously published results for "RNP + AAV6" and "+ GSE56"[22] is shown (n = 5, 6). Mean ± SEM. For all panels with statistical analyses: LME followed by post-hoc analysis. All statistical tests are two-tailed. n indicate independent animals.

Enhanced gene editing supports polyclonal human graft without perturbing clonal behavior

We then assessed clonal composition and dynamics of host repopulation by enhancer edited HSPCs. Addition of Ad5-E4orf6/7 when editing cells with standard or GSE56-comprising protocols, while increasing the proportion of HDR-edited cells (see Fig. 4c), did not significantly increase the number of dominant BARs compared to respective controls in limiting dose experiments (**Fig. 5a**). This finding is consistent with the lower human engraftment obtained with the GSE56/Ad5-E4orf6/7 combination as compared to GSE56 alone (see Fig. 4b). Polyclonal reconstitution after GSE56/Ad5-E4orf6/7 treatment was also confirmed by analyzing PBMCs in the saturating-dose experiment (**Fig. 5b**). The clonality of HDR-edited cells decreased over time independently from the treatment, as also noted in previous experiments (see Fig. 1h, k), possibly reflecting exhaustion of short-term progenitors. The number of dominant BARs correlated with the percentage of edited cells in PB of recipient mice at early and late timepoints (**Fig. 5c**), confirming that our treatments did not markedly alter the average clonal output of repopulating HSPCs. Enhanced polyclonal composition was confirmed in B and myeloid cell compartments of GSE56/Ad5-E4orf6/7 mice, while T cells remained oligoclonal (**Fig. 5d** and **Extended Data Fig. 5a, b**), suggesting constraints of the model rather than the editing treatment. At the end of the experiment, the majority of dominant repopulating clones showed multilineage output. Few dominant BARs were shared across repopulated mice also upon enhanced editing, indicating the likely occurrence of *ex vivo* duplication of HDR-edited HSPCs (**Extended Data Fig. 5a**). Of note, comparable number of BARs were retrieved in mice transplanted with saturating doses of HSPCs edited in presence or absence of GSE56, further indicating that GSE56 treatment does not alter the growth properties of repopulating HSPC (**Extended Data Fig. 5c**).

To assess the impact of the editing procedure also on HSPCs undergoing NHEJ-mediated repair of DNA DSBs, we deep sequenced the *AAVS1* locus in the long-term human graft and measured indel diversity (Clement *et al*, 2019). GSE56 increased the number of unique indels,

while Ad5-E4orf6/7 protein *per se* did not affect indel diversity (**Fig. 5e-g**). Moreover, the fraction of NHEJ-edited alleles within the non-HDR edited subset was tendentially higher in presence of GSE56 (**Fig. 5h** and **Extended Data Fig. 5d**). These findings support the contention that editing-induced DDR shrinks clonal repertoire of the edited human graft independently of the pathway engaged for DNA DSB repair and that our enhanced editing protocol rescues its polyclonal composition.

HDR-edited LT-HSCs perform symmetrical and asymmetrical divisions in xenotransplantation settings

To assess self-renewal and clonal dynamics of HDR-edited HSPCs, we performed clonal tracking on secondary transplanted mice from Fig. 4f, g. We observed a strong contraction in the total number of dominant clones in PB of secondary recipients, uncovering a "bottleneck" effect during engraftment of human HSPCs. About 80% of dominant BARs were recaptured from those retrieved in PBMC at long term in primary recipients, while remaining BARs were either identified as dominant within sorted cell lineages or within the "rare" BAR populations (**Fig. 5i, j**). These results confirmed that individual HDR-edited LT-HSCs retain self-renewing capacity in serial transplantation. Remarkably, ~44% of the BARs identified in secondary recipients where shared among different mice (**Fig. 5k**), suggesting that some HDR-edited HSPCs underwent symmetric self-renewing divisions in primary recipients. These clones robustly contributed to hematopoietic lineages and were present within $CD34^+$ progenitors in the BM (**Extended Data Fig. 5e**). Overall, these data provide stringent evidence at single cell level that human HDR-edited HSPCs are able to perform symmetric and asymmetric divisions long term after transplantation.

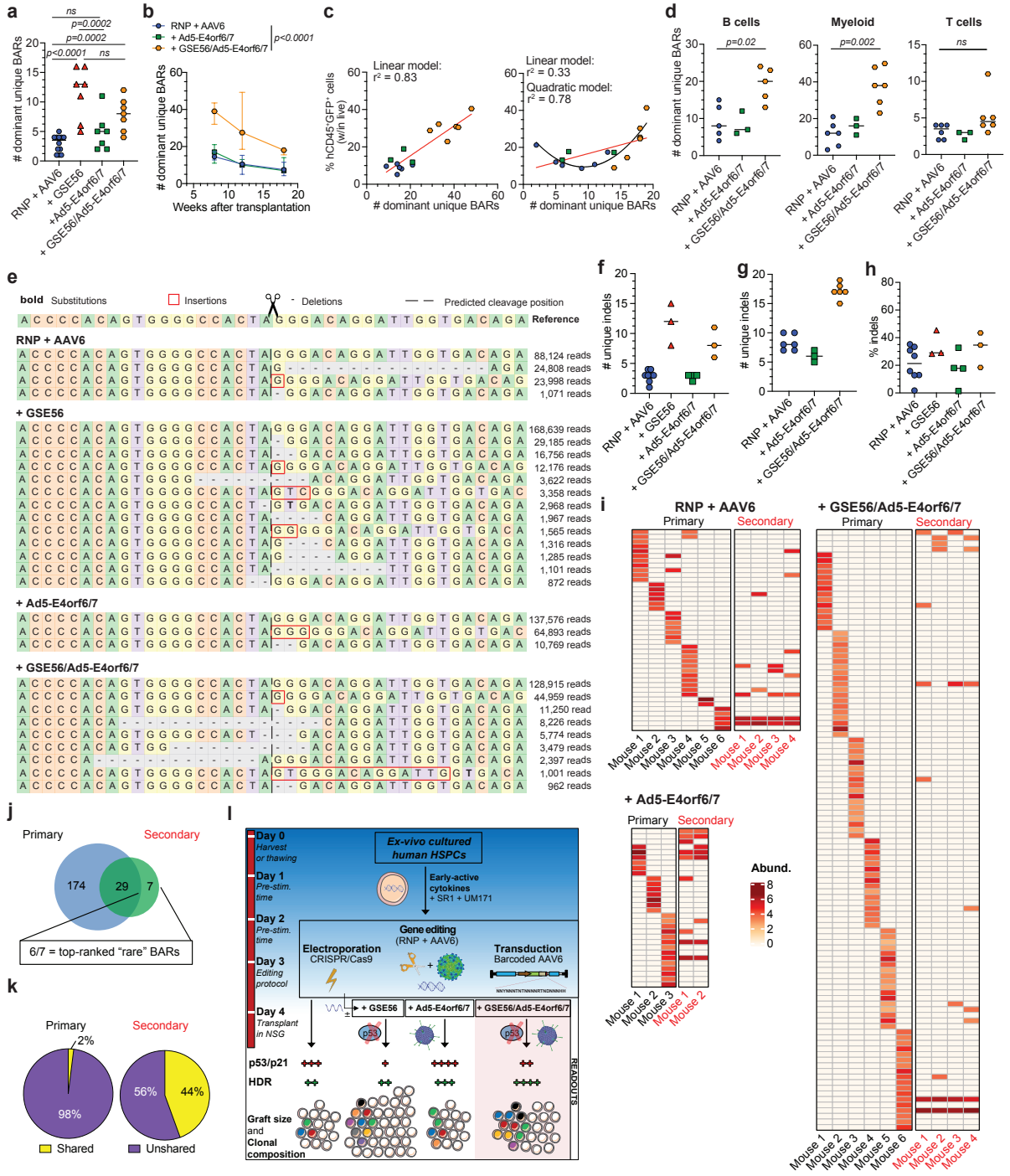


Figure 5. Editing enhancers allow polyclonal composition of the human edited graft without perturbing clonal dynamics. a, Number of dominant unique BARs in human splenocytes of mice in Fig. 4b (n = 12, 7, 7, 7). Median. GLMER for count data. **b**, Longitudinal PBMC analysis showing the number of dominant unique BARs in mice from one experiment in Fig. 4a (n = 6, 3, 6). Median with IQR. GLMER for count data. **c**, Linear/quadratic regression showing the relationship between the number of dominant unique BARs from Fig. 5b and the percentage of hCD45^{+}GFP^{+} cells at the 8 weeks (left) and 18 weeks (right) (n = 6, 3, 6). **d**, Number

of dominant unique BARs in sorted hCD45^{+} cell lineages of mice from one experiment in Fig. 4a (n = 6, 3, 6). Median. Mann-Whitney test. **e**, Deep sequencing analysis of *AAVS1* in human edited splenocytes from one experiment in Fig. 4b. Dashed line indicates Cas9 cleavage site. The reference wild type allele and representative plots for one mouse/group are shown. **f-g**, Number of unique indels in human splenocytes of mice from one experiment at starting-matched limiting (**f**) (n = 8, 3, 4, 3) and saturating (**g**) (n = 6, 3, 6) HSPC doses. Median. **h**, Percentage of NHEJ-edited alleles within the non-HDR edited fraction from Fig. 5f (n = 8, 3, 4, 3). Median. **i**, Heatmaps showing dominant unique BARs (rows) and relative abundances in PBMCs of primary (18 weeks) and secondary (9 weeks) transplant from Fig. 4a, f. **j**, Venn diagram showing the number of dominant unique BARs shared between PBMCs of primary and secondary recipients. **k**, Pie charts showing the percentage of shared/unshared BARs in primary and secondary recipients. Fisher's exact test ($p<0.0001$). **l)** Schematic summary of the editing strategies and their outcomes in HSPCs. All statistical tests are two-tailed. n indicates independent animals.

DISCUSSION

Our findings elucidate and overcome two major biological barriers to efficient HDR-mediated gene editing in HSPC and show by clonal tracking that our enhanced editing protocol preserves their multilineage and self-renewal capacity long term after serial transplant (see schematic in **Fig. 5l**).

The substantially lower number of repopulating HSPC clones well explains the lower human engraftment reported after transplanting edited vs. untreated cells (Schiroli *et al*, 2019). The mechanism underlying this loss remains to be fully understood, although the robust activation of p53 pathway and its downstream effectors, such as p21, p14 and p16 suggests induction of detrimental processes like permanent growth arrest, senescence and apoptosis (Milyavsky *et al*, 2010). Although we measured some increase in apoptosis among treated HSPCs, its extent was limited and could not explain the several-fold loss in engrafting clones. Because clonal dynamics was not different among all treatments, there might be a threshold of p53 activation(van den Berg *et al*, 2018) leading to all or none outcome when LT-HSCs are treated for editing, *i.e.* full preservation or irreversible loss of repopulation potential. In support of this hypothesis are the increased indels frequency, diversity and biallelic HDR targeting when GSE56 is added to the treatment, suggesting preferential rescue of cells undergoing higher DDR burden from multiple DNA DSBs and/or increased template uptake. It should be mentioned that our clonal dynamics analysis could not investigate quiescence and short-lived progenitors providing limited output and was limited to dominant clones within the edited cell graft. However, if we consider that dominant clones accounted for 1 every $2x10^3$-$2x10^4$ edited $CD34^+$ cells throughout our study, such frequency is consistent with previous estimates of SCID-repopulating cells in cultured CB $CD34^+$ cells assayed by limiting dilution transplantation (Wang *et al*, 1997; Zonari *et al*, 2017; Wagenblast *et al*, 2019; Bai *et al*, 2019), suggesting preservation of the normal repopulation capacity by individual HDR/NHEJ-edited HSPC.

Our data clearly show that cell cycle regulation represents a fundamental rate-limiting step for HDR-editing in HSPCs. However, despite the fraction of cells in S/G2 was similar at the time of editing between bulk and $CD90^+$ HSPCs, HDR efficiency was always lower in the latter cells, as previously reported (Hoban *et al*, 2015; Schiroli *et al*, 2019). This observation suggests that, beside the requirement for progression to S/G2, other factors account for lower HDR efficiency in $CD90^+$ cells, such as low expression and activity of HDR machinery (Beerman *et al*, 2014; Schiroli *et al*, 2019) and delayed transit through the G1/S checkpoint (Laurenti *et al*, 2015), which receives multiple inputs to adjust metabolic regulation of growth rate to cell size

and cell cycle progression. Ad5-E4orf6/7 is known to bind and stably recruit active E2F transcription factors to the adenoviral E2 and cellular E2F-1 promoters and activate downstream gene expression (Schaley *et al*, 2000, 2005). Transient expression of Ad5-E4orf6/7 in HSPCs triggered an E2F-driven pleiotropic response coupling promotion of G1/S transition (Stanelle *et al*, 2002) and enhanced expression of HDR machinery, which increased HDR efficiency preferentially in the most primitive cells. Such pervasive modulation of highly integrated cellular networks by a viral protein naturally evolved to capture the benefits of cell proliferation for viral infection might be difficult to replicate with small drugs or other strategies targeting individual genes engaged in the process.

Notably, the HDR increase by Ad5-E4orf6/7 was further enhanced by combination with p53 inhibition, which can be explained by counteracting the p21 and p14 mediated negative feedback triggered by E2F activation, a previously reported finding (Komori *et al*, 2005; Radhakrishnan *et al*, 2004) also shown by our data. This feedback might also explain why Ad5-E4orf6/7 did not increase engraftment of standard edited cells and lowered the GSE56-dependent graft increase. Of note, the number of clones upon Ad5-E4orf6/7 addition might be underestimated if upregulation of the HDR machinery increased the proportion of cells undergoing template integration before replication of the targeted locus, thus producing two clones with the same BAR. Intriguingly, granular inspection of Fig. 5c shows that Ad5-E4orf6/7-treated long-term engrafting clones tend to have higher output than their experimental counterparts, as shown by better fit of data to a quadratic regression model. The decreased percentage and MFI of $CD90^{+}$ cells upon Ad5-E4orf6/7 treatment is likely due to the observed transcriptional downregulation of the *CD90* gene rather than differentiation. This proposition is further supported by the observation that other LT-HSC markers, such as CD133 (*PROM1*) (Yin *et al*, 1997), CD49f (*ITGA6*) (Notta *et al*, 2011) and CD201 (*EPCR*) (Fares *et al*, 2017), were not downregulated by Ad5-E4orf6/7 treatments.

The detrimental effects of p53 activation might confer selective advantage to rare $p53^{-/-}$ cells (Haapaniemi *et al*, 2018; Ihry *et al*, 2018). Limited and transient inhibition of the editing-induced p53 response would reduce the risk of selecting for p53 mutant clones and mono/oligoclonal expansion. Robust p53-dependent transcriptional activation of the DNA cytidine deaminase *APOBEC3H* upon editing (Schiroli *et al*, 2019) might also raise concerns for mutagenesis targeting single-stranded genomic DNA intermediates during repair, replication and transcription (Sakofsky *et al*, 2019). GSE56 and Ad5-E4orf6/7 together nearly abolished *APOBEC3H* induction, thus potentially protecting edited cells from a further source of genotoxicity. Importantly, the use of mRNA for transient expression of the p53 inhibitor and Ad5-E4orf6/7 rules

out the risk of genomic integration of these potentially transforming factors. As first readout of genomic alterations, we did not detect increased occurrence by single or combined addition of GSE56 and Ad5-E4orf6/7 of a chromosomal translocation specifically traceable to the activity of an editing nuclease.

An unexpected benefit of Ad5-E4orf6/7 treatment is the downregulation of some immune response/chemokine genes, which may contribute to the immune evasive strategy of the parental virus. This response might also decrease the risk of antigen presentation and immune effector recruitment by the administered HSPCs, which shortly after editing still contain immunogenic proteins of bacterial and viral origin, such as Cas nuclease and AAV capsid proteins.

Overall, the gains in clonal repertoire and percentage of edited HSPCs obtained by our enhanced protocol are relevant for clinical translation. Indeed, oligoclonal composition might delay hematopoietic recovery after conditioning and limit the size, long-term stability and safety of the engineered cell graft. Moreover, the higher the proportion of HDR-edited cells in the cell product the less is the competition with unedited and residual HSPCs in the host to achieve sufficient chimerism for therapeutic benefit. These benefits may well balance the inherent risk of first-in-human clinical testing in suitable disease contexts, such as primary immunodeficiencies, where HSPC gene editing may eventually provide effective treatment.

REFERENCES

Abarrategi A, Foster K, Hamilton A, Mian SA, Passaro D, Gribben J, Mufti G & Bonnet D (2017) Versatile humanized niche model enables study of normal and malignant human hematopoiesis. *J Clin Invest*

Abbott A (1992) Italians first to use stem cells. *Nature* doi:10.1038/356465a0

Abraham A, Hsieh M, Eapen M, Fitzhugh C, Carreras J, Keesler D, Guilcher G, Kamani N, Walters MC, Boelens JJ, *et al* (2017) Relationship between Mixed Donor–Recipient Chimerism and Disease Recurrence after Hematopoietic Cell Transplantation for Sickle Cell Disease. *Biol Blood Marrow Transplant*

Adikusuma F, Piltz S, Corbett MA, Turvey M, McColl SR, Helbig KJ, Beard MR, Hughes J, Pomerantz RT & Thomas PQ (2018) Large deletions induced by Cas9 cleavage. *Nature*

Aiuti A, Slavin S, Aker M, Ficara F, Deola S, Mortellaro A, Morecki S, Andolfi G, Tabucchi A, Carlucci F, *et al* (2002) Correction of ADA-SCID by stem cell gene therapy combined with nonmyeloablative conditioning. *Science (80-)*

Akashi K, Traver D, Miyamoto T & Weissman IL (2000) A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature*

Akinsheye I, Alsultan A, Solovieff N, Ngo D, Baldwin CT, Sebastiani P, Chui DHK & Steinberg MH (2011) Fetal hemoglobin in sickle cell anemia. *Blood* doi:10.1182/blood-2011-03-325258

Aleem E, Kiyokawa H & Kaldis P (2005) Cdc2-cyclin E complexes regulate the G1/S phase transition. *Nat Cell Biol*

Allers K, Hütter G, Hofmann J, Loddenkemper C, Rieger K, Thiel E & Schneider T (2011) Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. *Blood*

Ara T, Tokoyoda K, Sugiyama T, Egawa T, Kawabata K & Nagasawa T (2003) Long-term hematopoietic stem cells require stromal cell-derived factor-1 for colonizing bone marrow during ontogeny. *Immunity*

Arnold SD, Bhatia M, Horan J & Krishnamurti L (2016) Haematopoietic stem cell transplantation for sickle cell disease – current practice and new approaches. *Br J Haematol* doi:10.1111/bjh.14167

Ataga KI, Kutlar A, Kanter J, Liles D, Cancado R, Friedrisch J, Guthrie TH, Knight-Madden J, Alvarez OA, Gordeuk VR, *et al* (2017) Crizanlizumab for the prevention of pain crises in sickle cell disease. *N Engl J Med*

Bai T, Li J, Sinclair A, Imren S, Merriam F, Sun F, O'Kelly MB, Nourigat C, Jain P, Delrow JJ, *et al* (2019) Expansion of primitive human hematopoietic stem cells by culture in a zwitterionic hydrogel. *Nat Med*

Bates D, Mächler M, Bolker BM & Walker SC (2015) Fitting linear mixed-effects models using lme4. *J Stat Softw*

Bauer DE, Kamran SC, Lessard S, Xu J, Fujiwara Y, Lin C, Shao Z, Canver MC, Smith EC, Pinello L, *et al* (2013) An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level. *Science (80-)*

Baum CM, Weissman IL, Tsukamoto AS, Buckle AM & Peault B (1992) Isolation of a candidate human hematopoietic stem-cell population. *Proc Natl Acad Sci U S A* 89: 2804–2808

Beerman I, Seita J, Inlay MA, Weissman IL & Rossi DJ (2014) Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. *Cell Stem Cell*

Benveniste P, Frelin C, Janmohamed S, Barbara M, Herrington R, Hyam D & Iscove NN (2010) Intermediate-Term Hematopoietic Stem Cells with Extended but Time-Limited Reconstitution Potential. *Cell Stem Cell* 6: 48–58

Berenson RJ, Andrews RG, Bensinger WI, Kalamasz D, Knitter G, Buckner CD & Bernstein ID (1988) Antigen CD34+ marrow cells engraft lethally irradiated baboons. *J Clin Invest* 81: 951–955

van den Berg J, Manjón AG, Kielbassa K, Feringa FM, Freire R & Medema RH (2018) A limited number of double-strand DNA breaks is sufficient to delay cell cycle progression. *Nucleic Acids Res*

Bernardo ME & Aiuti A (2016) The Role of Conditioning in Hematopoietic Stem-Cell Gene Therapy. *Hum Gene Ther*

Bernitz JM, Kim HS, MacArthur B, Sieburg H & Moore K (2016) Hematopoietic Stem Cells Count and Remember Self-Renewal Divisions. *Cell* 167: 1296-1309.e10

