CRISPR/Cas9-based correction of ELANE mutations in severe congenital neutropenia (CN) patients with no response to G-CSF and high risk to develop leukemia

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EIDESSTATTLICHE ERKLÄRUNG

Ich, Masoud Nasri versichere, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe; aus fremden Quellen entnommene Passagen und Gedanken sind als solche kenntlich gemacht.

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Contents

ABBREVIATIONS

AD	Autosomal dominant	**CD**	Cluster of differentiation
AKT	Protein kinase B	**C/EBP**	CCAAT/enhancer-binding protein
AML	Acute myeloid leukemia	**CFU**	Colony forming unit
ANC	Absolute neutrophil count	**CLP**	Common lymphoid progenitor
AR	Autosomal recessive	**CMP**	Common myeloid progenitor
ATF	Activating transcription factor	**CRISPR**	Clustered regularly interspaced short palindromic repeats
AZU1	Azurocidin	**crRNA**	CRISPR RNA
BCL-X	BCL associated X protein	**CSF3R**	Receptor for colony stimulating factor 3
BIP	Binding immunoglobulin protein	**CXCR2**	C-X-C chemokine receptor 2
BM	Bone marrow	**CXCR4**	C-X-C chemokine receptor 4
BMT	Bone marrow transplantation	**CXCL8**	C-X-C motif chemokine ligand 8
BRET	Bioluminescence resonance energy transfer	**CYP1B1**	Cytochrome P450 family 1 subfamily B member 1
C5a	Complement factor 5a	**CYPE21**	Cytochrome P450 family 2 subfamily E member 1
CAMT	Congenital amegakaryocytic thrombocytopenia	**DBA**	Diamond blackfan anemia

Cas	CRISPR associated protein	**DNA**	Desoxyribonucleic acid
DEG	Differentially expressed gene	**GFP**	Green fluorescent protein
dpf	Days post fertilization	**GMP**	Granulocyte-monocyte progenitor
DSB	Double strand break	**gRNA**	Guide RNA
EB	Embryoid body	**GvHD**	Graft vs. host disease
ECP	Eosinophil cationic protein	**G6PC3**	Glucose-6-phosphatase catalytic subunit 3
EDN	Eosinophil-derived neurotoxin	**h**	Hour
e.g.	For example	**H2AX**	H2A histone family member X
ELANE	Neutrophil elastase	**HAX1**	HCLS1-associated protein X-1
EPO	Eosinophil peroxidase	**HDR**	Homology-directed repair
ER	Endoplasmic reticulum	**hpf**	Hours post fertilization
FA	Fanconi anemia	**HOXD13**	Homeobox D13
FACS	Fluorescence activated cell sorting	**HS(P)C**	Hematopoietic stem (and progenitor) cells
fMLP	Formyl methionyl-leucyl-phenylalanine	**IBMF**	Inherited bone marrow failure syndorme
GADD45B	Growth arrest and DNA-damage-inducible 45, beta	**IGFBP4**	Insulin like growth factor binding protein 4
G-CSF	Granulocyte-colony stimulating factor	**IL-**	Interleukin-
G-CSFR	G-CSF receptor	**Indel**	Insertion deletion

GFI1	Growth factor independent 1 transcriptional repressor	**iPSCs**	Induced pluripotent stem cells
JAGN1	Jagunal homolog 1	**NETs**	Neutrophil extracellular traps
K	Lysine	**NHEJ**	Non-homologous end joining
kDa	Kilo dalton	**NK**	Natural killer cell
KLF4	Kruppel-like factor 4	**OCT4**	Octamer-binding transcription factor 4
KO	Knockout	**OSKM**	OCT4, SOX2, KLF4, MYC
LPS	Lipopolysaccharide	**PB**	Peripheral blood
LMO2	LIM domain only 2	**PLS**	Papillon–Lefèvre syndrome
LTB4	Leukotriene B4	**PRDM1**	PR/SET domain
LT-HSC	Long-term repopulating HSC	**RASL11B**	RAS like family 11 member B
MBP	Major basic protein	**RBC**	Red blood cell
MDS	Myelodysplasia syndrome	**RFLP**	Restriction fragment length polymorphism
min	Minutes	**RNA**	Ribonucleic acid
ml	Milliliter	**RNP**	Ribonucleoprotein
MPO	Myeloperoxidase	**ROS**	Reactive oxygen species
mRNA	Messenger RNA	**SCN/CN**	Congenital neutropenia
MyD118	Myeloid differentiation primary response 118	**SDS**	Schwachman diamond syndrome
NAD+	Nicotinamide adenine dinucleotide	**SEM**	Scanning electron microscopy
NADPH	Nicotinamide adenine dinucleotide phosphate	**SIRT**	Sirtuin
NAMPT	Nicotinamide phosphoribosyltransferase	**SOX2**	SRY (sex determining region Y)-box 2

NE	Neutrophil elastase	**SOX7**	SRY-box transcription factor 7
SOX12	SRY-box transcription factor 12		
ssODN	single-stranded donor oligonucleotides		
T-ALL	T-cell acute lymphoblastic leukaemia		
TAR	Thrombocytopenia with absent radii		
TEM	Transmission electron microscopy		
TF	Transcription factor		
TGFα	Transforming growth factor alpha		
TGFβ	Transforming growth factor beta		
TIDE	Tracking of indels by decomposition		
TNFa	Tumor necrosis factor alpha		
TRAF1	TNF receptor associated factor 1		
tracrRNA	Trans-activating CRISPR RNA		
UPR	Unfolded protein response		
UV	Ultra violet		
Y	Tyrosine		