Bhatia M, Wang JCY, Kapp U, Bonnet D & Dick JE (1997) Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. *Proc Natl Acad Sci U S A* 94: 5320–5325

Bibikova M, Carroll D, Segal DJ, Trautman JK, Smith J, Kim Y-G & Chandrasegaran S (2001) Stimulation of Homologous Recombination through Targeted Cleavage by Chimeric Nucleases. *Mol Cell Biol*

Biechonski S, Olender L, Zipin-Roitman A, Yassin M, Aqaqe N, Marcu-Malina V, Rall-Scharpf M, Trottier M, Meyn MS, Wiesmüller L, *et al* (2018) Attenuated DNA damage responses and increased apoptosis characterize human hematopoietic stem cells exposed to irradiation. *Sci Rep* 8

Bitinaite J, Wah DA, Aggarwal AK & Schildkraut I (1998) Foki dimerization is required for dna cleavage. *Proc Natl Acad Sci U S A* 95: 10570–10575

Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, Shearer G, Chang L, Chiang Y, Tolstoshev P, *et al* (1995) T lymphocyte-directed gene therapy for ADA-SCID: Initial trial results after 4 years. *Science (80-)*

Boitano AE, Wang J, Romeo R, Bouchez LC, Parker AE, Sutton SE, Walker JR, Flaveny CA, Perdew GH, Denison MS, *et al* (2010) Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. *Science (80-)*

Borducchi EN, Liu J, Nkolola JP, Cadena AM, Yu WH, Fischinger S, Broge T, Abbink P, Mercado NB, Chandrashekar A, *et al* (2018) Antibody and TLR7 agonist delay viral rebound in SHIV-infected monkeys. *Nature*

Bothmer A, Robbiani DF, Feldhahn N, Gazumyan A, Nussenzweig A & Nussenzweig MC (2010) 53BP1 regulates DNA resection and the choice between classical and alternative end joining during class switch recombination. *J Exp Med* 207: 855–865

Boulais PE & Frenette PS (2015) Making sense of hematopoietic stem cell niches. *Blood*

Braun CJ, Boztug K, Paruzynski A, Witzel M, Schwarzer A, Rothe M, Modlich U, Beier R, Göhring G, Steinemann D, *et al* (2014) Gene therapy for Wiskott-Aldrich syndrome-long-term efficacy and genotoxicity. *Sci Transl Med*

Buckley RH (2011) Transplantation of hematopoietic stem cells in human severe combined immunodeficiency: Longterm outcomes. *Immunol Res* doi:10.1007/s12026-010-8191-9

Bunting SF, Callén E, Wong N, Chen HT, Polato F, Gunn A, Bothmer A, Feldhahn N, Fernandez-Capetillo O, Cao L, *et al* (2010) 53BP1 inhibits homologous recombination in brca1-deficient cells by blocking resection of DNA breaks. *Cell* 141: 243–254

Burma S, Chen BPC & Chen DJ (2006) Role of non-homologous end joining (NHEJ) in maintaining genomic integrity. *DNA Repair (Amst)* 5: 1042–1048 doi:10.1016/j.dnarep.2006.05.026

Cameron P, Fuller CK, Donohoue PD, Jones BN, Thompson MS, Carter MM, Gradia S, Vidal B, Garner E, Slorach EM, *et al* (2017) Mapping the genomic landscape of CRISPR-Cas9 cleavage. *Nat Methods* 14: 600–606

Cannan WJ & Pederson DS (2016) Mechanisms and Consequences of Double-Strand DNA

Break Formation in Chromatin. *J Cell Physiol* doi:10.1002/jcp.25048
Canny MD, Moatti N, Wan LCK, Fradet-Turcotte A, Krasner D, Mateos-Gomez PA, Zimmermann M, Orthwein A, Juang Y-C, Zhang W, *et al* (2017) Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR–Cas9 genome-editing efficiency. *Nat Biotechnol*
Carroll D (2014) Genome Engineering with Targetable Nucleases. *Annu Rev Biochem*
Carter CA & Ehrlich LS (2008) Cell biology of HIV-1 infection of macrophages. *Annu Rev Microbiol* doi:10.1146/annurev.micro.62.081307.162758
Casini A, Olivieri M, Petris G, Montagna C, Reginato G, Maule G, Lorenzin F, Prandi D, Romanel A, Demichelis F, *et al* (2018) A highly specific SpCas9 variant is identified by in vivo screening in yeast. *Nat Biotechnol*
Cathomen T & Keith Joung J (2008) Zinc-finger nucleases: The next generation emerges. *Mol Ther* 16: 1200–1207 doi:10.1038/mt.2008.114
Cavazza A, Moiani A & Mavilio F (2013) Mechanisms of retroviral integration and mutagenesis. *Hum Gene Ther* doi:10.1089/hum.2012.203
Cavazzana-Calvo M, Hacein-Bey S, De Saint Basile G, Gross F, Yvon E, Nusbaum P, Selz F, Hue C, Certain S, Casanova JL, *et al* (2000) Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. *Science (80-)*
Cavazzana-Calvo M, Thrasher A & Mavillo F (2004) The future of gene therapy. *Nature* doi:10.1038/427779a
Cavazzana M, Six E, Lagresle-Peyrou C, André-Schmutz I & Hacein-Bey-Abina S (2016) Gene Therapy for X-Linked Severe Combined Immunodeficiency: Where Do We Stand? *Hum Gene Ther*
Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia N V., Bogdanove AJ & Voytas DF (2011) Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. *Nucleic Acids Res* 39
Chad M, Rivella S, Callegari J, Heller G, Gaensler KML, Luzzatto L & Sadelain M (2000) Therapeutic haemoglobin synthesis in β-thalassaemic mice expressing lentivirus-encoded human β-globin. *Nature*
Chang HHY, Pannunzio NR, Adachi N & Lieber MR (2017) Non-homologous DNA end joining and alternative pathways to double-strand break repair. *Nat Rev Mol Cell Biol* doi:10.1038/nrm.2017.48
Chang HHY, Watanabe G & Lieber MR (2015) Unifying the DNA end-processing roles of the artemis nuclease: Ku-dependentartemis resection atbluntdnaends. *J Biol Chem*
Charlesworth CT, Camarena J, Cromer MK, Vaidyanathan S, Bak RO, Carte JM, Potter J, Dever DP & Porteus MH (2018) Priming Human Repopulating Hematopoietic Stem and Progenitor Cells for Cas9/sgRNA Gene Targeting. *Mol Ther - Nucleic Acids*
Charpentier M, Khedher AHY, Menoret S, Brion A, Lamribet K, Dardillac E, Boix C, Perrouault L, Tesson L, Geny S, *et al* (2018) CtIP fusion to Cas9 enhances transgene integration by homology-dependent repair. *Nat Commun*
Chatterjee N & Walker GC (2017) Mechanisms of DNA damage, repair, and mutagenesis. *Environ Mol Mutagen* 58: 235–263 doi:10.1002/em.22087
Chen F, Pruett-Miller SM & Davis GD (2015) Gene editing using ssODNs with engineered endonucleases. *Methods Mol Biol*
Chen Z, Yang H & Pavletich NP (2008) Mechanism of homologous recombination from the RecA-ssDNA/dsDNA structures. *Nature* 453: 489–494
Cheng H, Zheng Z & Cheng T (2020) New paradigms on hematopoietic stem cell differentiation. *Protein Cell* 11: 34–44 doi:10.1007/s13238-019-0633-0
Cho SW, Kim S, Kim Y, Kweon J, Kim HS, Bae S & Kim JS (2014) Analysis of off-target effects of CRISPR/Cas-derived RNA-guided endonucleases and nickases. *Genome Res*

Chu VT, Weber T, Wefers B, Wurst W, Sander S, Rajewsky K & Kühn R (2015) Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat Biotechnol* 33: 543–548

Cicalese MP, Ferrua F, Castagnaro L, Pajno R, Barzaghi F, Giannelli S, Dionisio F, Brigida I, Bonopane M, Casiraghi M, *et al* (2016) Update on the safety and efficacy of retroviral gene therapy for immunodeficiency due to adenosine deaminase deficiency. *Blood*

Cieri N, Camisa B, Cocchiarella F, Forcato M, Oliveira G, Provasi E, Bondanza A, Bordignon C, Peccatori J, Ciceri F, *et al* (2013) IL-7 and IL-15 instruct the generation of human memory stem T cells from naive precursors. *Blood*

Civin CI, Strauss LC, Brovall C, Fackler MJ, Schwartz JF & Shaper JH (1984) Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J Immunol* 133: 157–65

Clement K, Rees H, Canver MC, Gehrke JM, Farouni R, Hsu JY, Cole MA, Liu DR, Joung JK, Bauer DE, *et al* (2019) CRISPResso2 provides accurate and rapid genome editing sequence analysis. *Nat Biotechnol* doi:10.1038/s41587-019-0032-3

Cole LJ, Habermeyer JG & Bond VP (1955) Recovery from acute radiation injury in mice following administration of rat bone marrow. *J Natl Cancer Inst*

Conti A & Di Micco R (2018) p53 activation: A checkpoint for precision genome editing? *Genome Med* doi:10.1186/s13073-018-0578-6

Copelan EA (2006) Hematopoietic stem-cell transplantation. *N Engl J Med* 354: 1813–1826 doi:10.1056/NEJMra052638

Copley MR, Beer PA & Eaves CJ (2012) Hematopoietic stem cell heterogeneity takes center stage. *Cell Stem Cell* 10: 690–697 doi:10.1016/j.stem.2012.05.006

Del Core Luca, Montini Eugenio, Di Serio Clelia CA (2018) Dealing with Data Evolution and Data Integration: An approach using Rarefaction. In *49th Scientific meeting of the Italian Statistical Society*

Cromer MK, Vaidyanathan S, Ryan DE, Curry B, Lucas AB, Camarena J, Kaushik M, Hay SR, Martin RM, Steinfeld I, *et al* (2018) Global Transcriptional Response to CRISPR/Cas9-AAV6-Based Genome Editing in CD34+ Hematopoietic Stem and Progenitor Cells. *Mol Ther*

Cullot G, Boutin J, Toutain J, Prat F, Pennamen P, Rooryck C, Teichmann M, Rousseau E, Lamrissi-Garcia I, Guyonnet-Duperat V, *et al* (2019) CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. *Nat Commun*

Dausset J, Colombani J & Evelin J (1958) Présence de l'antigène Rh (D) dans les leucocytes et les plaquettes humaines. *Vox Sang*

Davis AJ, Chen BPC & Chen DJ (2014) DNA-PK: A dynamic enzyme in a versatile DSB repair pathway. *DNA Repair (Amst)*

Deltcheva E, Chylinski K, Sharma CM, Gonzales K, Chao Y, Pirzada ZA, Eckert MR, Vogel J & Charpentier E (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. *Nature* 471: 602–607

Dever DP, Bak RO, Reinisch A, Camarena J, Washington G, Nicolas CE, Pavel-Dinu M, Saxena N, Wilkens AB, Mantri S, *et al* (2016) CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. *Nature* 539: 384–389

DeWitt MA, Magis W, Bray NL, Wang T, Berman JR, Urbinati F, Heo SJ, Mitros T, Muñoz DP, Boffelli D, *et al* (2016) Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. *Sci Transl Med*

Didigu CA, Wilen CB, Wang J, Duong J, Secreto AJ, Danet-Desnoyers GA, Riley JL, Gregory PD, June CH, Holmes MC, *et al* (2014) Simultaneous zinc-finger nuclease editing of the HIV coreceptors ccr5 and cxcr4 protects CD4+ T cells from HIV-1 infection. *Blood*

Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K & Sadelain M (2018) Gene therapy comes of age. *Science (80-)* doi:10.1126/science.aan4672

Enache OM, Rendo V, Abdusamad M, Lam D, Davison D, Pal S, Currimjee N, Hess J, Pantel S, Nag A, *et al* (2020) Cas9 activates the p53 pathway and selects for p53-inactivating mutations. *Nat Genet*

Essers MAG, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA & Trumpp A (2009) IFNα activates dormant haematopoietic stem cells in vivo. *Nature*

Fares I, Chagraoui J, Gareau Y, Gingras S, Ruel R, Mayotte N, Csaszar E, Knapp DJHFHF, Miller P, Ngom M, *et al* (2014) Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal. *Science (80-)* 345: 1509–1512

Fares I, Chagraoui J, Lehnertz B, MacRae T, Mayotte N, Tomellini E, Aubert L, Roux PP & Sauvageau G (2017) EPCR expression marks UM171-expanded CD34 + cord blood stem cells. *Blood*: blood-2016-11-750729

Ferrari S, Jacob A, Beretta S, Unali G, Albano L, Vavassori V, Cittaro D, Lazarevic D, Brombin C, Cugnata F, *et al* (2020) Efficient gene editing of human long-term hematopoietic stem cells validated by clonal tracking. *Nat Biotechnol*

Ferrua F, Cicalese MP, Galimberti S, Giannelli S, Dionisio F, Barzaghi F, Migliavacca M, Bernardo ME, Calbi V, Assanelli AA, *et al* (2019) Lentiviral haemopoietic stem/progenitor cell gene therapy for treatment of Wiskott-Aldrich syndrome: interim results of a non-randomised, open-label, phase 1/2 clinical study. *Lancet Haematol*

Fitzhugh CD, Hsieh MM, Bolan CD, Saenz C & Tisdale JF (2009) Granulocyte colony-stimulating factor (G-CSF) administration in individuals with sickle cell disease: Time for a moratorium? *Cytotherapy*

Frese KK, Lee SS, Thomas DL, Latorre IJ, Weiss RS, Glaunsinger BA & Javier RT (2003) Selective PDZ protein-dependent stimulation of phosphatidylinositol 3-kinase by the adenovirus E4-ORF1 oncoprotein. *Oncogene*

Friedmann T & Roblin R (1972) Gene therapy for human genetic disease? *Science (80-)*

Frock RL, Hu J, Meyers RM, Ho YJ, Kii E & Alt FW (2015) Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases. *Nat Biotechnol*

Fu Y, Sander JD, Reyon D, Cascio VM & Joung JK (2014) Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. *Nat Biotechnol*

Gabriel R, Lombardo A, Arens A, Miller JC, Genovese P, Kaeppel C, Nowrouzi A, Bartholomae CC, Wang J, Friedman G, *et al* (2011) An unbiased genome-wide analysis of zinc-finger nuclease specificity. *Nat Biotechnol*

Gaj T, Gersbach CA & Barbas CF (2013) ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 31: 397–405 doi:10.1016/j.tibtech.2013.04.004

Ganor Y, Real F, Sennepin A, Dutertre CA, Prevedel L, Xu L, Tudor D, Charmeteau B, Couedel-Courteille A, Marion S, *et al* (2019) HIV-1 reservoirs in urethral macrophages of patients under suppressive antiretroviral therapy. *Nat Microbiol*

Gatti RA, Meuwissen HJ, Allen HD, Hong R & Good RA (1968) Immunological reconstitution of sex-linked lymphopenic immunological deficiency. *Lancet*

Genovese P, Schiroli G, Escobar G, Di Tomaso T, Firrito C, Calabria A, Moi D, Mazzieri R, Bonini C, Holmes MC, *et al* (2014) Targeted genome editing in human repopulating haematopoietic stem cells. *Nature* 510: 235–240

Gersbach CA (2014) Genome engineering: The next genomic revolution. *Nat Methods* 11: 1009–1011

Ginn SL, Liao SH, Dane AP, Hu M, Hyman J, Finnie JW, Zheng M, Cavazzana-Calvo M, Alexander SI, Thrasher AJ, *et al* (2010) Lymphomagenesis in SCID-X1 mice following lentivirus-mediated phenotype correction independent of insertional mutagenesis and γc overexpression. *Mol Ther*

Greiner DL, Shultz LD, Yates J, Appel MC, Perdrizet G, Hesselton RM, Schweitzer I, Beamer WG, Shultz KL, Pelsue SC, *et al* (1995) Improved engraftment of human spleen cells in

NOD/LtSz-scid/scid mice as compared with C.B-17-scid/scid mice. *Am J Pathol* 146: 888–902

Le Guiner C, Servais L, Montus M, Larcher T, Fraysse B, Moullec S, Allais M, François V, Dutilleul M, Malerba A, *et al* (2017) Long-term microdystrophin gene therapy is effective in a canine model of Duchenne muscular dystrophy. *Nat Commun*

Gupta RK, Abdul-Jawad S, McCoy LE, Mok HP, Peppa D, Salgado M, Martinez-Picado J, Nijhuis M, Wensing AMJ, Lee H, *et al* (2019) HIV-1 remission following CCR5Δ32/Δ32 haematopoietic stem-cell transplantation. *Nature*

Gupta RK, Peppa D, Hill AL, Gálvez C, Salgado M, Pace M, McCoy LE, Griffith SA, Thornhill J, Alrubayyi A, *et al* (2020) Evidence for HIV-1 cure after CCR5Δ32/Δ32 allogeneic haemopoietic stem-cell transplantation 30 months post analytical treatment interruption: a case report. *Lancet HIV*

Gutschner T, Haemmerle M, Genovese G, Draetta GF & Chin L (2016) Post-translational Regulation of Cas9 during G1 Enhances Homology-Directed Repair. *Cell Rep*

Gwiazda KS, Grier AE, Sahni J, Burleigh SM, Martin U, Yang JG, Popp NA, Krutein MC, Khan IF, Jacoby K, *et al* (2016) High Efficiency CRISPR/Cas9-mediated Gene Editing in Primary Human T-cells Using Mutant Adenoviral E4orf6/E1b55k 'Helper' Proteins. *Mol Ther* 24: 1–11

Haapaniemi E, Botla S, Persson J, Schmierer B & Taipale J (2018) CRISPR-Cas9 genome editing induces a p53-mediated DNA damage response. *Nat Med*

Hacein-Bey-Abina S, Garrigue A, Wang GP, Soulier J, Lim A, Morillon E, Clappier E, Caccavelli L, Delabesse E, Beldjord K, *et al* (2008) Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest*

Hacein-Bey-Abina S, Von Kalle C, Schmidt M, McCormack MP, Wulffraat N, Leboulch P, Lim A, Osborne CS, Pawliuk R, Morillon E, *et al* (2003) LMO2-Associated Clonal T Cell Proliferation in Two Patients after Gene Therapy for SCID-X1. *Science (80-)*

Hacein-Bey-Abina S, Pai SY, Gaspar HB, Armant M, Berry CC, Blanche S, Bleesing J, Blondeau J, De Boer H, Buckland KF, *et al* (2014) A modified γ-retrovirus vector for X-linked severe combined immunodeficiency. *N Engl J Med*

Haddad E, Logan BR, Griffith LM, Buckley RH, Parrott RE, Prockop SE, Small TN, Chaisson J, Dvorak CC, Murnane M, *et al* (2018) SCID genotype and 6-month posttransplant CD4 count predict survival and immune recovery. *Blood*

Hanawa H, Yamamoto M, Zhao H, Shimada T & Persons DA (2009) Optimized lentiviral vector design improves titer and transgene expression of vectors containing the chicken β-globin locus HS4 insulator element. *Mol Ther*

Hanlon KS, Kleinstiver BP, Garcia SP, Zaborowski MP, Volak A, Spirig SE, Muller A, Sousa AA, Tsai SQ, Bengtsson NE, *et al* (2019) High levels of AAV vector integration into CRISPR-induced DNA breaks. *Nat Commun*

Hao QL, Thiemann FT, Petersen D, Smogorzewska EM & Crooks GM (1996) Extended long-term culture reveals a highly quiescent and primitive human hematopoietic progenitor population. *Blood* 88: 3306–3313

Harrington LB, Paez-Espino D, Staahl BT, Chen JS, Ma E, Kyrpides NC & Doudna JA (2017) A thermostable Cas9 with increased lifetime in human plasma. *Nat Commun*

Hartweger H, McGuire AT, Horning M, Taylor JJ, Dosenovic P, Yost D, Gazumyan A, Seaman MS, Stamatatos L, Jankovic M, *et al* (2019) HIV-specific humoral immune responses by CRISPR/Cas9-edited B cells. *J Exp Med*

Hendel A, Bak RO, Clark JT, Kennedy AB, Ryan DE, Roy S, Steinfeld I, Lunstad BD, Kaiser RJ, Wilkens AB, *et al* (2015) Chemically modified guide RNAs enhance CRISPR-Cas genome editing in human primary cells. *Nat Biotechnol*

Hendrie PC & Russell DW (2005) Gene targeting with viral vectors. *Mol Ther*

doi:10.1016/j.ymthe.2005.04.006
Heyer WD, Ehmsen KT & Liu J (2010) Regulation of homologous recombination in eukaryotes. *Annu Rev Genet* doi:10.1146/annurev-genet-051710-150955
Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP & Butler KS (2020) Gene editing and CRISPR in the clinic: Current and future perspectives. *Biosci Rep* doi:10.1042/BSR20200127
Hirsch ML (2015) Adeno-associated virus inverted terminal repeats stimulate gene editing. *Gene Ther*
Ho AD (2005) Kinetics and symmetry of divisions of hematopoietic stem cells. *Exp Hematol* doi:10.1016/j.exphem.2004.09.004
Hoban MD, Cost GJ, Mendel MC, Romero Z, Kaufman ML, Joglekar A V., Ho M, Lumaquin D, Gray D, Lill GR, *et al* (2015) Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. *Blood*
Hoban MD, Orkin SH & Bauer DE (2016) Genetic treatment of a molecular disorder: gene therapy approaches to sickle cell disease. *Blood* doi:10.1182/blood-2015-09-618587
Hoeijmakers JHJ (2009) DNA damage, aging, and cancer
Hoggatt J, Singh P, Sampath J & Pelus LM (2009) Prostaglandin E2 enhances hematopoietic stem cell homing, survival, and proliferation. *Blood* 113: 5444–5455
Howe SJ, Mansour MR, Schwarzwaelder K, Bartholomae C, Hubank M, Kempski H, Brugman MH, Pike-Overzet K, Chatters SJ, De Ridder D, *et al* (2008) Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest*
Hsieh MM, Fitzhugh CD, Weitzel RP, Link ME, Coles WA, Zhao X, Rodgers GP, Powell JD & Tisdale JF (2014) Nonmyeloablative HLA-matched sibling allogeneic hematopoietic stem cell transplantation for severe sickle cell phenotype. *JAMA - J Am Med Assoc*
Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, *et al* (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol*
Hu JH, Miller SM, Geurts MH, Tang W, Chen L, Sun N, Zeina CM, Gao X, Rees HA, Lin Z, *et al* (2018) Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. *Nature*
Huang MM & Hearing P (1989) The adenovirus early region 4 open reading frame 6/7 protein regulates the DNA binding activity of the cellular transcription factor, E2F, through a direct complex. *Genes Dev* 3: 1699–1710
Hustedt N & Durocher D (2017) The control of DNA repair by the cell cycle. *Nat Cell Biol* doi:10.1038/ncb3452
Hutter G, Nowak D, Mossner M, Ganepola S, Müß A, Allers K, Schneider T, Hofmann J, Külcherer C, Blau O, *et al* (2009) Long-term control of HIV by CCR5 delta32/delta32 stem-cell transplantation. *N Engl J Med*
Ihry RJ, Worringer KA, Salick MR, Frias E, Ho D, Theriault K, Kommineni S, Chen J, Sondey M, Ye C, *et al* (2018) P53 inhibits CRISPR-Cas9 engineering in human pluripotent stem cells. *Nat Med*
Isalan M (2012) Zinc-finger nucleases: How to play two good hands. *Nat Methods* doi:10.1038/nmeth.1805
Ishikawa F, Yasukawa M, Lyons B, Yoshida S, Miyamoto T, Yoshimoto G, Watanabe T, Akashi K, Shultz LD & Harada M (2005) Development of functional human blood and immune systems in NOD/SCID/IL2 receptor γ chainnull mice. *Blood*
Izquierdo-Useros N, Naranjo-Gómez M, Archer J, Hatch SC, Erkizia I, Blanco J, Borràs FE, Puertas MC, Connor JH, Fernádez-Figueras MT, *et al* (2009) Capture and transfer of HIV-1 particles by mature dendritic cells converges with the exosome-dissemination pathway. *Blood*
Jacobson LO, Simmons EL, Marks EK & Eldredge JH (1951) Recovery from radiation injury.

Science (80-)
Javier RT & Rice AP (2011) Emerging Theme: Cellular PDZ Proteins as Common Targets of Pathogenic Viruses. *J Virol*
Jayavaradhan R, Pillis DM, Goodman M, Zhang F, Zhang Y, Andreassen PR & Malik P (2019) CRISPR-Cas9 fusion to dominant-negative 53BP1 enhances HDR and inhibits NHEJ specifically at Cas9 target sites. *Nat Commun*
Jenq RR & Van Den Brink MRM (2010) Allogeneic haematopoietic stem cell transplantation: Individualized stem cell and immune therapy of cancer. *Nat Rev Cancer* 10: 213–221 doi:10.1038/nrc2804
Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA & Charpentier E (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science (80-)*
Joung JK & Sander JD (2013) TALENs: A widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol* 14: 49–55 doi:10.1038/nrm3486
Kamel-Reid S & Dick JE (1988) Engraftment of immune-deficient mice with human hematopoietic stem cells. *Science (80-)* 242: 1706–1709
Kan Y, Ruis B, Takasugi T & Hendrickson EA (2017) Mechanisms of precise genome editing using oligonucleotide donors. *Genome Res*
Kanter J, Walters MC, Hsieh MM, Krishnamurti L, Kwiatkowski J, Kamble RT, von Kalle C, Kuypers FA, Cavazzana M, Leboulch P, *et al* (2016) Interim Results from a Phase 1/2 Clinical Study of Lentiglobin Gene Therapy for Severe Sickle Cell Disease. *Blood*
Karikó K, Muramatsu H, Ludwig J & Weissman D (2011) Generating the optimal mRNA for therapy: HPLC purification eliminates immune activation and improves translation of nucleoside-modified, protein-encoding mRNA. *Nucleic Acids Res*
Kim D, Bae S, Park J, Kim E, Kim S, Yu HR, Hwang J, Kim J Il & Kim JS (2015) Digenome-seq: Genome-wide profiling of CRISPR-Cas9 off-target effects in human cells. *Nat Methods* 12: 237–243
Kim D, Luk K, Wolfe SA & Kim JS (2019) Evaluating and enhancing target specificity of gene-editing nucleases and deaminases. *Annu Rev Biochem*
Kleinstiver BP, Pattanayak V, Prew MS, Tsai SQ, Nguyen NT, Zheng Z & Joung JK (2016) High-fidelity CRISPR-Cas9 nucleases with no detectable genome-wide off-target effects. *Nature*
Kobayashi H, Morikawa T, Okinaga A, Hamano F, Hashidate-Yoshida T, Watanuki S, Hishikawa D, Shindou H, Arai F, Kabe Y, *et al* (2019) Environmental Optimization Enables Maintenance of Quiescent Hematopoietic Stem Cells Ex Vivo. *Cell Rep*
Kohn DB, Booth C, Kang EM, Pai SY, Shaw KL, Santilli G, Armant M, Buckland KF, Choi U, De Ravin SS, *et al* (2020) Lentiviral gene therapy for X-linked chronic granulomatous disease. *Nat Med* doi:10.1038/s41591-019-0735-5
Komori H, Enomoto M, Nakamura M, Iwanaga R & Ohtani K (2005) Distinct E2F-mediated transcriptional program regulates p14ARF gene expression. *EMBO J*
Kondo M, Weissman IL & Akashi K (1997) Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell*
Kordelas L, Verheyen J & Esser S (2014) Shift of HIV tropism in stem-cell transplantation with CCR5 delta32 mutation. *N Engl J Med* doi:10.1056/NEJMc1405805
Korkmaz S & Altuntas F (2017) What is the role of biosimilar G-CSF agents in hematopoietic stem cell mobilization at present? *Transfus Apher Sci* 56: 795–799
Kosicki M, Tomberg K & Bradley A (2018) Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol*
Kraus AP, Koch B & Burckett L (1961) Two families showing interaction of haemoglobin c or thalassaemia with high foetal haemoglobin in adults. *Br Med J*

Kumar S & Geiger H (2017) HSC Niche Biology and HSC Expansion Ex Vivo. *Trends Mol Med* doi:10.1016/j.molmed.2017.07.003

Kunisaki Y, Bruns I, Scheiermann C, Ahmed J, Pinho S, Zhang D, Mizoguchi T, Wei Q, Lucas D, Ito K, *et al* (2013) Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* 502: 637–643

Kuo CY, Long JD, Campo-Fernandez B, de Oliveira S, Cooper AR, Romero Z, Hoban MD, Joglekar A V., Lill GR, Kaufman ML, *et al* (2018) Site-Specific Gene Editing of Human Hematopoietic Stem Cells for X-Linked Hyper-IgM Syndrome. *Cell Rep*

Lapidot T, Pflumio F, Doedens M, Murdoch B, Williams DE & Dick JE (1992) Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID Mice. *Science (80-)* 255: 1137–1141

Lassmann T (2015) TagDust2: A generic method to extract reads from sequencing data. *BMC Bioinformatics*

Laurenti E, Frelin C, Xie S, Ferrari R, Dunant CF, Zandi S, Neumann A, Plumb I, Doulatov S, Chen J, *et al* (2015) CDK6 levels regulate quiescence exit in human hematopoietic stem cells. *Cell Stem Cell*

Laurenti E & Göttgens B (2018) From haematopoietic stem cells to complex differentiation landscapes. *Nature* 553: 418–426 doi:10.1038/nature25022

Lee CM, Cradick TJ & Bao G (2016) The neisseria meningitidis CRISPR-Cas9 system enables specific genome editing in mammalian cells. *Mol Ther*

Lee JK, Jeong E, Lee J, Jung M, Shin E, Kim Y hoon, Lee K, Jung I, Kim D, Kim S, *et al* (2018) Directed evolution of CRISPR-Cas9 to increase its specificity. *Nat Commun*

Levasseur DN, Ryan TM, Pawlik KM & Townes TM (2003) Correction of a mouse model of sickle cell disease: Lentiviral/ antisickling β-globin gene transduction of unmobilized, purified hematopoietic stem cells. *Blood*

Levasseur DN, Ryan TM, Reilly MP, McCune SL, Asakura T & Townes TM (2004) A recombinant human hemoglobin with anti-sickling properties greater than fetal hemoglobin. *J Biol Chem*

Lidonnici MR & Ferrari G (2018) Gene therapy and gene editing strategies for hemoglobinopathies. *Blood Cells, Mol Dis* doi:10.1016/j.bcmd.2017.12.001

Lim Y, Bak SY, Sung K, Jeong E, Lee SH, Kim JS, Bae S & Kim SK (2016) Structural roles of guide RNAs in the nuclease activity of Cas9 endonuclease. *Nat Commun*

Lindahl T (1993) Instability and decay of the primary structure of DNA. *Nature* 362: 709–715 doi:10.1038/362709a0

Liu C, Srihari S, Cao KAL, Chenevix-Trench G, Simpson PT, Ragan MA & Khanna KK (2014) A fine-scale dissection of the DNA double-strand break repair machinery and its implications for breast cancer therapy. *Nucleic Acids Res* 42: 6106–6127 doi:10.1093/nar/gku284

Liu J, Guo YM, Hirokawa M, Iwamoto K, Ubukawa K, Michishita Y, Fujishima N, Tagawa H, Takahashi N, Xiao W, *et al* (2012) A synthetic double-stranded RNA, poly I: C, induces a rapid apoptosis of human CD34+ cells. *Exp Hematol*

Liu M, Rehman S, Tang X, Gu K, Fan Q, Chen D & Ma W (2019) Methodologies for improving HDR efficiency. *Front Genet* doi:10.3389/fgene.2018.00691

Lombardo A, Cesana D, Genovese P, Di Stefano B, Provasi E, Colombo DF, Neri M, Magnani Z, Cantore A, Lo Riso P, *et al* (2011) Site-specific integration and tailoring of cassette design for sustainable gene transfer. *Nat Methods* 8: 861–869

Lombardo A, Genovese P, Beausejour CM, Colleoni S, Lee YL, Kim KA, Ando D, Urnov FD, Galli C, Gregory PD, *et al* (2007) Gene editing in human stem cells using zinc finger nucleases and integrase-defective lentiviral vector delivery. *Nat Biotechnol*

Lubeck D, Agodoa I, Bhakta N, Danese M, Pappu K, Howard R, Gleeson M, Halperin M & Lanzkron S (2019) Estimated Life Expectancy and Income of Patients With Sickle Cell

Disease Compared With Those Without Sickle Cell Disease. *JAMA Netw open*
Maier DA, Brennan AL, Jiang S, Binder-Scholl GK, Lee G, Plesa G, Zheng Z, Cotte J, Carpenito C, Wood T, *et al* (2013) Efficient clinical scale gene modification via zinc finger nuclease-targeted disruption of the HIV co-receptor CCR5. *Hum Gene Ther* 24: 245–258
Manches O, Frleta D & Bhardwaj N (2014) Dendritic cells in progression and pathology of HIV infection. *Trends Immunol* doi:10.1016/j.it.2013.10.003
Mani M, Smith J, Kandavelou K, Berg JM & Chandrasegaran S (2005) Binding of two zinc finger nuclease monomers to two specific sites is required for effective double-strand DNA cleavage. *Biochem Biophys Res Commun* 334: 1191–1197
Marcus JL, Leyden WA, Alexeeff SE, Anderson AN, Hechter RC, Hu H, Lam JO, Towner WJ, Yuan Q, Horberg MA, *et al* (2020) Comparison of Overall and Comorbidity-Free Life Expectancy Between Insured Adults With and Without HIV Infection, 2000-2016. *JAMA Netw open*
Mari PO, Florea BI, Persengiev SP, Verkaik NS, Brüggenwirth HT, Modesti M, Giglia-Mari G, Bezstarosti K, Demmers JAA, Luider TM, *et al* (2006) Dynamic assembly of end-joining complexes requires interaction between Ku70/80 and XRCC4. *Proc Natl Acad Sci U S A*
Marktel S, Scaramuzza S, Cicalese MP, Giglio F, Galimberti S, Lidonnici MR, Calbi V, Assanelli A, Bernardo ME, Rossi C, *et al* (2019) Intrabone hematopoietic stem cell gene therapy for adult and pediatric patients affected by transfusion-dependent ß-thalassemia. *Nat Med*
Martins CP, Brown-Swigart L & Evan GI (2006) Modeling the Therapeutic Efficacy of p53 Restoration in Tumors. *Cell*
Maruyama T, Dougan SK, Truttmann MC, Bilate AM, Ingram JR & Ploegh HL (2015) Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat Biotechnol*
Masiuk KE, Zhang R, Osborne K, Hollis RP, Campo-Fernandez B & Kohn DB (2019) PGE2 and Poloxamer Synperonic F108 Enhance Transduction of Human HSPCs with a β-Globin Lentiviral Vector. *Mol Ther - Methods Clin Dev*
Matthys P, Hatse S, Vermeire K, Wuyts A, Bridger G, Henson GW, De Clercq E, Billiau A & Schols D (2001) AMD3100, a Potent and Specific Antagonist of the Stromal Cell-Derived Factor-1 Chemokine Receptor CXCR4, Inhibits Autoimmune Joint Inflammation in IFN- Receptor-Deficient Mice. *J Immunol*
Maurer HS, Behrman RE & Honig GR (1970) Dependence of the oxygen affinity of blood on the presence of foetal or adult haemoglobin. *Nature*
Maximow AA (1909) The lymphocyte as a stem cell, common to different blood elements in embryonic development and during the post-fetal life of mammals. *Folia Haematol*
May C, Rivella S, Chadburn A & Sadelain M (2002) Successful treatment of murine β-thalassemia intermedia by transfer of the human β-globin gene. *Blood*
Mazzolari E, Forino C, Guerci S, Imberti L, Lanfranchi A, Porta F & Notarangelo LD (2007) Long-term immune reconstitution and clinical outcome after stem cell transplantation for severe T-cell immunodeficiency. *J Allergy Clin Immunol*
McIntosh BE, Brown ME, Duffin BM, Maufort JP, Vereide DT, Slukvin II & Thomson JA (2015) Nonirradiated NOD,B6.SCID Il2rγ-/- kitW41/W41 (NBSGW) mice support multilineage engraftment of human hematopoietic cells. *Stem Cell Reports* 4: 171–180
Menendez D, Nguyen T-AA, Snipe J & Resnick MA (2017) The cytidine deaminase APOBEC3 family is subject to transcriptional regulation by p53. *Mol Cancer Res*
Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, Schurra C, Garré M, Giovanni Nuciforo P, Bensimon A, *et al* (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature*
Milyavsky M, Gan OI, Trottier M, Komosa M, Tabach O, Notta F, Lechman E, Hermans KG,

Eppert K, Konovalova Z, *et al* (2010) A Distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal. *Cell Stem Cell* 7: 186–197

Mjelle R, Hegre SA, Aas PA, Slupphaug G, Drabløs F, Sætrom P & Krokan HE (2015) Cell cycle regulation of human DNA repair and chromatin remodeling genes. *DNA Repair (Amst)*

Mock U, MacHowicz R, Hauber I, Horn S, Abramowski P, Berdien B, Hauber J & Fehse B (2015) mRNA transfection of a novel TAL effector nuclease (TALEN) facilitates efficient knockout of HIV co-receptor CCR5. *Nucleic Acids Res*

Morbitzer R, Elsaesser J, Hausner J & Lahaye T (2011) Assembly of custom TALE-type DNA binding domains by modular cloning. *Nucleic Acids Res* 39: 5790–5799

Moreau-Gaudry F, Xia P, Jiang G, Perelman NP, Bauer G, Ellis J, Surinya KH, Mavilio F, Shen CK & Malik P (2001) High-level erythroid-specific gene expression in primary human and murine hematopoietic cells with self-inactivating lentiviral vectors. *Blood*

Morgan RA, Unti MJ, Aleshe B, Brown D, Osborne KS, Koziol C, Smith OB, O'Brien R, Tam C, Miyahira E, *et al* (2019) Improved Titer and Gene Transfer by Lentiviral Vectors Using Novel, Small β-Globin Locus Control Region Elements. *Mol Ther*

Morrison SJ, Uchida N & Weissman IL (1995) The biology of hematopoietic stem cells. *Annu Rev Cell Dev Biol* doi:10.1146/annurev.cb.11.110195.000343

Moscou MJ & Bogdanove AJ (2009) A simple cipher governs DNA recognition by TAL effectors. *Science (80-)* 326: 1501

Mu W, Tang N, Cheng C, Sun W, Wei X & Wang H (2019) In vitro transcribed sgRNA causes cell death by inducing interferon release. *Protein Cell* doi:10.1007/s13238-018-0605-9

Müller M, Lee CM, Gasiunas G, Davis TH, Cradick TJ, Siksnys V, Bao G, Cathomen T & Mussolino C (2016) Streptococcus thermophilus CRISPR-Cas9 systems enable specific editing of the human genome. *Mol Ther*

Nakamura-Ishizu A, Takizawa H & Suda T (2014) The analysis, roles and regulation of quiescence in hematopoietic stem cells. *Dev* 141: 4656–4666

Naldini L (2015) Gene therapy returns to centre stage. *Nature*

Naldini L (2019) Genetic engineering of hematopoiesis: current stage of clinical translation and future perspectives. *EMBO Mol Med*

Nelson CE, Wu Y, Gemberling MP, Oliver ML, Waller MA, Bohning JD, Robinson-Hamm JN, Bulaklak K, Castellanos Rivera RM, Collier JH, *et al* (2019) Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. *Nat Med* 25: 427–432

Nishimasu H, Shi X, Ishiguro S, Gao L, Hirano S, Okazaki S, Noda T, Abudayyeh OO, Gootenberg JS, Mori H, *et al* (2018) Engineered CRISPR-Cas9 nuclease with expanded targeting space. *Science (80-)*

Noguchi M, Yi H, Rosenblatt HM, Filipovich AH, Adelstein S, Modi WS, McBride OW & Leonard WJ (1993) Interleukin-2 receptor γ chain mutation results in X-linked severe combined immunodeficiency in humans. *Cell*

Notredame C, Higgins DG & Heringa J (2000) T-coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol*

Notta F, Doulatov S, Laurenti E, Poeppl A, Jurisica I & Dick JE (2011) Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science (80-)*

Nowak J (2008) Role of HLA in hematopoietic SCT. *Bone Marrow Transplant* doi:10.1038/bmt.2008.288

Obert S, O'Connor RJ, Schmid S & Hearing P (1994) The adenovirus E4-6/7 protein transactivates the E2 promoter by inducing dimerization of a heteromeric E2F complex. *Mol Cell Biol* 14: 1333–46

van der Oost J, Jore MM, Westra ER, Lundgren M & Brouns SJJ (2009) CRISPR-based

adaptive and heritable immunity in prokaryotes. *Trends Biochem Sci* 34: 401–407 doi:10.1016/j.tibs.2009.05.002

Osborn MJ, Starker CG, McElroy AN, Webber BR, Riddle MJ, Xia L, Defeo AP, Gabriel R, Schmidt M, Von Kalle C, *et al* (2013) TALEN-based gene correction for epidermolysis bullosa. *Mol Ther* 21: 1151–1159

Pabo CO & Sauer RT (1983) Protein-DNA recognition. *Annu Rev Biochem*

Palchaudhuri R, Saez B, Hoggatt J, Schajnovitz A, Sykes DB, Tate TA, Czechowicz A, Kfoury Y, Ruchika FNU, Rossi DJ, *et al* (2016) Non-genotoxic conditioning for hematopoietic stem cell transplantation using a hematopoietic-cell-specific internalizing immunotoxin. *Nat Biotechnol* 34: 738–745

Passegué E & Ernst P (2009) IFN-α wakes up sleeping hematopoietic stem cells. *Nat Med* doi:10.1038/nm0609-612

Pattabhi S, Lotti SN, Berger MP, Singh S, Lux CT, Jacoby K, Lee C, Negre O, Scharenberg AM & Rawlings DJ (2019) In Vivo Outcome of Homology-Directed Repair at the HBB Gene in HSC Using Alternative Donor Template Delivery Methods. *Mol Ther - Nucleic Acids*

Pavel-Dinu M, Wiebking V, Dejene BT, Srifa W, Mantri S, Nicolas CE, Lee C, Bao G, Kildebeck EJ, Punjya N, *et al* (2019) Gene correction for SCID-X1 in long-term hematopoietic stem cells. *Nat Commun*

Pavletich NP & Pabo CO (1991) Zinc finger-DNA recognition: Crystal structure of a Zif268-DNA complex at 2.1 Å. *Science (80-)* 252: 809–817

Pawliuk R, Westerman KA, Fabry ME, Payen E, Tighe R, Bouhassira EE, Acharya SA, Ellis J, London IM, Eaves CJ, *et al* (2001) Correction of sickle cell disease in transgenic mouse models by gene therapy. *Science (80-)*

Pelus LM & Broxmeyer HE (2018) Peripheral Blood Stem Cell Mobilization: a Look Ahead. *Curr Stem Cell Reports* doi:10.1007/s40778-018-0141-9

Perumbeti A, Higashimoto T, Urbinati F, Franco R, Meiselman HJ, Witte D & Malik P (2009) A novel human gamma-globin gene vector for genetic correction of sickle cell anemia in a humanized sickle mouse model: Critical determinants for successful correction. *Blood*

Pestina TI, Hargrove PW, Jay D, Gray JT, Boyd KM & Persons DA (2009) Correction of murine sickle cell disease using γ-globin lentiviral vectors to mediate high-level expression of fetal hemoglobin. *Mol Ther*

Petrillo C, Cesana D, Piras F, Bartolaccini S, Naldini L, Montini E & Kajaste-Rudnitski A (2015) Cyclosporin A and rapamycin relieve distinct lentiviral restriction blocks in hematopoietic stem and progenitor cells. *Mol Ther*

Petrillo C, Thorne LG, Unali G, Schiroli G, Giordano AMS, Piras F, Cuccovillo I, Petit SJ, Ahsan F, Noursadeghi M, *et al* (2018) Cyclosporine H Overcomes Innate Immune Restrictions to Improve Lentiviral Transduction and Gene Editing In Human Hematopoietic Stem Cells. *Cell Stem Cell*

Piel FB, Steinberg MH & Rees DC (2017) Sickle cell disease. *N Engl J Med* doi:10.1056/NEJMra1510865

Pike BL & Robinson WA (1970) Human bone marrow colony growth in agar-gel. *J Cell Physiol*

Pinheiro J, Bates D, DebRoy S, Sarkar D & Team the RC (2007) The nlme Package: Linear and Nonlinear Mixed Effects Models. *R-project*

Pinho S & Frenette PS (2019) Haematopoietic stem cell activity and interactions with the niche. *Nat Rev Mol Cell Biol* 20: 303–320 doi:10.1038/s41580-019-0103-9

Piras F & Kajaste-Rudnitski A (2020) Antiviral immunity and nucleic acid sensing in haematopoietic stem cell gene engineering. *Gene Ther* doi:10.1038/s41434-020-0175-3

Piras F, Riba M, Petrillo C, Lazarevic D, Cuccovillo I, Bartolaccini S, Stupka E, Gentner B, Cittaro D, Naldini L, *et al* (2017) Lentiviral vectors escape innate sensing but trigger p53 in

human hematopoietic stem and progenitor cells. *EMBO Mol Med*

Povirk LF, Zhou T, Zhou R, Cowan MJ & Yannone SM (2007) Processing of 3′-phosphoglycolate-terminated DNA double strand breaks by artemis nuclease. *J Biol Chem*

Provasi E, Genovese P, Lombardo A, Magnani Z, Liu PQ, Reik A, Chu V, Paschon DE, Zhang L, Kuball J, *et al* (2012) Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nat Med*

Publicover A, Richardson DS, Davies A, Hill KS, Hurlock C, Hutchins D, Jenner MW, Johnson PW, Lamb J, Launders H, *et al* (2013) Use of a biosimilar granulocyte colony-stimulating factor for peripheral blood stem cell mobilization: An analysis of mobilization and engraftment. *Br J Haematol* 162: 107–111

Querido E, Blanchette P, Yan Q, Kamura T, Morrison M, Boivin D, Kaelin WG, Conaway RC, Conaway JW & Branton PE (2001) Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev* 15: 3104–3117

Radhakrishnan SK, Feliciano CS, Najmabadi F, Haegebarth A, Kandel ES, Tyner AL & Gartel AL (2004) Constitutive expression of E2F-1 leads to p21-dependent cell cycle arrest in S phase of the cell cycle. *Oncogene*

Rahman AH, Taylor DK & Turka LA (2009) The contribution of direct TLR signaling to T cell responses. *Immunol Res* doi:10.1007/s12026-009-8113-x

Ran FA, Cong L, Yan WX, Scott DA, Gootenberg JS, Kriz AJ, Zetsche B, Shalem O, Wu X, Makarova KS, *et al* (2015) In vivo genome editing using Staphylococcus aureus Cas9. *Nature*

Ratajczak MZ (2018) Circulating Stem Cells in Physiology and Pathology - Recent Studies Published in Stem Cell Reviews and Reports. *Stem Cell Rev Reports* doi:10.1007/s12015-018-9842-9

De Ravin SS, Li L, Wu X, Choi U, Allen C, Koontz S, Lee J, Theobald-Whiting N, Chu J, Garofalo M, *et al* (2017) CRISPR-Cas9 gene repair of hematopoietic stem cells from patients with X-linked chronic granulomatous disease. *Sci Transl Med*

De Ravin SS, Reik A, Liu P-Q, Li L, Wu X, Su L, Raley C, Theobald N, Choi U, Song AH, *et al* (2016) Targeted gene addition in human CD34(+) hematopoietic cells for correction of X-linked chronic granulomatous disease. *Nat Biotechnol* 34: 1–8

Reyon D, Tsai SQ, Khgayter C, Foden JA, Sander JD & Joung JK (2012) FLASH assembly of TALENs for high-throughput genome editing. *Nat Biotechnol* 30: 460–465

Ribeil JA, Hacein-Bey-Abina S, Payen E, Magnani A, Semeraro M, Magrin E, Caccavelli L, Neven B, Bourget P, El Nemer W, *et al* (2017) Gene therapy in a patient with sickle cell disease. *N Engl J Med*

Robert F, Barbeau M, Éthier S, Dostie J & Pelletier J (2015) Pharmacological inhibition of DNA-PK stimulates Cas9-mediated genome editing. *Genome Med*

Romero Z, Campo-Fernandez B, Wherley J, Kaufman ML, Urbinati F, Cooper AR, Hoban MD, Baldwin KM, Lumaquin D, Wang X, *et al* (2015) The human ankyrin 1 promoter insulator sustains gene expression in a β-globin lentiviral vector in hematopoietic stem cells. *Mol Ther - Methods Clin Dev*

Romero Z, Lomova A, Said S, Miggelbrink A, Kuo CY, Campo-Fernandez B, Hoban MD, Masiuk KE, Clark DN, Long J, *et al* (2019) Editing the Sickle Cell Disease Mutation in Human Hematopoietic Stem Cells: Comparison of Endonucleases and Homologous Donor Templates. *Mol Ther*

Romero Z, Urbinati F, Geiger S, Cooper AR, Wherley J, Kaufman ML, Hollis RP, De Assin RR, Senadheera S, Sahagian A, *et al* (2013) β-globin gene transfer to human bone marrow for sickle cell disease. *J Clin Invest*

Roth TL, Puig-Saus C, Yu R, Shifrut E, Carnevale J, Li PJ, Hiatt J, Saco J, Krystofinski P, Li H, *et al* (2018) Reprogramming human T cell function and specificity with non-viral genome

targeting. *Nature*
Russell S, Bennett J, Wellman JA, Chung DC, Yu ZF, Tillman A, Wittes J, Pappas J, Elci O, McCague S, *et al* (2017) Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. *Lancet*
Saag MS, Benson CA, Gandhi RT, Hoy JF, Landovitz RJ, Mugavero MJ, Sax PE, Smith DM, Thompson MA, Buchbinder SP, *et al* (2018) Antiretroviral drugs for treatment and prevention of HIV infection in adults: 2018 recommendations of the international antiviral society-USA panel. In *JAMA - Journal of the American Medical Association*
Sadelain M, Rivière I & Riddell S (2017) Therapeutic T cell engineering. *Nature* doi:10.1038/nature22395
Sakofsky CJ, Saini N, Klimczak LJ, Chan K, Malc EP, Mieczkowski PA, Burkholder AB, Fargo D & Gordenin DA (2019) Repair of multiple simultaneous double-strand breaks causes bursts of genome-wide clustered hypermutation. *PLoS Biol*
Salinas Cisneros G & Thein SL (2020) Recent Advances in the Treatment of Sickle Cell Disease. *Front Physiol* doi:10.3389/fphys.2020.00435
San Filippo J, Sung P & Klein H (2008) Mechanism of Eukaryotic Homologous Recombination. *Annu Rev Biochem* 77: 229–257
Saunders KO, Wiehe K, Tian M, Acharya P, Bradley T, Munir Alam S, Go EP, Scearce R, Sutherland L, Henderson R, *et al* (2019) Targeted selection of HIV-specific antibody mutations by engineering B cell maturation. *Science (80-)*
Sauvageau G, Iscove NN & Humphries RK (2004) In vitro and in vivo expansion of hematopoietic stem cells. *Oncogene* 23: 7223–7232 doi:10.1038/sj.onc.1207942
Schaley J, O'Connor RJ, Taylor LJ, Bar-Sagi D & Hearing P (2000) Induction of the Cellular E2F-1 Promoter by the Adenovirus E4-6/7 Protein. *J Virol*
Schaley JE, Polonskaia M & Hearing P (2005) The Adenovirus E4-6/7 Protein Directs Nuclear Localization of E2F-4 via an Arginine-Rich Motif. *J Virol*
Schiroli G, Conti A, Ferrari S, della Volpe L, Jacob A, Albano L, Beretta S, Calabria A, Vavassori V, Gasparini P, *et al* (2019) Precise Gene Editing Preserves Hematopoietic Stem Cell Function following Transient p53-Mediated DNA Damage Response. *Cell Stem Cell*
Schiroli G, Ferrari S, Conway A, Jacob A, Capo V, Albano L, Plati T, Castiello MC, Sanvito F, Gennery AR, *et al* (2017) Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1. *Sci Transl Med*
Scully R, Panday A, Elango R & Willis NA (2019) DNA double-strand break repair-pathway choice in somatic mammalian cells. *Nat Rev Mol Cell Biol* 20: 698–714
Seandel M, Butler JM, Kobayashi H, Hooper AT, White IA, Zhang F, Vertes EL, Kobayashi M, Zhang Y, Shmelkov S V., *et al* (2008) Generation of a functional and durable vascular niche by the adenoviral E4ORF1 gene. *Proc Natl Acad Sci* 105: 19288–19293
Seita J & Weissman IL (2010) Hematopoietic stem cell: Self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med* 2: 640–653 doi:10.1002/wsbm.86
Sengupta S & Siliciano RF (2018) Targeting the Latent Reservoir for HIV-1. *Immunity* doi:10.1016/j.immuni.2018.04.030
Shin JJ, Schröder MS, Caiado F, Wyman SK, Bray NL, Bordi M, Dewitt MA, Vu JT, Kim WT, Hockemeyer D, *et al* (2020) Controlled Cycling and Quiescence Enables Efficient HDR in Engraftment-Enriched Adult Hematopoietic Stem and Progenitor Cells. *Cell Rep*
Shultz LD, Lyons BL, Burzenski LM, Gott B, Chen X, Chaleff S, Kotb M, Gillies SD, King M, Mangada J, *et al* (2005) Human Lymphoid and Myeloid Cell Development in NOD/LtSz-scid IL2R γ null Mice Engrafted with Mobilized Human Hemopoietic Stem Cells . *J Immunol*
Slaymaker IM, Gao L, Zetsche B, Scott DA, Yan WX & Zhang F (2016) Rationally engineered Cas9 nucleases with improved specificity. *Science (80-)*

Smith J (2000) Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucleic Acids Res* 28: 3361–3369

Sorek R, Kunin V & Hugenholtz P (2008) CRISPR - A widespread system that provides acquired resistance against phages in bacteria and archaea. *Nat Rev Microbiol* 6: 181–186

Soutoglou E, Dorn JF, Sengupta K, Jasin M, Nussenzweig A, Ried T, Danuser G & Misteli T (2007) Positional stability of single double-strand breaks in mammalian cells. *Nat Cell Biol*

Spagnolo L, Rivera-Calzada A, Pearl LH & Llorca O (2006) Three-Dimensional Structure of the Human DNA-PKcs/Ku70/Ku80 Complex Assembled on DNA and Its Implications for DNA DSB Repair. *Mol Cell*

Srivastava M, Nambiar M, Sharma S, Karki SS, Goldsmith G, Hegde M, Kumar S, Pandey M, Singh RK, Ray P, *et al* (2012) An inhibitor of nonhomologous end-joining abrogates double-strand break repair and impedes cancer progression. *Cell* 151: 1474–1487

Stanelle J, Stiewe T, Theseling CC, Peter M & Pützer BM (2002) Gene expression changes in response to E2F1 activation. *Nucleic Acids Res*

Steensma DP (2018) Myelodysplastic syndromes current treatment algorithm 2018. *Blood Cancer J*

Stein S, Ott MG, Schultze-Strasser S, Jauch A, Burwinkel B, Kinner A, Schmidt M, Krämer A, Schwäble J, Glimm H, *et al* (2010) Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. *Nat Med*

Täuber B & Dobner T (2001) Adenovirus early E4 genes in viral oncogenesis. *Oncogene* 20: 7847–7854 doi:10.1038/sj/onc/1204914

Tebas P, Stein D, Tang WW, Frank I, Wang SQ, Lee G, Spratt SK, Surosky RT, Giedlin MA, Nichol G, *et al* (2014) Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med* 370: 901–910

Thomas ED, Lochte HL, Lu WC & Ferrebee JW (1957) Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. *N Engl J Med*

Thompson AA, Walters MC, Kwiatkowski J, Rasko JEJ, Ribeil JA, Hongeng S, Magrin E, Schiller GJ, Payen E, Semeraro M, *et al* (2018) Gene therapy in patients with transfusion-dependent β-thalassemia. *N Engl J Med*

Touzot F, Moshous D, Creidy R, Neven B, Frange P, Cros G, Caccavelli L, Blondeau J, Magnani A, Luby JM, *et al* (2015) Faster T-cell development following gene therapy compared with haploidentical HSCT in the treatment of SCID-X1. *Blood*

Truong LN, Li Y, Shi LZ, Hwang PYH, He J, Wang H, Razavian N, Berns MW & Wu X (2013) Microhomology-mediated End Joining and Homologous Recombination share the initial end resection step to repair DNA double-strand breaks in mammalian cells. *Proc Natl Acad Sci U S A*

Tsai SQ, Nguyen NT, Malagon-Lopez J, Topkar V V., Aryee MJ & Joung JK (2017) CIRCLE-seq: a highly sensitive in vitro screen for genome-wide CRISPR–Cas9 nuclease off-targets. *Nat Methods* 14

Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar V V., Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, *et al* (2015) GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nat Biotechnol*

Turnell AS & Grand RJ (2012) DNA viruses and the cellular DNA-damage response. *J Gen Virol* doi:10.1099/vir.0.044412-0

Uchida N, Dykstra B, Lyons KJ, Leung FYK & Eaves CJ (2003) Different in vivo repopulating activities of purified hematopoietic stem cells before and after being stimulated to divide in vitro with the same kinetics. *Exp Hematol* 31: 1338–1347

Uchida N, Hsieh MM, Raines L, Haro-Mora JJ, Demirci S, Bonifacino AC, Krouse AE, Metzger ME, Donahue RE & Tisdale JF (2019) Development of a forward-oriented

therapeutic lentiviral vector for hemoglobin disorders. *Nat Commun*
Urnov FD, Miller JC, Lee YL, Beausejour CM, Rock JM, Augustus S, Jamieson AC, Porteus MH, Gregory PD & Holmes MC (2005) Highly efficient endogenous human gene correction using designed zinc-finger nucleases. *Nature*
Urnov FD, Rebar EJ, Holmes MC, Zhang HS & Gregory PD (2010) Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 11: 636–646 doi:10.1038/nrg2842
Uy GL, Rettig MP & Cashen AF (2008) Plerixafor, a CXCR4 antagonist for the mobilization of hematopoietic stem cells. *Expert Opin Biol Ther*
Vakulskas CA, Dever DP, Rettig GR, Turk R, Jacobi AM, Collingwood MA, Bode NM, McNeill MS, Yan S, Camarena J, *et al* (2018) A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. *Nat Med*
Velten L, Haas SF, Raffel S, Blaszkiewicz S, Islam S, Hennig BP, Hirche C, Lutz C, Buss EC, Nowak D, *et al* (2017) Human haematopoietic stem cell lineage commitment is a continuous process. *Nat Cell Biol* 19: 271–281
Vichinsky E, Hoppe CC, Ataga KI, Ware RE, Nduba V, El-Beshlawy A, Hassab H, Achebe MM, Alkindi S, Brown RC, *et al* (2019) A Phase 3 randomized trial of voxelotor in sickle cell disease. *N Engl J Med*
Wagenblast E, Azkanaz M, Smith SA, Shakib L, McLeod JL, Krivdova G, Araújo J, Shultz LD, Gan OI, Dick JE, *et al* (2019) Functional profiling of single CRISPR/Cas9-edited human long-term hematopoietic stem cells. *Nat Commun*
Walasek MA, van Os R & de Haan G (2012) Hematopoietic stem cell expansion: Challenges and opportunities. *Ann N Y Acad Sci* 1266: 138–150
Walton RT, Christie KA, Whittaker MN & Kleinstiver BP (2020) Unconstrained genome targeting with near-PAMless engineered CRISPR-Cas9 variants. *Science (80-)*
Wang CX & Cannon PM (2016) The clinical applications of genome editing in HIV. *Blood* doi:10.1182/blood-2016-01-678144
Wang J, Exline CM, DeClercq JJ, Llewellyn GN, Hayward SB, Li PW-LL, Shivak DA, Surosky RT, Gregory PD, Holmes MC, *et al* (2015) Homology-driven genome editing in hematopoietic stem and progenitor cells using ZFN mRNA and AAV6 donors. *Nat Biotechnol* 33: 1256–1263
Wang JCY, Doedens M & Dick JE (1997) Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. *Blood*
Wang X, Wang Y, Wu X, Wang J, Wang Y, Qiu Z, Chang T, Huang H, Lin RJ & Yee JK (2015) Unbiased detection of off-target cleavage by CRISPR-Cas9 and TALENs using integrase-defective lentiviral vectors. *Nat Biotechnol* 33: 175–179
Watson JD & Crick FHC (1953) Molecular structure of nucleic acids: A structure for deoxyribose nucleic acid. *Nature*
Weissman IL & Shizuru JA (2008) The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases. *Blood* 112: 3543–3553
Wienert B, Wyman SK, Richardson CD, Yeh CD, Akcakaya P, Porritt MJ, Morlock M, Vu JT, Kazane KR, Watry HL, *et al* (2019) Unbiased detection of CRISPR off-targets in vivo using DISCOVER-Seq. *Science (80-)*
Wilen CB, Wang J, Tilton JC, Miller JC, Kim KA, Rebar EJ, Sherrill-Mix SA, Patro SC, Secreto AJ, Jordan APO, *et al* (2011) Engineering HIV-resistant human CD4+ T cells with CXCR4-specific zinc-finger nucleases. *PLoS Pathog*
Wilson A, Laurenti E, Oser G, van der Wath RC, Blanco-Bose W, Jaworski M, Offner S, Dunant CF, Eshkind L, Bockamp E, *et al* (2008) Hematopoietic Stem Cells Reversibly Switch

from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell*

Wilson RC & Carroll D (2019) The Daunting Economics of Therapeutic Genome Editing. *Cris J*

Woods NB, Bottero V, Schmidt M, Von Kalle C & Verma IM (2006) Gene therapy: Therapeutic gene causing lymphoma. *Nature*

Wu Y, Zeng J, Roscoe BP, Liu P, Yao Q, Lazzarotto CR, Clement K, Cole MA, Luk K, Baricordi C, *et al* (2019) Highly efficient therapeutic gene editing of human hematopoietic stem cells. *Nat Med*

Xie SZ, Garcia-Prat L, Voisin V, Ferrari R, Gan OI, Wagenblast E, Kaufmann KB, Zeng AGX, Takayanagi S ichiro, Patel I, *et al* (2019) Sphingolipid Modulation Activates Proteostasis Programs to Govern Human Hematopoietic Stem Cell Self-Renewal. *Cell Stem Cell*

Xu K, Ren C, Liu Z, Zhang T, Zhang T, Li D, Wang L, Yan Q, Guo L, Shen J, *et al* (2015) Efficient genome engineering in eukaryotes using Cas9 from Streptococcus thermophilus. *Cell Mol Life Sci*

Xu L, Wang J, Liu Y, Xie L, Su B, Mou D, Wang L, Liu T, Wang X, Zhang B, *et al* (2019) CRISPR-edited stem cells in a patient with HIV and acute lymphocytic leukemia. *N Engl J Med*

Yao X, Wang X, Hu X, Liu Z, Liu J, Zhou H, Shen X, Wei Y, Huang Z, Ying W, *et al* (2017) Homology-mediated end joining-based targeted integration using CRISPR/Cas9. *Nat Publ Gr*: 1–14

Yeh CD, Richardson CD & Corn JE (2019) Advances in genome editing through control of DNA repair pathways. *Nat Cell Biol* 21: 1468–1478

Yin AH, Miraglia S, Zanjani ED, Almeida-Porada G, Ogawa M, Leary AG, Olweus J, Kearney J & Buck DW (1997) AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood*

Yukl SA, Boritz E, Busch M, Bentsen C, Chun TW, Douek D, Eisele E, Haase A, Ho YC, Hütter G, *et al* (2013) Challenges in Detecting HIV Persistence during Potentially Curative Interventions: A Study of the Berlin Patient. *PLoS Pathog*

Zeng J, Wu Y, Ren C, Bonanno J, Shen AH, Shea D, Gehrke JM, Clement K, Luk K, Yao Q, *et al* (2020) Therapeutic base editing of human hematopoietic stem cells. *Nat Med*

Zetsche B, Gootenberg JS, Abudayyeh OO, Slaymaker IM, Makarova KS, Essletzbichler P, Volz SE, Joung J, Van Der Oost J, Regev A, *et al* (2015) Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. *Cell*

Zhao H, Dahlö M, Isaksson A, Syvänen AC & Pettersson U (2012) The transcriptome of the adenovirus infected cell. *Virology*

Zonari E, Desantis G, Petrillo C, Boccalatte FE, Lidonnici MR, Kajaste-Rudnitski A, Aiuti A, Ferrari G, Naldini L & Gentner B (2017) Efficient Ex Vivo Engineering and Expansion of Highly Purified Human Hematopoietic Stem and Progenitor Cell Populations for Gene Therapy. *Stem Cell Reports* 8: 977–990

MATERIALS & METHODS

Vectors and nucleases

AAV6 donor templates were generated from a construct containing AAV2 inverted terminal repeats (ITRs), produced at the TIGEM Vector Core (Pozzuoli (NA), Italy) by triple-transfection method and purified by ultracentrifugation on a cesium chloride gradient. Design of the non-barcoded AAV6 donor templates carrying homologies for *AAVS1* or *IL2RG* (both encompassing a PGK.GFP reporter cassette) were previously reported[15]. Design of the AAV6 donor template with homologies for *CD40LG* will be reported elsewhere. The barcoded vector was obtained by subcloning a degenerated BAR sequence downstream of the GFP reporter cassette in the reference AAV backbone for *AAVS1* editing. For molecular cloning of the barcoded AAV, a single stranded ODN embedding the 22-bp BAR sequence flanked by unique restriction sites (Bsu36I and SphI, New England Biolabs) was purchased from Sigma Aldrich. Theoretical complexity of the ssODN was estimated in $2.9x10^{10}$. BAR consensus sequence was designed to contain some invariant positions (7, 9, 15) and others limited to few bases (3, 14, 17, 21, 22) to avoid generating Bsu36I and SphI restriction sites. To generate the complementary strand, 50 pmol of the ssODN underwent 10 PCR cycle with Easy-A High-Fidelity enzyme (Agilent Technologies) using the appropriate primers (see **Supplementary Table 3**) and according to manufacturer instruction. The amplified product was purified with MinElute PCR Purification kit (QIAGEN), digested with the restriction enzymes and verified by capillary electrophoresis. 2 µg of this purified product were ligated with the digested reference backbone (molar ratio 7:1) using T4 DNA Ligase (New England Biolabs) by scaling up the manufacturer protocol. XL-10 Gold Ultracompetent Cells (Agilent Technologies) were transformed with the ligation product, plated and incubated for 12 h at 30°C to minimize the occurrence of recombination events. Colonies were scraped, mixed, grown in LB medium for additional 6 h and processed with NucleoBond Xtra MaxiPrep (Machery Nagel) according to manufacturer instruction. The plasmid prep was screened with MscI and XmaI restriction enzymes (New England Biolabs) for ITRs and plasmid integrity.

IDLV donor was generated using HIV-derived, third-generation self-inactivating transfer construct and the IDLV stock was prepared by transient transfection of HEK293T, as previously described (Petrillo *et al*, 2018). At 30 hours post-transfection, vector-containing supernatant was collected, filtered, clarified, DNAse treated and loaded on a DEAE-packed column for Anion Exchange Chromatography. The vector-containing peak was collected, subjected to a second round of DNAse treatment, concentration by Tangential Flow Filtration and a final Size

Exclusion Chromatography separation followed by sterilizing filtration and titration of the purified stock as previously described (Petrillo *et al*, 2018).

Sequences of the gRNAs were designed using an online tool (Hsu *et al*, 2013) and selected for predicted specificity score and on-target activity. Genomic sequences recognized by the gRNAs were previously reported (*AAVS1*, *IL2RG*)(Schiroli *et al*, 2019) or will be reported elsewhere (*CD40LG*). RNP complexes were assembled by incubating at 1:1.5 molar ratio *Streptococcus pyogenes* (*Sp*)Cas9 protein (Aldevron) with pre-annealed synthetic Alt-R® crRNA:tracrRNA (Integrated DNA Technologies) for 10' at 25°C. together with 0.1 nmol of Alt-R® Cas9 Electroporation Enhancer (Integrated DNA Technologies) was added prior to electroporation according to manufacturer's instructions.

Vector maps were designed with SnapGene software v5.0.7 (from GSL Biotech; available at snapgene.com) or Vector NTI® Express v1.6.2 (from Thermo Fisher Scientific, available at thermofisher.com).

Multiple sequences alignment

Multiple sequences alignments were performed with E4orf1 and E4orf6/7 variants derived from different Ad serotypes using the T-Coffee algorithm (Notredame *et al*, 2000).

mRNA *in vitro* transcription

The GSE56 construct was previously described(Schiroli *et al*, 2019). For other constructs, DNA coding sequences were synthetized (GeneArt™, Thermo Fisher) using *Homo sapiens* codon-usage optimized Ad-E4orf1 and E4orf6/7 sequences. Coding sequences were subcloned in "pVax" plasmids under the control of the T7 promoter and followed by WHP posttranscriptional regulatory element (WPRE) and a 64-bp polyA sequence. For GSE56 and Ad5-E4orf6/7 co-expression, we used separate mRNA in initial setup experiments and a fusion construct, in which the coding sequences were part of the same 'open reading frame' (ORF) and separated by a nucleotide sequence encoding for the P2A self-cleaving peptide in most of the follow-up experiments (**Supplementary Table 4**). For mRNA *in vitro* transcription, pVax plasmids were linearized with SpeI (New England Biolabs) restriction enzyme and purified by phenol-chloroform extraction. mRNA was *in vitro* transcribed using the commercial 5X MEGAscript T7 kit (Thermo Fisher) and capped with the Anti-Reverse Cap Analog (ARCA) 3′-O-Me-mG(5′)ppp(5′)G (New England Biolabs). mRNA was purified using RNeasy Plus Mini Kit (Qiagen)

followed by HPLC purification (ADS BIOTEC WAVE® System) and Amicon Ultra-15 (30K) tube (Millipore) concentration. mRNA productions were aliquoted and stored at -80°C.

Cell lines and primary cell culture

HEK293T cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Corning) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Euroclone), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2% glutamine.

Primary T cells were isolated from healthy male donors' peripheral blood mononuclear cells (PBMCs) purified from buffy coats by sequential centrifugations in a Ficoll gradient according to a protocol approved by the Ospedale San Raffaele Scientific Institute bioethical committee (TIGET-HPCT). $CD3^+$ T cells were stimulated using magnetic beads (1:3 cell:beads ratio) conjugated with anti-CD3/anti-CD28 antibodies (Dynabeads human T-activator CD3/CD28, Thermo Fisher). Cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Corning) supplemented with 10% heat-inactivated FBS, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2% glutamine, 5 ng/ml hIL-7 (PreproTech) and 5 ng/ml hIL-15 (PreproTech)(Provasi *et al*, 2012). Dynabeads were removed after 6 days of culture.

CB $CD34^+$ HSPCs were purchased frozen from Lonza upon approval by the Ospedale San Raffaele Bioethical Committee (TIGET-HPCT) and were seeded at the concentration of $5x10^5$ cells/ml in serum-free StemSpan medium (StemCell Technologies) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2% glutamine, 100 ng/ml hSCF (PeproTech), 100 ng/ml hFlt3-L (PeproTech), 20 ng/ml hTPO (PeproTech), and 20 ng/ml hIL-6 (PeproTech) and 10 µM PGE_2 (at the beginning of the culture; Cayman). Culture medium was also supplemented with 1 µM SR1 (Biovision) and 50 nM UM171 (STEMCell Technologies), unless otherwise specified.

G-CSF mPB $CD34^+$ HSPCs were purified with the CliniMACS CD34 Reagent System (Miltenyi Biotec) from Mobilized Leukopak (AllCells) upon approval by the Ospedale San Raffaele Bioethical Committee (TIGET-HPCT) according to manufacturer's instructions. HSPCs were seeded at the concentration of $5x10^5$ cells/ml in serum-free StemSpan medium (StemCell Technologies) supplemented with 100 IU/ml penicillin, 100 µg/ml streptomycin, 2% glutamine, 300 ng/ml hSCF, 300 ng/ml hFlt3-L, 100 ng/ml hTPO and 10 µM PGE2 (at the beginning of the culture). Culture medium was also supplemented with 1 µM SR1 and 35 nM UM171.
All cells were cultured in a 5% CO_2 humidified atmosphere at 37°C.

Gene editing of human T cells and analyses

After three days of stimulation, 5-10x10^5 T cells were electroporated using P3 Primary Cell 4D-Nucleofector X Kit and program DS-130 (Lonza). Cells were electroporated with RNP at final concentration 1.25 μM (Integrated DNA Technologies) together with 0.1 nmol of Alt-R® Cas9 Electroporation Enhancer (Integrated DNA Technologies) and transduced with 5x10^4 vg/cell of AAV6 15 min after electroporation. Where specified, mRNAs were added to the electroporation mixture at the final concentrations reported in **Supplementary Table 4**. T cells were expanded for 14 days to perform flow cytometry.

Gene editing of human HSPCs and analyses

For AAV6-based gene editing, after 3 days of stimulation 1-5x10^5 cells were washed with Dulbecco's Phosphate-Buffered Saline without Ca^{2+} and Mg^{2+} (DPBS, Corning) and electroporated using P3 Primary Cell 4D-Nucleofector X Kit and program EO-100 (Lonza). Cells were electroporated with RNPs at final concentration 1.25-2.5 μM together with 0.1 nmol of Alt-R® Cas9 Electroporation Enhancer (Integrated DNA Technologies), according to manufacturer's instructions. AAV6 transduction was performed at a dose of 1-2x10^4 vg/cell 15 min after electroporation. For IDLV-based gene editing, after 2 days of stimulation 1-5x10^5 cells were treated with 8 μM cyclosporin H (CsH, Sigma) or DMSO vehicle and transduced 2 hours later with purified IDLV at multiplicity of infection of 100-200 (vector concentration = 1.1x10^{10} Transducing Units^{293T}/mL). After 24 h, cells were washed with DPBS and electroporated using P3 Primary Cell 4D-Nucleofector X Kit and program EO-100 (Lonza), as described above.

Additional mRNAs were added to the electroporation mixture at the final concentrations reported in **Supplementary Table 4**. Three/four days after editing procedure cells were harvested to analyze by flow cytometry the percentage of cells expressing the GFP marker within HSPC subpopulations and to extract genomic DNA for molecular analyses, unless otherwise indicated.

CFU-C assay was performed 24 h after editing procedure by plating 600 cells in methylcellulose-based medium (MethoCult H4434, StemCell Technologies) supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin. Three technical replicates were performed for each condition. Two weeks after plating, colonies were counted and identified according to morphological criteria.

Mice

All experiments and procedures involving animals were performed with the approval of the Animal Care and Use Committee of the San Raffaele Hospital (IACUC: #749) and authorized by the Italian Ministry of Health and local authorities accordingly to Italian law. NOD-SCID-IL2Rg$^{-/-}$ (NSG) female mice (The Jackson Laboratory) were held in specific pathogen-free (SPF) conditions.

CD34^{+} HSPC xenotransplantation experiments in NSG mice

For transplantation of CB and G-CSF mPB CD34^{+} HSPCs, the outgrowth of 1-3x10^{5} and 1x10^{6} HSPCs, respectively, at the start of the culture were injected intravenously 24 h after editing into sub-lethally irradiated NSG mice (150-180 cGy). Sample size for each experiment was determined by the total number of available treated cells. Mice were randomly distributed to each experimental group. Human CD45^{+} cell engraftment and the presence of edited cells were monitored by serial collection of blood from the mouse tail and, at the end of the experiment (>18 weeks after transplantation), BM and SPL were harvested for end-point analyses.

Secondary transplantation was performed upon injection of 2x10^{6} beads purified human CD34^{+} cells (Miltenyi Biotec) harvested from the BM of primary engrafted NSG mice and pooled for each experimental group.

Flow cytometry

Immunophenotypic analyses were performed on FACS Canto II (BD Pharmingen) or CytoFLEX LX Flow Cytometer (Beckman Coulter). From 0.5 to 2x10^{5} cells (either from culture or mouse samples) were analyzed by flow cytometry. Cells were stained for 15 min at 4°C with antibodies listed in the **Reporting Summary** in a final volume of 100 µl and then washed with DPBS + 2% heat inactivated FBS. Single stained and FMO stained cells were used as controls. Live/Dead Fixable Dead Cell Stain Kit (Thermo Fisher) or 7-Aminoactinomycin D (Sigma Aldrich) were included during sample preparation according to the manufacturer's instructions to identify dead cells. Apoptosis analysis was performed as previously described(Genovese *et al*, 2014). Cell sorting was performed on a BD FACSAria Fusion (BD Biosciences) using BDFACS Diva software and equipped with four lasers: blue (488 nm), yellow/green (561 nm), red (640 nm) and violet (405 nm). Cells were sorted with an 85 mm nozzle. Sheath fluid pressure was set at 45 psi. A highly pure sorting modality (4-way purity sorting) was chosen. Sorted cells were collected in 1.5 ml Eppendorf tubes containing 500 µl of DPBS. Gating strategies

for flow cytometry analyses are provided in **Supplementary Figure 2**. Data were analyzed with FCS Express 6 Flow.

Molecular analyses

For molecular analyses, genomic DNA was isolated with QIAamp DNA Micro Kit (QIAGEN) according to manufacturer's instructions. Nuclease activity was measured by mismatch-sensitive endonuclease T7 assay (New England Biolabs) or Surveyor® mutation detection kit (IDT) on PCR-based amplification products of the targeted locus, as previously described(Schiroli *et al*, 2017). Digested DNA fragments were resolved and quantified by capillary electrophoresis on LabChip® GX Touch HT (Perkin Elmer) or 4200 TapeStation System (Agilent) according to the manufacturer's instructions.

For HDR digital droplet PCR (ddPCR) analysis, 5-50 ng of genomic DNA were analyzed using the QX200 Droplet Digital PCR System (Bio-Rad) according to the manufacturer's instructions. HDR ddPCR primers and probes were designed on the junction between the vector sequence and the targeted locus. Human *TTC5* (Bio-Rad) was used for normalization. The percentage of cells harboring biallelic integration was calculated with the following formula: (# of *AAVS1*$^+$ droplets / # of *TTC5*$^+$ droplets x 200) - % GFP$^+$ cells. The percentage of monoallelic integration was then calculated with the following formula: % GFP$^+$ cells - % cells with biallelic integration. For chromosomes X-14 translocation, ddPCR was performed as previously reported(Schiroli *et al*, 2019).

For gene expression analyses, total RNA was extracted using RNeasy Plus Micro Kit (QIAGEN), according to the manufacturer's instructions and DNase treatment was performed using RNase-free DNase Set (QIAGEN). cDNA was synthetized with SuperScript VILO IV cDNA Synthesis Kit (Thermo Fisher) with EzDNase treatment. cDNA was then used for qPCR in a Viia7 Real-time PCR thermal cycler using TaqMan Gene Expression Assays (Applied Biosystems) mapping to genes listed in **Supplementary Table 3**. Data were analyzed with QuantStudio™ Real-Time PCR software v1.1 (Applied Biosystem). Relative expression of each target gene was first normalized to *HPRT* and then represented as fold changes ($2^{-\Delta\Delta Ct}$) relative to the untreated cells.

For ddPCR array card, CD34$^+$CD133$^+$CD90$^+$ cells were sorted 12 h after HSPC editing in presence or absence of Ad5-E4orf6/7. After RNA isolation and reverse transcription as described above, gene expression was performed with "Cell Cycle Generic H384" predesigned

384-well panel (PrimePCR Arrays, Bio-Rad) with SYBR Green system. Data were analyzed with QuantaSoft™ Software v1.7.4 (Bio-Rad).

BAR-Seq library preparation

PCR amplicons for individual samples were generated by nested PCR using primers listed in **Supplementary Table 3** and starting from >50-100 ng of purified gDNA. The first PCR step was performed with GoTaq G2 DNA Polymerase (Promega) according to manufacturer instruction using the following amplification protocol: 95°C x 5 min, (95°C x 0.5 min, 60°C x 0.5 min, 72°C x 0.5 min) x 20 cycles, 72°C x 5 min. Forward primer was designed to bind donor template upstream the BAR sequence, while the reverse primer annealed outside the homology arm, thus amplifying 328 bp of the on-target integrated cassette. For targeted deep sequencing of the plasmid and AAV libraries, the reverse primer annealed to the homology arm. The second PCR step was performed with GoTaq G2 DNA Polymerase (Promega) according to manufacturer instruction using 5 µl of the first-step PCR product and the following amplification protocol: 95°C x 5 min, (95°C x 0.5 min, 60°C x 0.5 min, 72°C x 0.5 min) x 20 cycles, 72°C x 5 min. Second-step PCR primers were endowed with tails containing P5/P7 sequences, i5/i7 Illumina tags to allow multiplexed sequencing and R1/R2 primer binding sites (**Supplementary Table 3**). PCR amplicons were separately purified using MinElute PCR Purification kit (QIAGEN) and AmpPure XP beads (Beckman Coulter). Library quality was assessed by Agilent Tapestation (Agilent Technologies). Amplicons were multiplexed and run on MiSeq 2x75bp or 2x150bp paired end (Illumina).

BAR-Seq analyses

BAR-Seq data were processed with TagDust(Lassmann, 2015) (v2.33) to identify and extract the BAR from each sample by taking advantage of the structural composition of the reads. Each putative BAR was then examined to filter out those having an incorrect nucleotide at the fixed positions or BAR length different from the expected one (22 bp). BAR abundance was quantified by summing the number of identical sequences. Since amplification and sequencing errors may produce highly similar barcodes, a graph-based procedure was employed. For each sample a graph structure was created in which BARs represent nodes and two nodes are linked with an edge if the corresponding sequences have edit distance < 3. Ego subnetworks, *i.e.* subgraphs focalized on highly abundant BARs, were iteratively identified and collapsed into a single node and, consequently, into a single BAR sequence. More precisely, nodes were ranked

based on their counts, and at each iteration the ego network composed by the most abundant BAR and its neighbors were merged into a single BAR (the focal node) and its nodes were removed from the graph. The rationale behind this approach was that, although sequencing errors could produce different sequences, the parental BAR, which constitutes the focal node of the network, would have the highest count. BARs with read count lower than 3 were discarded and the remaining set of BARs were identified as the valid BARs of this sample. To verify that all the samples used in the analysis were informative after the filtering process, we employed a previously described approach to estimate the richness of each sample(Del Core Luca, Montini Eugenio, Di Serio Clelia, 2018), verifying that such value was above the threshold of 95% in all the samples. After BAR ranking from the most to the least abundant, a saturation-based approach was implemented. The dominant set of BARs for each sample was defined as the pool of BARs representing >90% of the total abundance of valid BARs, while the remaining <10% were comprised of rare BARs.

Total RNA-Seq library preparation and analysis

Whole transcriptomic analysis was performed on a pool of HSPCs derived from 5 CB donors. All conditions were performed in triplicate. Total RNA was isolated at 12 h after editing using miRNeasy Micro Kit (QIAGEN), and DNase treatment was performed using RNase-free DNase Set (QIAGEN), according to the manufacturer's instructions. RNA was quantified with The Qubit 2.0 Fluorometer (ThermoFisher) and its quality was assessed by a 2100 Agilent Bioanalyser (Agilent Technologies). Minimum quality was defined as RNA integrity number (RIN)>8. 300 ng of total RNA were used for library preparation with TruSeq Stranded mRNA (Illumina) and sequenced on a NextSeq 500 High 75 (Illumina). Read quality was determined using FastQC and low-quality sequences were trimmed using trimmomatic. Reads were then aligned to the human reference genome (GRCh38/hg38) using STAR, with standard input parameters, and gene counts were produced using Subread featureCounts and Genecode v31 as gene annotation. Transcript counts were processed by R/Bioconductor package edgeR, normalizing for library size using trimmed mean of M-values, and correcting p-values using FDR.

Cell cycle analysis

Cell cycle was analyzed by flow cytometry 12-24 or 96 h after editing by collecting 1-2x10^5 bulk cultured HSPCs. Cells were stained for >1 h at room temperature with 5 µl of solution 1 µg/µl Hoechst 33342 (Sigma-Aldrich) in a final volume of 100 µl and then washed with DPBS

+ 2% heat-inactivated FBS. Gating strategy for flow cytometry analysis is provided in **Supplementary Figure 6**. Data were analyzed with FCS Express 6 Flow.

Indels-based clonal tracking library preparation

PCR amplicons for individual samples were generated by nested PCR using primers listed in **Supplementary Table 3** and starting from >50-100 ng of purified gDNA. The first PCR step was performed with GoTaq G2 DNA Polymerase (Promega) according to manufacturer instruction using the following amplification protocol: 95°C x 5' min, (95°C x 0.5 min, 60°C x 0.5 min, 72°C x 0.25 min) x 20 cycles, 72°C x 5 min. The second PCR step was performed with GoTaq G2 DNA Polymerase (Promega) according to manufacturer instruction using 5 µl of the first-step PCR product and the following amplification protocol: 95°C x 5 min, (95°C x 0.5 min, 60°C x 0.5 min, 72°C x 0.3 min) x 20 cycles, 72°C x 5 min. Second-step PCR primers were endowed with tails containing P5/P7 sequences, i5/i7 Illumina tags to allow multiplexed sequencing and R1/R2 primer binding sites (**Supplementary Table 3**). PCR amplicons were separately purified performing double-side selection with AmpPure XP beads (Beckman Coulter). Library quality was assessed by LabChip® GX Touch HT (Perkin Elmer). Amplicons were multiplexed and sequenced by GeneWiz on MiSeq 2x300bp paired end sequencing (Illumina).

Indels-based clonal tracking analyses

Samples for Indels-based clonal tracking were analyzed with CRISPResso2(Clement *et al*, 2019), a suite of software developed to detect and quantify insertions, mutations and deletions in reads from gene editing experiments. In details, the CRISPRessoBatch pipeline was used to filter NGS reads relying on the phred33 score, getting rid of low-quality sequences, and to remove Illumina TruSeq3-PE adapters using Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic). Then, each couple of paired-end reads was merged using FLASH to produce a single sequence, which was mapped to the input amplicon reference sequence using a global alignment method. The gRNA sequence was uploaded in CRISPResso2 to focus the analysis on the target region. Quantification window was set to 10 nts. As suggested in CRISPResso2 guidelines, the sgRNA was provided without including the PAM sequence. For each sample, identified alleles were quantified by measuring the number of reads and their relative abundance based on total read counts. Alleles showing a relative abundance lower than the false positive threshold (set at 0.3%, based on untreated control) were filtered out.

Quantifications and statistical analyses

"n" indicates biologically independent samples/animals/experiments. For some experiments, different HSPC donors were pooled. Data were summarized as median (± interquartile range) or mean ± SEM depending on data distribution. Inferential techniques were applied in presence of adequate sample sizes ($n \geq 5$), otherwise only descriptive statistics are reported. Two-sided tests were performed. Association between categorical variables was evaluated by means of Fisher's Exact test. Spearman's correlation coefficient was calculated to evaluate the presence of a monotonic relationship between variables. Linear and quadratic regression models were fitted to test for the presence of linear/nonlinear relationships. Mann-Whitney test was performed to compare two independent groups, while in presence of more than two independent groups Kruskal-Wallis test followed by post-hoc analysis using Dunn's test was used. In presence of dependent observations, Friedman test with Dunn's multiple comparisons or Linear/Generalized Mixed-Effects models (LME/GLMER) (Pinheiro *et al*, 2007; Bates *et al*, 2015) were performed. The latter procedures were applied to properly account for the dependence structure among observations, by including additional (nested) random-effect terms, thus considering in the model unobservable sources of heterogeneity among experimental units. When analyzing time courses, treatment group indicator and time variables, along with their interaction, were included as covariates in the model to identify potential differences in growth dynamics of treatment groups. A random intercept model was estimated and, when necessary, nested random effects were considered (e.g., to account for repeated measures of cells/mouse within experiments). GLMER models were applied to properly analyze count data: in particular Poisson mixed models were estimated. Logarithmic and square root transformations of the outcome were also considered to satisfy underlying model assumptions. Post-hoc analysis after LME was performed, considering all the pairwise comparisons of treatment groups at a fixed time point. *p*-values were adjusted using Bonferroni's correction. In all the analyses, the significance threshold was set at 0.05, while ''NS'' means "non significance". Analyses were performed using GraphPad Prism v8.4.2 (GraphPad) and R statistical software. Detailed results of statistical analyses are shown in **Supplementary Table 5**.

Data and software availability

All relevant data are included in the manuscript. BAR-Seq and RNA-Seq data are deposited in GEO with the following access codes: GSE143995 (for RNA-Seq) and GSE144340 (BAR-

Seq). The reagents described in this manuscript are available under a material transfer agreement with IRCCS Ospedale San Raffaele and Fondazione Telethon; requests for materials should be addressed to LN.

Code availability

The BAR-Seq pipeline is freely available at https://bitbucket.org/bereste/bar-seq. To facilitate the analyses, we developed a user-friendly web-application available at http://www.bioinfotiget.it/barseq, which can perform the whole pipeline remotely upon the upload of the input sequencing files and the description of the amplicon structure by specifying the conserved sequences flanking the BAR.

METHODS-ONLY REFERENCES

Bates D, Mächler M, Bolker BM & Walker SC (2015) Fitting linear mixed-effects models using lme4. *J Stat Softw*

Clement K, Rees H, Canver MC, Gehrke JM, Farouni R, Hsu JY, Cole MA, Liu DR, Joung JK, Bauer DE, *et al* (2019) CRISPResso2 provides accurate and rapid genome editing sequence analysis. *Nat Biotechnol* doi:10.1038/s41587-019-0032-3

Del Core Luca, Montini Eugenio, Di Serio Clelia CA (2018) Dealing with Data Evolution and Data Integration: An approach using Rarefaction. In *49th Scientific meeting of the Italian Statistical Society*

Genovese P, Schiroli G, Escobar G, Di Tomaso T, Firrito C, Calabria A, Moi D, Mazzieri R, Bonini C, Holmes MC, *et al* (2014) Targeted genome editing in human repopulating haematopoietic stem cells. *Nature* 510: 235–240

Hsu PD, Scott DA, Weinstein JA, Ran FA, Konermann S, Agarwala V, Li Y, Fine EJ, Wu X, Shalem O, *et al* (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nat Biotechnol*

Lassmann T (2015) TagDust2: A generic method to extract reads from sequencing data. *BMC Bioinformatics*

Notredame C, Higgins DG & Heringa J (2000) T-coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol*

Petrillo C, Thorne LG, Unali G, Schiroli G, Giordano AMS, Piras F, Cuccovillo I, Petit SJ, Ahsan F, Noursadeghi M, *et al* (2018) Cyclosporine H Overcomes Innate Immune Restrictions to Improve Lentiviral Transduction and Gene Editing In Human Hematopoietic Stem Cells. *Cell Stem Cell*

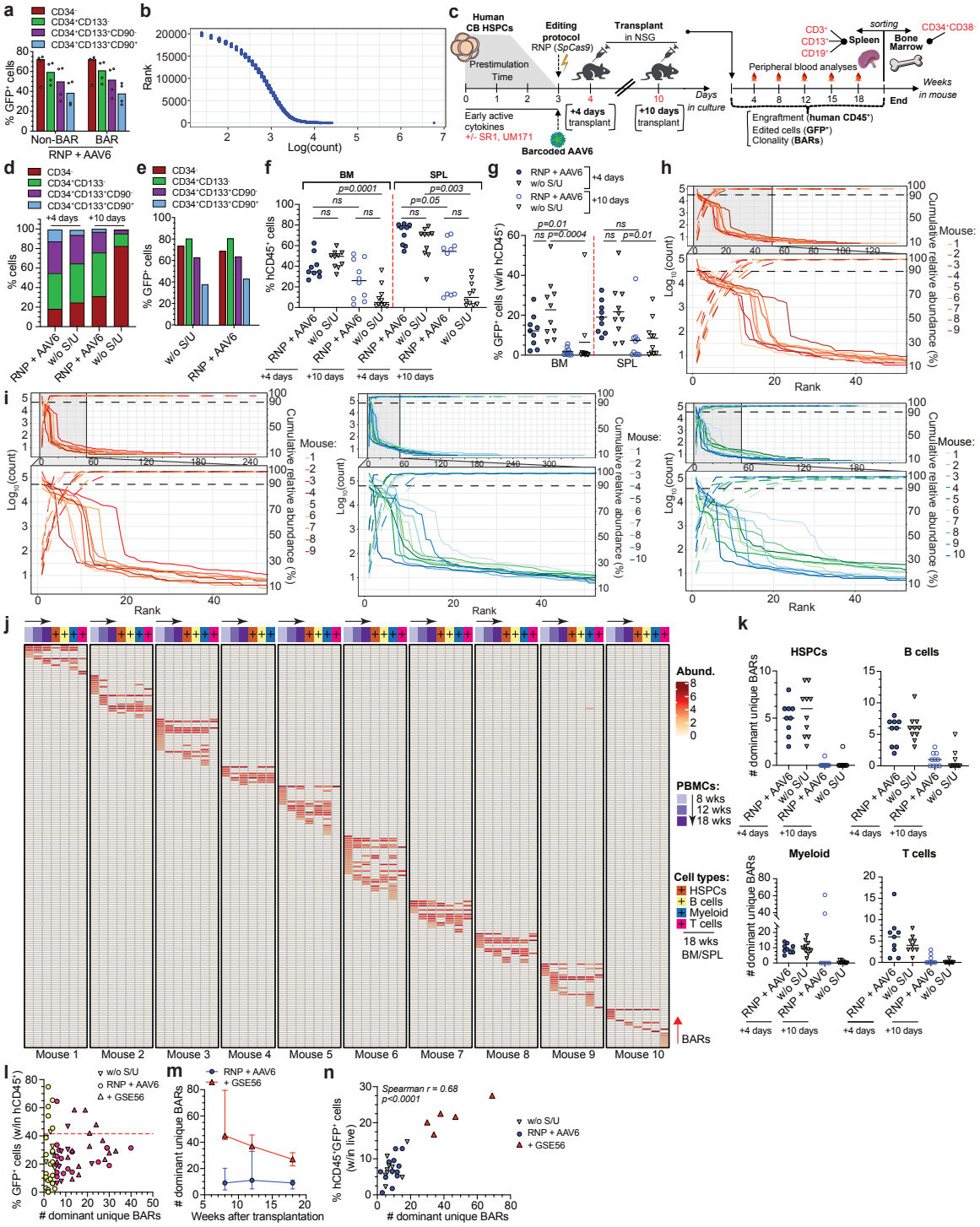
Pinheiro J, Bates D, DebRoy S, Sarkar D & Team the RC (2007) The nlme Package: Linear and Nonlinear Mixed Effects Models. *R-project*

Provasi E, Genovese P, Lombardo A, Magnani Z, Liu PQ, Reik A, Chu V, Paschon DE, Zhang L, Kuball J, *et al* (2012) Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nat Med*

Schiroli G, Conti A, Ferrari S, della Volpe L, Jacob A, Albano L, Beretta S, Calabria A, Vavassori V, Gasparini P, *et al* (2019) Precise Gene Editing Preserves Hematopoietic Stem Cell Function following Transient p53-Mediated DNA Damage Response. *Cell Stem Cell*

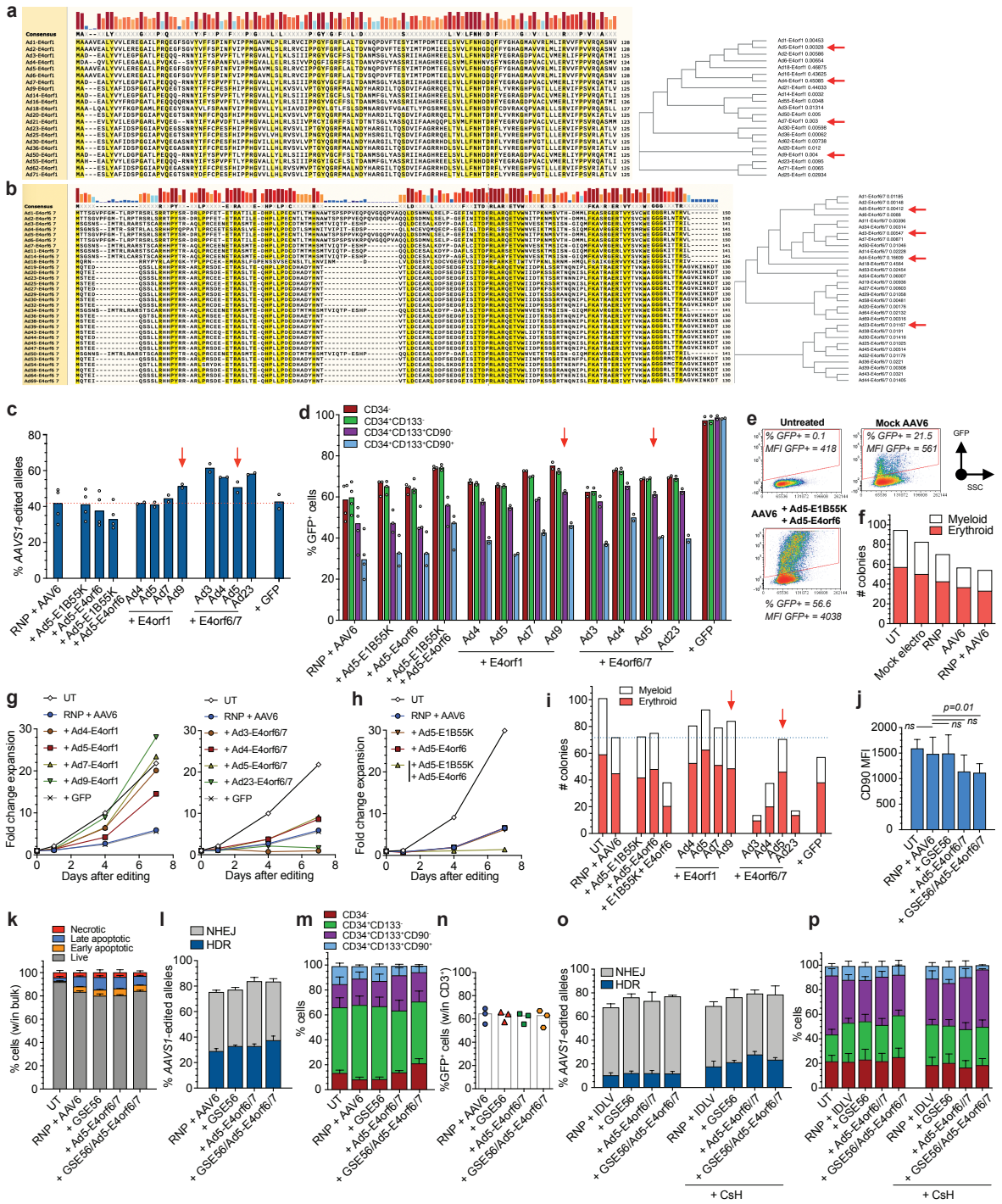
Schiroli G, Ferrari S, Conway A, Jacob A, Capo V, Albano L, Plati T, Castiello MC, Sanvito F, Gennery AR, *et al* (2017) Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1. *Sci Transl Med* 9: eaan0820

EXTENDED DATA



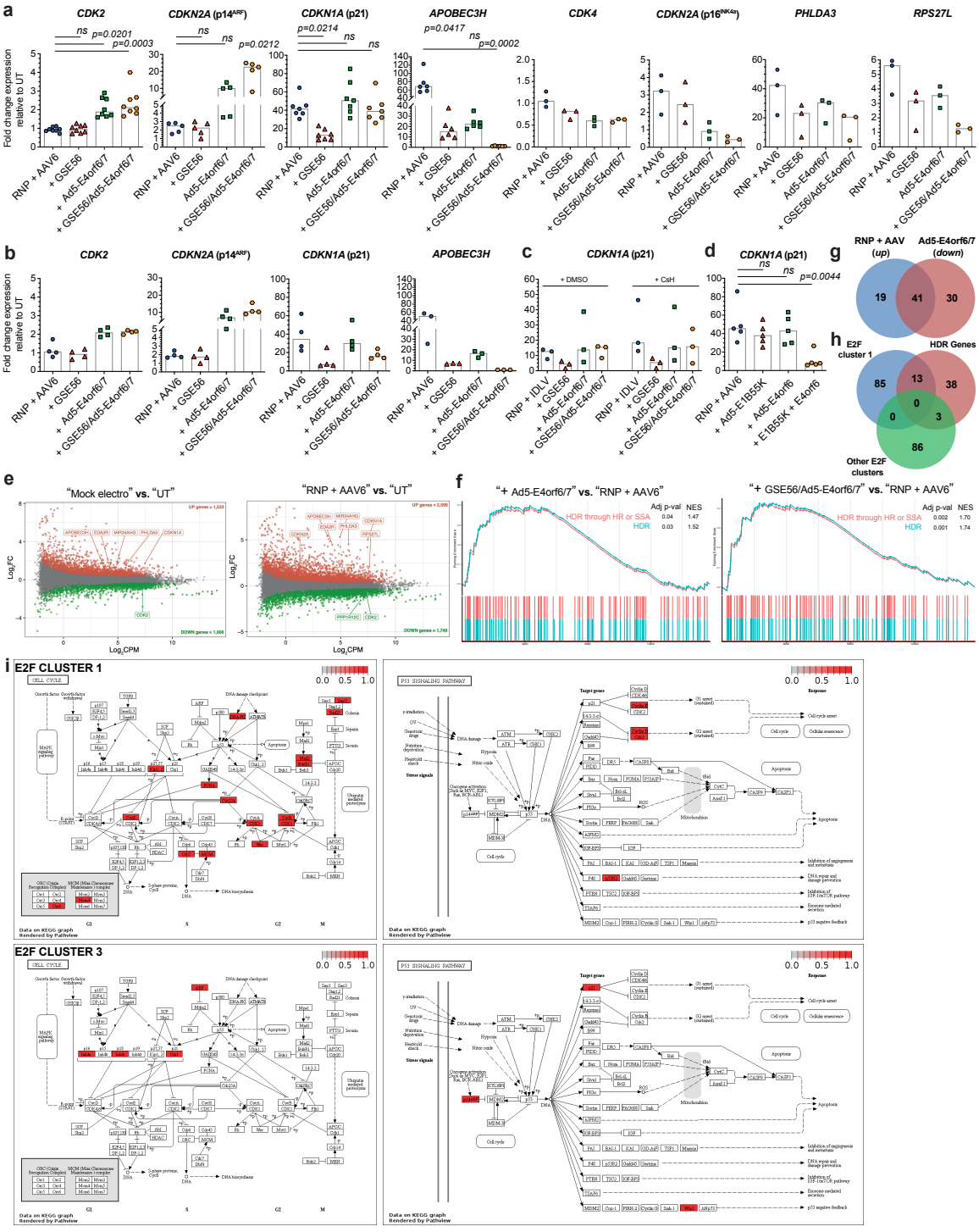
Extended Data Figure 1. BAR-Seq dissects clonal dynamics of HDR-edited cells. a, Percentage of GFP$^+$ cells within subpopulations 96 h after *AAVS1* editing with the barcoded or non-barcoded AAV6 (3 HSPC donors; n = 4). Median. **b**, Number of unique BARs and relative abundances in bulk cultured HSPCs 72 h after editing. One representative sample out of two is shown. **c**, Experimental scheme. **d-e**, Culture composition (**d**) and percentage of GFP$^+$ cells

within subpopulations (**e**) of *AAVS1* edited HSPCs with the indicated treatments at the time of transplant and 96 h after editing, respectively (10 HSPC donors; n = 1). **f-g**, Percentage of $hCD45^+$ cells (**f**) and GFP^+ cells within human graft (**g**) in BM or spleen (SPL) of mice from Fig. 1c (n = 9, 10, 6, 3). Median. Kruskal-Wallis test. **h-i**, Abundance of ranked BARs from PBMCs collected at 8 (**h**) and 12 (**i**) weeks after transplant, as in Fig. 1e. **j**, Heatmap as in Fig. 1f for "w/o S/U (+4 days)"-transplanted mice. **k**, Number of dominant unique BARs in sorted $hCD45^+$ cell lineages and HSPCs of mice from Fig. 1c. Mice with % of circulating $hCD45^+GFP^+$ cells at 18 weeks timepoints < 0.1 were plotted with BAR count = 0 (n = 9, 10, 10, 10). Median. **l**, Correlation between the percentage of GFP^+ cells (within $hCD45^+$) and the number of dominant unique BARs in "w/o S/U", "RNP + AAV6" and "+ GSE56" mice of this study (n = 71). Each dot represents one mouse. Mice with number of dominant unique BARs ≥6 (arbitrary threshold) are shown in magenta (coefficient of variation (CV) = 0.51); mice with number of dominant unique BARs <5 are shown in yellow (CV = 0.87). Dashed line indicates the median percentage of GFP^+ cells within $CD90^+$ HSPCs in the *in vitro* outgrown of transplanted edited cells. **m**, Longitudinal PBMC analysis as in Fig. 1k but including in the analysis >95% of total BAR reads (n = 4, 5). Median. **n**, Correlation as in Fig. 1l at 8 weeks after transplant (n = 28). Spearman correlation coefficient was calculated. All statistical tests are two-tailed. n indicate independent animals.



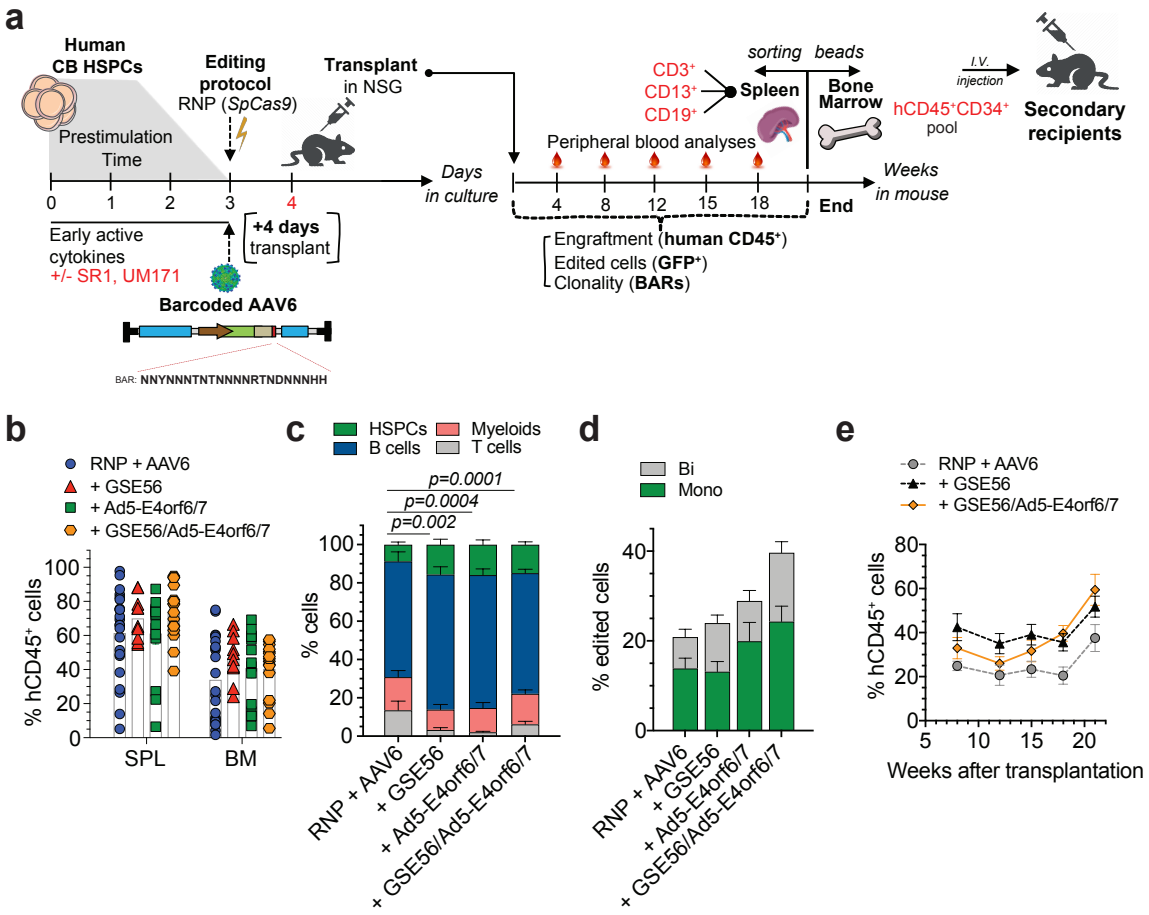
Extended Data Figure 2. Identification of Ad protein variants improving HDR efficiency. **a-b**, Multiple sequences alignment of E4orf1 (**a**) and E4orf6/7 (**b**) Ad variants. Sequences were collected from online RCSB Protein Data Bank and UniProt. **c-d**, Percentage of HDR-edited alleles (**c**) and GFP$^+$ cells within subpopulations (**d**) 96 h after *AAVS1* editing in bulk CB HSPCs with indicated treatments (n = 4, 4, 4, 4; other treatments: n = 2). Median. **e**, FACS plots of untreated (UT) and AAV6-transduced HSPCs in absence ("Mock AAV6") or presence of Ad5-

E1B55K+Ad5-E4orf6 measured 24 h after treatments. The results of one representative experiment out of three is shown. **f**, Number of colonies from bulk edited HSPCs in the indicated treatments (n = 2). Mean. **g-h**, Fold change expansion of live HSPCs after indicated treatments from Extended Data Fig. 2c (n = 2). Median. **i**, Number of colonies from bulk edited HSPCs with the indicated treatments (n = 2). Mean. **j**, CD90 MFI in edited HSPCs measured 96 h after editing with indicated treatments (n = 6). Median with IQR. Friedman test with two-tailed Dunn's multiple comparisons. **k**, Percentage of live, early/late apoptotic and necrotic bulk HSPCs 24 h after editing with the indicated treatments (7 HSPC donors; n = 3). Mean ± SEM. **l-m**, Percentage of HDR/NHEJ-edited alleles (**l**) and culture composition (**m**) 96 h after editing of bulk mPB HSPCs from Fig. 2h (n = 3). Mean ± SEM. **n)** Percentage of GFP^+ T cells 14 days after *AAVS1* editing with indicated treatments (n = 3). Median. **o-p**, Percentage of HDR/NHEJ-edited alleles (**o**) and culture composition (**p**) 96 h after IDLV-based editing of bulk CB HSPCs from Fig. 2i (n = 3). Mean ± SEM. Red arrows indicate Ad protein variants selected for further investigation. n indicate independent experiments.

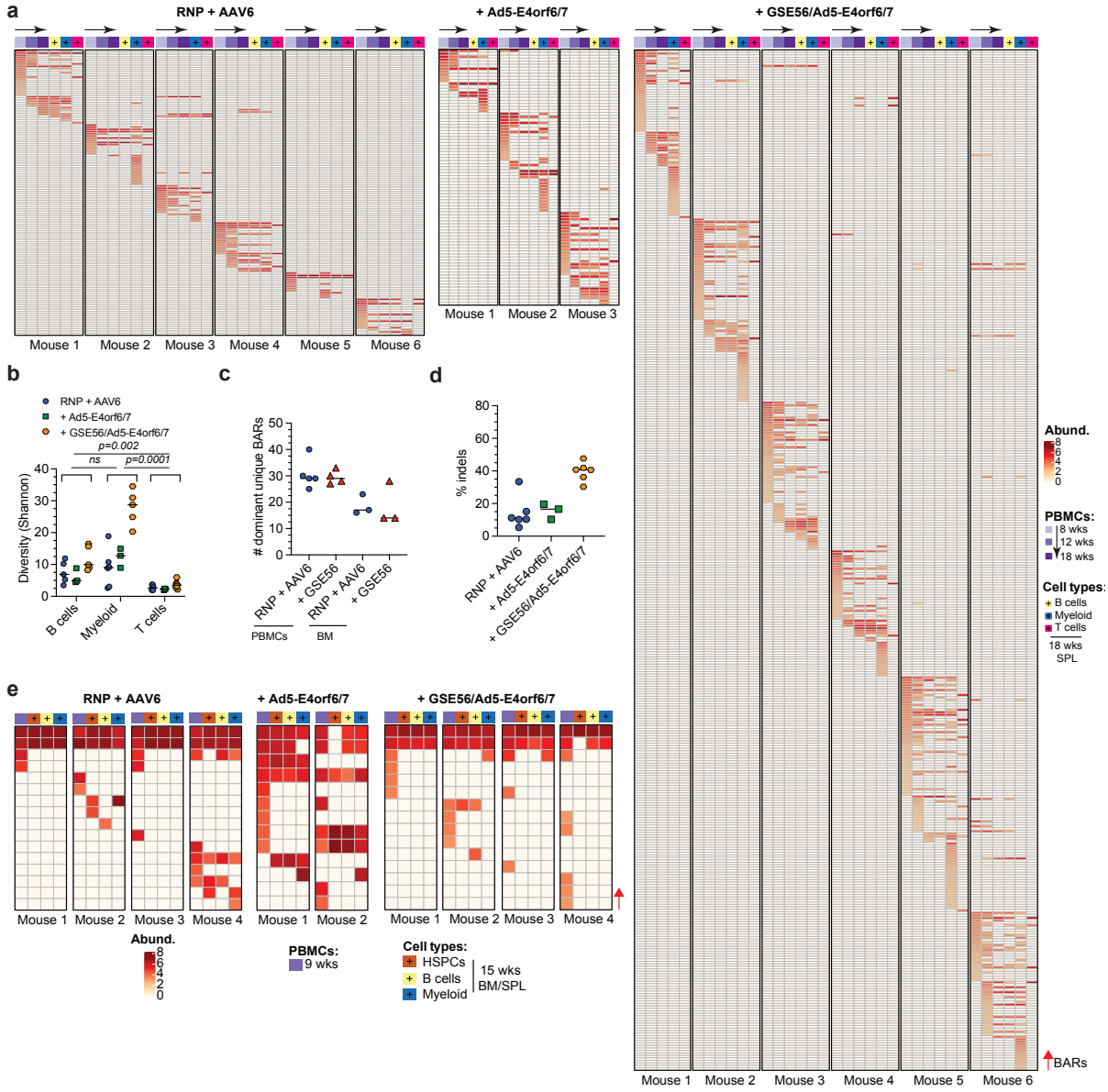


Extended Data Figure 3. Investigating the transcriptional response upon enhanced editing. a-b, Fold change expression of cell cycle related genes relative to UT 24 h after AAV-based editing with the indicated treatments in CB (**a**) or mPB (**b**) HSPCs (CB: n = 8, 5, 7, 6, 3, 3, 3, 3; mPB: n = 4, 4, 4, 3). Median. **c**, Fold change expression of *CDKN1A* relative to UT 24 h after IDLV-based editing with indicated treatments in CB HSPCs (n = 3). Median. **d**, Fold change expression of *CDKN1A* relative to UT at 24 h after AAV-based editing with indicated

treatments in CB HSPCs (n = 5). Median. **e,** MA plots showing significant down- (green) and up- (red) regulated genes after *AAVS1* editing in mock electroporated (left) and standard edited (right) compared to UT (n = 3). *PPP1R12C*, the *AAVS1* hosting gene appears among the down-regulated genes, concordantly with previous reports showing transient transcriptional repression at the site of DNA DSB[15]. **f,** Random walk plots for the indicated Reactome categories. Relative adjusted p-values and NES are shown. **g,** Venn diagram showing the number of genes related to the "Allograft rejection" category upregulated upon standard editing and downregulated in presence of "+ Ad5-E4orf6/7" treatment. **h,** Venn diagram showing the number of HDR genes ("Homology directed repair" category from Reactome database) shared with E2F pathway target genes (Hallmark gene set) from cluster 1 or other clusters from Fig. 3e. **i)** Schematic of "cell cycle" and "p53 pathway" KEGG gene ontologies highlighting genes (red) belonging to clusters 1 (top) and 3 (bottom) of Fig. 3e. For all panels with statistical analysis: Friedman test with two-tailed Dunn's multiple comparisons. n indicate independent experiments, except for Extended Data Fig. 3e where n indicates independent samples.

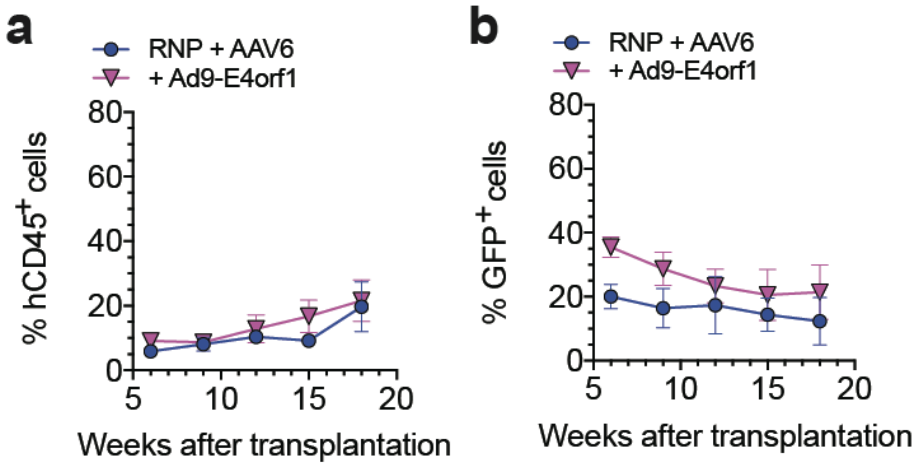


Extended Data Figure 4. Transplantation of enhancer edited HSPCs in NSG mice. a, Experimental workflow. **b**, Percentage of hCD45$^+$ cells in SPL and BM of mice from Fig. 4a and 4b (n = 23, 11, 15, 16). LME followed by post-hoc analysis. Mean ± SEM. **c**, BM cell composition in mice from Fig. 4a and 4b. LME followed by post-hoc analysis for HSPCs (n = 23, 11, 15, 16). Mean ± SEM. **d**, Percentage of cells harboring monoallelic or biallelic integration(s) in SPL of mice from Fig. 4a and 4b (n = 23, 11, 15, 16). Mean ± SEM. **e**, Percentage of circulating hCD45$^+$ cells in mice transplanted with CB HSPCs *IL2RG*-edited in presence of GSE56 and Ad5-E4orf6/7 (n = 4). Comparison with the previously published results for "RNP + AAV6" and "+ GSE56" groups[22] is shown (n = 5, 6). All statistical tests are two-tailed. n indicate independent animals.

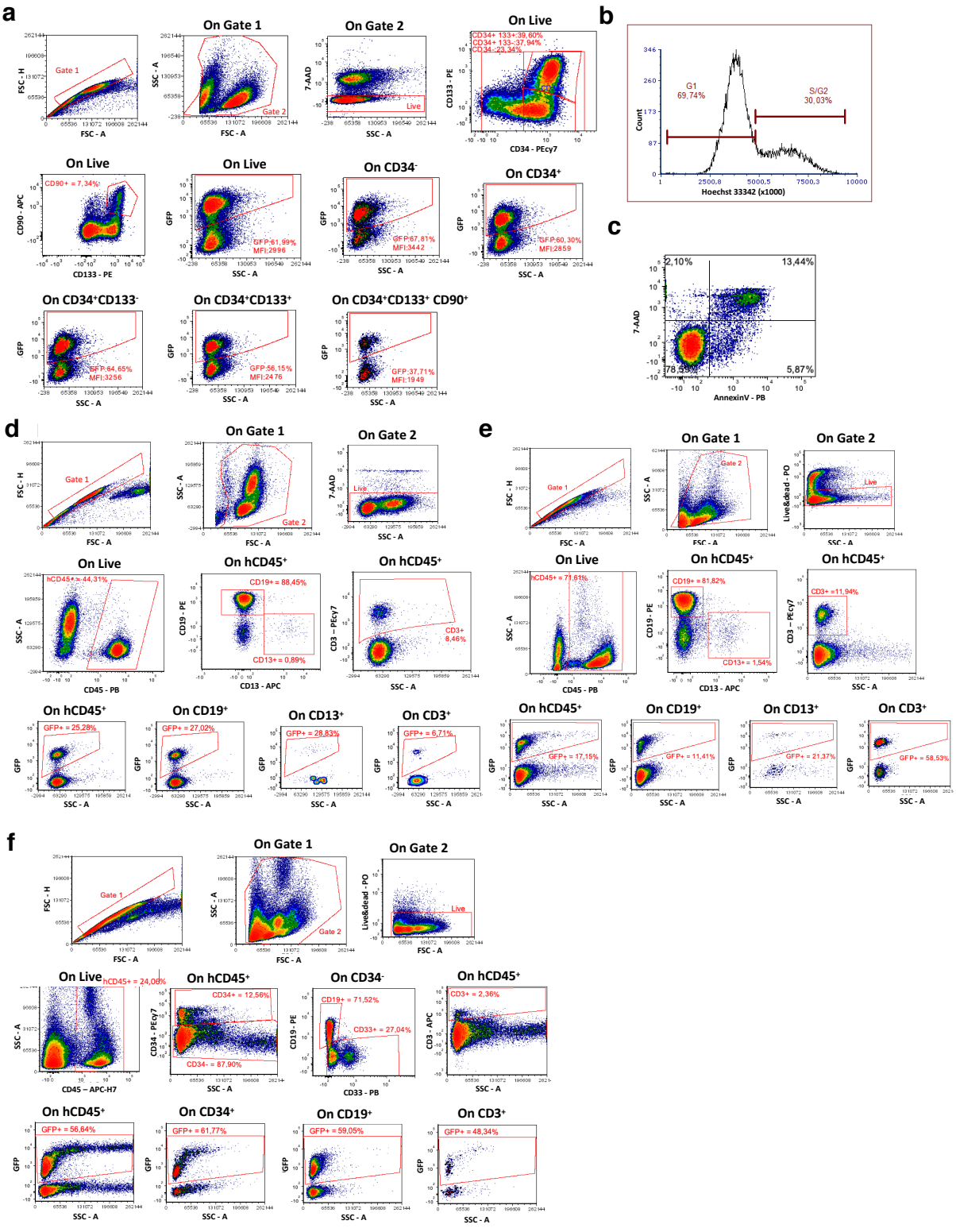


Extended Data Figure 5. Enhanced editing preserves multilineage repopulation capacity and self-renewing potential of individual edited HSPC clones. a, Heatmap showing the abundance (red-scaled palette) of dominant unique BARs (rows) retrieved in PBMCs at indicated times after transplant and sorted hCD45$^+$ cell lineages of mice from one experiment of Fig. 4a (separated columns). **b**, Clonal diversity within sorted hCD45$^+$ cell lineages in mice from Extended Data Fig. 5a (B cells: n = 5, 3, 5; Myeloid and T cells: n= 6, 3, 3). Median. Two-tailed Friedman test with Dunn's multiple comparisons. Experimental groups were unified for statistical analysis. **c**, Number of dominant unique BARs in PBMCs or BM of mice from one experiment in Fig. 4a (PBMCs: n = 5, 4; BM: n = 3, 3). Median. **d**, Percentage of NHEJ-edited alleles within the non-HDR edited fraction from Fig. 5g (n = 6, 3, 6). Median. **e**, Heatmaps as in Extended Data Fig. 5a showing the dominant unique BARs in 9-weeks PBMCs and in sorted hCD45$^+$ cell lineages (15 weeks) of secondary recipients. n indicate independent animals.

SUPPLEMENTARY FIGURES



Supplementary Figure 1. Transplantation of HSPCs edited in presence of Ad9-E4orf1 in NSG mice. a-b, Percentage of circulating hCD45$^+$ (**a**) and GFP$^+$ cells within human graft (**b**) in mice transplanted with the outgrown progeny of starting-matched limiting doses of CB HSPCs edited in *AAVS1* with indicated treatments (n = 4). When we evaluated *in vivo* the potential benefit of adding Ad9-E4orf1 to the HSPC editing treatment we found no improvement in human cell engraftment and only a minor increase in the percentage of HDR-edited cells in the graft, thus prompting no further investigation. Mean ± SEM. n indicates independent animals.



Supplementary Figure 2. Gating strategies for flow cytometry analyses. a, HSPC phenotype 96 h after treatments; **b**, Cell cycle phases 24/96 h after treatments; **c**, Apoptotic/necrotic cells 24 h after treatments; **d**, Human PBMCs in transplanted mice; **e**, Human cells within BM of transplanted mice; **f**, Human cells within SPL of transplanted mice.

CHAPTER III

Ex vivo editing of the human genome with programmable nucleases holds great potential for the treatment of several human blood disorders. Site-specific gene correction and restoration of physiological expression and regulation allows to design and tailor strategies that extend beyond the capabilities of semi random gene addition. The main advances of our study over current knowledge and published work are the following:

- The first to our knowledge adoption of an *ad hoc* designed clonal tracking strategy to stringently monitor HSC dynamics *in vivo* upon HDR-mediated gene editing. Technical challenges for developing such strategy were the identical reconstituted sequence at the target site upon editing and the need to barcode the AAV repair template achieving adequately high complexity of the library.
- The uncovering, by using such clonal tracking approach, of a significant "cost" of the editing procedure, which lowers substantially the number of clones engrafting in transplanted hosts. We previously reported robust p53 pathway activation upon HDR-mediated gene editing of HSPC impacting hematopoietic reconstitution and its rescue by transient expression of a dominant negative p53 mutant protein. It remained unknown whether such outcome was due to altered growth properties of the treated cells or improved preservation of repopulating cells during editing. Here we prove that inhibiting the p53 response to gene editing rescues polyclonal reconstitution by edited HSC without altering the size and lineage composition of their output.
- The development of an optimized protocol which overcomes the major biological barriers to HDR editing in HSC. Whereas it has long been contended that the quiescent feature of primitive HSC hinders HDR-mediated gene editing, the underlying mechanism of such block remained unclear. By forcing cell cycle progression, we show that the crucial enabling factor is pervasive upregulation of the HDR machinery accompanying engagement in S/G2 phases. Intriguingly, such complex choreography, which we mechanistically describe by global transcriptomic analysis, could be set in motion by the transient expression of an adenoviral protein (Ad5-E4orf6/7) naturally evolved to plug directly into the master cell cycle regulator E2F. By combining transient expression of this newly found adenoviral effector with the previously reported p53 inhibition, we reach HDR editing rates in long-term repopulating human HSC, which surpass those reported until now in the literature. Such outcome was reproducible

across several HSC donors and sources, genomic loci and conceivably portable to most types of editing platforms.

- The stringent validation of the improved protocol by tracking the clonal dynamics and individual output of treated HSC, which show full preservation of *in vivo* clonogenic output, multipotency and self-renewal capacity. Indeed, our data provide the first direct evidence that HDR-edited human HSC can undergo multiple rounds of symmetric and asymmetric divisions in primary and secondary xenogeneic host.

Despite the described improvements in HSC editing efficiency, complete HDR-mediated editing of a manipulated HSC sample is still far from reach. Yet, each disease requires a different correction threshold for therapeutic benefit, which in some case may well fall within the reach of current technologies: while as little as 10% functional HSC may suffice for correcting SCID-X1 (Schiroli *et al*, 2017), higher thresholds are expected to be required for other diseases, such as SCD (Abraham *et al*, 2017). Moreover, some aspects need to be further investigated before moving our enhanced gene editing protocol "from-bench-to-bedside", such as: i) the capacity of edited HSC to persist long-term in vivo and actively contribute to multi-lineage hematopoiesis and/or return to quiescence; ii) the genotoxic risk of the editing procedure.

Gene Editing impacts long-term engrafting HSCs

HSC gene addition require short-term protocols of *ex vivo* culture (1-2 days) before vector transduction, when primitive HSCs are characterized by low expression of DDR and DSB repair genes (Beerman *et al*, 2014; Biechonski *et al*, 2018). Thus, gene correction strategy requires more prolonged and extensive manipulation (3-4 days) to increase permissiveness to HDR and engage cell cycle progression (Genovese *et al*, 2014; Zonari *et al*, 2017). Culture conditions must then be tailored to preserve the repopulation capacity of edited HSCs. If the HSC harvest is limiting, expansion of the initial cell population may be an option (Kumar & Geiger, 2017). The addition in the culture media of SR1 and UM171 improved the early phase of hematopoietic reconstitution post-transplant by increasing the number of short-term contributing clones and the overall extent of repopulation after prolonged *ex vivo* culture. Our data indicate that SR1 and UM171 preserve edited HSPCs, while suggesting that further efforts are needed to successfully expand long-term HSCs. Recent identification of novel stem-cell preserving compounds (Xie *et al*, 2019) and the implementation of 3D structure and/or low-oxygen

culture systems (Kobayashi *et al*, 2019; Bai *et al*, 2019) might substantially improve the potential of *ex vivo* HSPC expansion.

Clonal tracking with BAR-seq show at single-cell resolution that HDR-edited HSPCs are functional, capable of long-term multi-lineage repopulation in xenograft mouse model and undergo multiple rounds of self-renewing divisions, as shown by the clonal recapturing among primary and secondary recipients belonging to the same experimental group. The substantial loss of repopulating clones among edited HSPCs well explains the lower levels of human engraftment reported after transplanting edited vs. untreated cells. The mechanism underlying this loss can be ascribed to the robust editing-induced activation of the p53 pathway and its downstream effectors, such as p21, p14 and p16, which suggest induction of detrimental processes on HSC biology like permanent growth arrest, senescence and apoptosis (Conti & Di Micco, 2018). Of note, although we measured some increase in apoptosis among treated primitive HSPCs, its extent was limited and cannot fully explain the several-fold loss in engrafting clones. Importantly, multilineage potential, clonal dynamics and long-term persistence of engrafting HDR-edited HSPCs was not different among all treatments tested. This finding suggests that a threshold level of activation of p53 response and p21 expression might dictate the full preservation or irreversible loss of edited-HSC repopulation potential (van den Berg *et al*, 2018). This hypothesis is supported by the rescue of cells undergoing higher DDR burden presenting higher indels frequency and diversity or bi-allelic HDR targeting in presence of p53 inhibitor.

Our data show that cell cycle regulation represents a fundamental rate-limiting step for HDR editing in HSPCs. However, despite the fraction of cells in S/G2 cell cycle phases was similar at the time of editing between bulk and $CD90^+$ HSPCs, HDR efficiency was always lower in the latter cells. This observation suggests that, beside the requirement for progression to S/G2 phases, other factors may account for the lower HDR efficiency in $CD90^+$ cells, such as the low basal expression and activity of the HDR machinery and delayed transit through the G1/S checkpoint (Laurenti *et al*, 2015). The transient expression of Ad5-E4orf6/7 promotes a pleiotropic cellular response upregulating the vast majority of E2F target genes, which are likely to be collectively responsible for HDR enhancement. Of note, this pervasive modulation of a highly integrated cellular network by a viral protein naturally evolved to capture the benefits of cell proliferation for viral infection might be difficult to replicate with small drugs or other strategies targeting individual genes engaged in the process. The consequent forced cell cycle progression *per se* did not reduce neither the clonogenic capacity and the clonal repertoire, nor the engraftment of treated HSPCs. Notably, the HDR increase by Ad5-E4orf6/7 was further enhanced by its combination with the GSE56, which can be explained by counteraction of the

p53-dependent negative feedback loop triggered by E2F activation, as also previously reported (Komori *et al*, 2005). In parallel, E2F directly promotes *CDKN1A* gene activation (p21) in a p53-independent manner (Radhakrishnan *et al*, 2004) that might also explain why Ad5-E4orf6/7 did not increase engraftment of standard edited cells and lowered the graft increase induced by GSE56, while achieving in both conditions a higher proportion of edited cells within similar graft size. Of note, the number of clones upon Ad5-E4orf6/7 addition might be underestimated if upregulation of the HDR machinery increased the proportion of cells undergoing template integration before replication of the targeted locus, thus producing two clones with the same BAR. Enrichment strategy of edited cells may be a valuable solution to further increase the proportion of edited cells, thus narrowing the gap towards the required therapeutic threshold (Dever *et al*, 2016), albeit at the expense of a lower absolute number of cells and clonal repertoire.

Importantly, BAR-Seq and indels-based clonal tracking analyses are limited to the graft outgrown from edited HSPCs including clones contributing up to 95% of such graft. Our analysis was also blind to certain aspects of hematopoietic dynamics, such as HSPC quiescence and the activity of short-lived committed progenitors providing limited output. Whereas these limitations do not affect the comparison of different editing protocols for their impact on robust repopulating HSPCs, we can only make inference on the reference values for untreated or unedited cells. If we consider that dominant repopulating clones accounted for 1 every $2x10^3$-$2x10^4$ edited $CD34^+$ cells (from the best to the least preserving conditions, in presence or absence of the p53 inhibitor) throughout these experiments, such frequency is consistent with previous estimates of SCID-repopulating cells in cultured CB $CD34^+$ cells assayed by limiting dilution transplantation (Wang *et al*, 1997; Zonari *et al*, 2017; Wagenblast *et al*, 2019; Bai *et al*, 2019).

The use of hematochimeric mice might be a theoretical limitation in these studies. Although xenograft in NSG mice is the state-of-the-art preclinical model to assess long-term persistence of *ex vivo* manipulated human HSPCs, the absence of a humanized niche, the limited functionality of the murine primary lymphatic organs (e.g. thymus) and the absence of human cytokines may potentially introduce confounding effects on hematopoietic reconstitution dynamics of human HSPCs. While advanced humanized *in vivo* models (e.g. ossicles) (Abarrategi *et al*, 2017) might extend and complement the knowledge at preclinical level on edited HSPC behavior, data from large animal models and clinical trials in humans would ultimately provide substantial indications about long-term persistence of edited HSPCs.

Genotoxic Risk of Genome Editing

Safety concerns of gene editing revolve around unwanted genomic events, such as off-targets, large deletions (Kosicki *et al*, 2018; Adikusuma *et al*, 2018; Cullot *et al*, 2019), translocations and random insertion of arbitrary DNA sequences (e.g. AAV or IDLV) (Hanlon *et al*, 2019; Nelson *et al*, 2019), chromothripsis. Up to now these events are difficult to precisely quantify and of uncertain clinical significance. Presumably, their consequences depend on the specific context. However, precaution dictates that efforts should be made towards minimizing their incidence. Indeed, we found low but detectable chromosomal translocations *in vitro* between on- and off- target alleles in edited HSPCs. These translocations were undetectable in the human graft, possibly suggesting a tendency toward counterselection of cells harboring abnormal chromosomal rearrangements or lower frequency of these events in long-term engrafting HSPCs. Our experimental conditions only partially inhibit p53 signaling within the first 24 hours post-editing, while maintaining proficient DNA repair. The detrimental effect of p53 activation could potentially confer selective advantage to rare $p53^{-/-}$ cells (Enache *et al*, 2020). Importantly, limited and transient inhibition by GSE56 of the editing-induced p53 response would reduce the risk of selecting for p53 mutant clones and mono/oligo-clonal expansion. We did not detect any evidence for increased occurrence of chromosomal aberrations and mutational burden when editing was performed in the presence of GSE56 and/or Ad5-E4orf6/7, although with the caveats of limited sensitivity of these analyses. In any case, even if oncogenic aberrations occur, prompt restoration of the p53 pathway may counter-select cells that have acquired them (Di Micco *et al*, 2006; Martins *et al*, 2006). Interestingly, GSE56 and Ad5-E4orf6/7 synergistically abolished the p53-dependent transcriptional activation of the DNA cytidine deaminase *APOBEC3H* upon editing procedure, thus potentially protecting the treated cells from a further source of genotoxicity (Sakofsky *et al*, 2019).

A growing set of evidence shows that other unwanted outcomes might occur at detectable frequencies upon editing. Recent reports showed that the repair of nuclease-induced DNA DSB might result in large deletions at the cleavage site in several cell types, including mouse hematopoietic progenitors (Kosicki *et al*, 2018). In our work, on-target trapping of AAV fragments, including ITRs, in long-term repopulating HSPCs was unexpected due to the transient expression of the nuclease and the rapid AAV dilution but consistent with previous findings in murine models of Duchenne Muscular Dystrophy treated with AAV expressing the CRISPR/Cas9 system (Nelson *et al*, 2019). Yet, the implication of large deletions, chromosomal rearrangements and trapping of AAV fragments on the safety profile and long-term genotoxic risk is still unknown and have to be fully elucidated. Another potential on target adverse event of gene editing

is chromothripsis, an extensive rearrangement affecting chromosomes harboring DNA breaks which generates abnormal nuclear structures, such as micronuclei and chromosome bridges. Whether this catastrophic event occurs during HSC editing and might even be increased upon enhanced conditions remains to be investigated, including whether any HSC undergoing such process would survive and remain capable of engrafting in vivo.

In the context of HDR-mediated therapeutic strategies, enrichment of edited cells may reduce to some extent the proportion of cells carrying undesired on-target events. Conversely, enrichment approaches or enhanced editing protocols would not *per se* decrease the proportion of cells carrying off-target edits.

Although detailed analyses and considerations on nuclease specificity are imperative, the presence of unwanted genomic events does not necessarily preclude gene editing from succeeding in translating to clinical applications. An unintended genomic event may in theory contribute to cancer, depending on its genomic location and its nature. However oncogenic transformation is multifaceted and multistep. Thus, the same mutations may or not give rise to tumors also depending on genetic background and the subsequent exposure to other genotoxic events. Furthermore, the consequences of off-target events are expected to be different according to the cell type and may be more tolerated by fully differentiated or short-lived cell types (Hirakawa *et al*, 2020).

Toward therapeutic Genome Editing

HSPC based therapy may be an option for a number of diseases that are not amenable to be corrected with differentiated cells. Ultimately, the rationale of testing novel gene editing-based strategies depends on the presumed benefit offered to the patient with respect to his prognosis with the best available therapy. Indeed, it is reasonable to offer gene editing based products at first to patients with no alternative options and a dismal prognosis, e.g. cancer, or to those for who the standard of care is presumed to be more toxic, such as congenital immunodeficiencies.

Clinical testing of gene editing approaches in these fields would provide a first detailed characterization of their safety profile. This would also allow to define the appropriate assays to follow the dynamics of unwanted genomic events in time and establish their clinical relevance, setting the thresholds to manage the genotoxic risk. This data would thus pave the way for their application to other diseases with a less dismal prognosis and alternative therapies, such as haemoglobinopathies and HIV. Ultimately, the road towards successful commercialization of these approaches will have to account for the alternative therapeutic options under development, as well as the health and monetary value attributable to the therapeutic intervention itself.

REFERENCES

Abarrategi A, Foster K, Hamilton A, Mian SA, Passaro D, Gribben J, Mufti G & Bonnet D (2017) Versatile humanized niche model enables study of normal and malignant human hematopoiesis. *J Clin Invest*

Abraham A, Hsieh M, Eapen M, Fitzhugh C, Carreras J, Keesler D, Guilcher G, Kamani N, Walters MC, Boelens JJ, *et al* (2017) Relationship between Mixed Donor–Recipient Chimerism and Disease Recurrence after Hematopoietic Cell Transplantation for Sickle Cell Disease. *Biol Blood Marrow Transplant*

Adikusuma F, Piltz S, Corbett MA, Turvey M, McColl SR, Helbig KJ, Beard MR, Hughes J, Pomerantz RT & Thomas PQ (2018) Large deletions induced by Cas9 cleavage. *Nature*

Bai T, Li J, Sinclair A, Imren S, Merriam F, Sun F, O'Kelly MB, Nourigat C, Jain P, Delrow JJ, *et al* (2019) Expansion of primitive human hematopoietic stem cells by culture in a zwitterionic hydrogel. *Nat Med*

Beerman I, Seita J, Inlay MA, Weissman IL & Rossi DJ (2014) Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. *Cell Stem Cell*

van den Berg J, Manjón AG, Kielbassa K, Feringa FM, Freire R & Medema RH (2018) A limited number of double-strand DNA breaks is sufficient to delay cell cycle progression. *Nucleic Acids Res*

Biechonski S, Olender L, Zipin-Roitman A, Yassin M, Aqaqe N, Marcu-Malina V, Rall-Scharpf M, Trottier M, Meyn MS, Wiesmüller L, *et al* (2018) Attenuated DNA damage responses and increased apoptosis characterize human hematopoietic stem cells exposed to irradiation. *Sci Rep* 8

Conti A & Di Micco R (2018) p53 activation: A checkpoint for precision genome editing?

Cullot G, Boutin J, Toutain J, Prat F, Pennamen P, Rooryck C, Teichmann M, Rousseau E, Lamrissi-Garcia I, Guyonnet-Duperat V, *et al* (2019) CRISPR-Cas9 genome editing induces megabase-scale chromosomal truncations. *Nat Commun*

Dever DP, Bak RO, Reinisch A, Camarena J, Washington G, Nicolas CE, Pavel-Dinu M, Saxena N, Wilkens AB, Mantri S, *et al* (2016) CRISPR/Cas9 β-globin gene targeting in human haematopoietic stem cells. *Nature*

Enache OM, Rendo V, Abdusamad M, Lam D, Davison D, Pal S, Currimjee N, Hess J, Pantel S, Nag A, *et al* (2020) Cas9 activates the p53 pathway and selects for p53-inactivating mutations. *Nat Genet*

Genovese P, Schiroli G, Escobar G, Di Tomaso T, Firrito C, Calabria A, Moi D, Mazzieri R, Bonini C, Holmes MC, *et al* (2014) Targeted genome editing in human repopulating haematopoietic stem cells. *Nature* 510: 235–240

Hanlon KS, Kleinstiver BP, Garcia SP, Zaborowski MP, Volak A, Spirig SE, Muller A, Sousa AA, Tsai SQ, Bengtsson NE, *et al* (2019) High levels of AAV vector integration into CRISPR-induced DNA breaks. *Nat Commun*

Hirakawa MP, Krishnakumar R, Timlin JA, Carney JP & Butler KS (2020) Gene editing and CRISPR in the clinic: Current and future perspectives. *Biosci Rep*

Kobayashi H, Morikawa T, Okinaga A, Hamano F, Hashidate-Yoshida T, Watanuki S, Hishikawa D, Shindou H, Arai F, Kabe Y, *et al* (2019) Environmental Optimization Enables Maintenance of Quiescent Hematopoietic Stem Cells Ex Vivo. *Cell Rep*

Komori H, Enomoto M, Nakamura M, Iwanaga R & Ohtani K (2005) Distinct E2F-mediated transcriptional program regulates p14ARF gene expression. *EMBO J*

Kosicki M, Tomberg K & Bradley A (2018) Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. *Nat Biotechnol*

Kumar S & Geiger H (2017) HSC Niche Biology and HSC Expansion Ex Vivo. *Trends Mol*

Med doi:10.1016/j.molmed.2017.07.003

Laurenti E, Frelin C, Xie S, Ferrari R, Dunant CF, Zandi S, Neumann A, Plumb I, Doulatov S, Chen J, *et al* (2015) CDK6 levels regulate quiescence exit in human hematopoietic stem cells. *Cell Stem Cell*

Martins CP, Brown-Swigart L & Evan GI (2006) Modeling the Therapeutic Efficacy of p53 Restoration in Tumors. *Cell*

Di Micco R, Fumagalli M, Cicalese A, Piccinin S, Gasparini P, Luise C, Schurra C, Garré M, Giovanni Nuciforo P, Bensimon A, *et al* (2006) Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature*

Nelson CE, Wu Y, Gemberling MP, Oliver ML, Waller MA, Bohning JD, Robinson-Hamm JN, Bulaklak K, Castellanos Rivera RM, Collier JH, *et al* (2019) Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. *Nat Med*

Nelson CE, Wu Y, Gemberling MP, Oliver ML, Waller MA, Bohning JD, Robinson-Hamm JN, Bulaklak K, Castellanos Rivera RM, Collier JH, *et al* (2019) Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. *Nat Med* 25: 427–432

Radhakrishnan SK, Feliciano CS, Najmabadi F, Haegebarth A, Kandel ES, Tyner AL & Gartel AL (2004) Constitutive expression of E2F-1 leads to p21-dependent cell cycle arrest in S phase of the cell cycle. *Oncogene*

Sakofsky CJ, Saini N, Klimczak LJ, Chan K, Malc EP, Mieczkowski PA, Burkholder AB, Fargo D & Gordenin DA (2019) Repair of multiple simultaneous double-strand breaks causes bursts of genome-wide clustered hypermutation. *PLoS Biol*

Schiroli G, Ferrari S, Conway A, Jacob A, Capo V, Albano L, Plati T, Castiello MC, Sanvito F, Gennery AR, *et al* (2017) Preclinical modeling highlights the therapeutic potential of hematopoietic stem cell gene editing for correction of SCID-X1. *Sci Transl Med*

Wagenblast E, Azkanaz M, Smith SA, Shakib L, McLeod JL, Krivdova G, Araújo J, Shultz LD, Gan OI, Dick JE, *et al* (2019) Functional profiling of single CRISPR/Cas9-edited human long-term hematopoietic stem cells. *Nat Commun*

Wang CX & Cannon PM (2016) The clinical applications of genome editing in HIV. *Blood*

Wang JCY, Doedens M & Dick JE (1997) Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. *Blood*

Xie SZ, Garcia-Prat L, Voisin V, Ferrari R, Gan OI, Wagenblast E, Kaufmann KB, Zeng AGX, Takayanagi S ichiro, Patel I, *et al* (2019) Sphingolipid Modulation Activates Proteostasis Programs to Govern Human Hematopoietic Stem Cell Self-Renewal. *Cell Stem Cell*

Zonari E, Desantis G, Petrillo C, Boccalatte FE, Lidonnici MR, Kajaste-Rudnitski A, Aiuti A, Ferrari G, Naldini L & Gentner B (2017) Efficient Ex Vivo Engineering and Expansion of Highly Purified Human Hematopoietic Stem and Progenitor Cell Populations for Gene Therapy. *Stem Cell Reports* 8: 977–990

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