SUMMARY

Severe congenital neutropenia (CN) is a monogenic bone marrow failure syndrome with an occurrence of 1:200,000. CN is characterized by an absolute neutrophil count (ANC) below 500 cells per microliter. Due to low ANC, patients with CN suffer from severe, fatal bacterial infections starting early after birth. The mechanisms that lead to "maturation arrest" in CN are not fully elucidated. Therapeutic options for CN patients are limited and, in some cases, not available. We aimed to design and develop novel and efficient alternative therapies for these patients. We first focused on CN-*ELANE* patients because autosomal dominant mutations in *ELANE* are the main disease-causing reason in approximately 45% of CN patients. We hypothesized that impaired granulopoiesis in CN patients could be restored by knockout of mutated *ELANE*. As mutated *ELANE* induces unfolded protein response (UPR) and endoplasmic reticulum (ER) stress in HSPCs of CN patients, *ELANE* knockout might restore a "maturation arrest" of granulopoiesis. To evaluate this hypothesis, we performed CRISPR/Cas9 RNP based knockout of *ELANE* in iPSCs and HSPCs of CN patients as well as healthy controls followed by *in vitro* granulocytic differentiation. Our results showed that CRISPR/Cas9 RNP based *ELANE* knockout significantly increased granulocytic differentiation resulting in the generation of fully functional mature neutrophils *in vitro*. Therefore, CRISPR/Cas9 based *ELANE* knockout in CN-*ELANE* patients might be an effective, safe, virus-, and DNA-free universal therapy option for CN. It is particularly true for patients who are G-CSF non-responders or needing high G-CSF dosages and for patients with unsuccessful bone marrow transplantation.

We further addressed the challenges of gene-editing in primary human HSPCs or iPSCs and investigated the strategies to improve the efficiency of the safe gene-editing in these cells. In this regard, we established a method of fluorescently labeling of CRISPR/Cas9 RNP and thus enriching the edited cell population by fluorescent activated cell sorting (FACS). We tested the applicability of our approach for genes with low-abundance transcripts and context-dependent inducible expression. Thus, we successfully deleted growth arrest and DNA-damage-inducible β (GADD45B) gene in the hard-to-transfect

leukemia cell line, primary human HSPCs, and human iPSCs. We were able to perform downstream functional studies using enriched GADD45B knockout cells.

Lastly, we focused on the analysis of the signal transduction pathways operating during hematopoietic differentiation and the leukemogenic transformation of HSPCs. Particularly, we studied the role of NAMPT - mediated deacetylation of the LIM domain only 2 (LMO2) transcription factor in hematopoiesis and leukemogenesis. We found that the deacetylation of LMO2 protein is crucial for the blood cell formation but also proliferation and survival of T-ALL blasts *in vitro* and *in vivo.* Our investigations elucidated that NAMPT and SIRT2 play an essential role in the activation of LMO2 by deacetylation. Moreover, inhibition of the NAMPT/SIRT2 - mediated LMO2 deacetylation pathway by selective small molecule inhibitors led to reduced proliferation and elevated apoptosis of T-ALL cells *in vitro* and *in vivo*. Thus, inhibition of NAMPT/SIRT2 deacetylation of LMO2 might be further elaborated as a therapeutic approach for T-ALL patients.

ZUSAMMENFASSUNG

Die kongenitale Neutropenie (CN) ist ein monogener Knochenmarkdefekt mit einer Häufigkeitsrate von 1:200.000. CN ist gekennzeichnet durch eine absolute Neutrophilenzahl (ANC) von unter 500 Zellen pro Mikroliter. Aufgrund der niedrigen ANC erleiden Patienten mit CN schwere bakterielle Infektionen, die bereits früh nach der Geburt auftreten. Die Mechanismen, die zu einem "Ausreifungsstopp" bei CN führen, sind nicht vollständig aufgeklärt. Die therapeutischen Möglichkeiten für CN Patienten sind begrenzt und in einigen Fällen sind die verfügbaren Therapien sogar wirkungslos. Das Ziel dieser Arbeit war es daher neue und effiziente Alternativtherapien für diese Patienten zu entwickeln. Wir haben uns zunächst auf die Gruppe der CN-*ELANE* Patienten konzentriert, da die autosomal dominanten Mutationen in *ELANE* bei etwa 45% der Patienten beschrieben sind und damit die häufigsten, mit CN assoziierten Mutationen sind. Wir stellten die Hypothese auf, dass eine beeinträchtigte Granulopoese bei CN-*ELANE* Patienten durch das Ausschalten des mutierten *ELANE* Gens wiederhergestellt werden könnte. Da mutiertes *ELANE* bei HSPCs von CN Patienten eine UPR (unfolded protein response) und einen Stress des endoplasmatischen Retikulums (ER) induziert, könnte das Ausschalten des *ELANE* Gens den "Reifungsstopp" der Granulopoese aufheben. Um unsere Hypothese experimentell zu überprüfen, führten wir einen CRISPR/Cas9 RNP-basierten Knockout von *ELANE* in iPSCs und HSPCs von CN Patienten sowie in gesunde Kontrollen durch, gefolgt von einer granulozytären Differenzierung *in vitro*. Unsere Ergebnisse zeigten, dass der CRISPR/Cas9 RNP-basierte *ELANE* Knockout die granulozytäre Differenzierung von HSPCS von CN Patienten signifikant erhöhte und *in vitro* voll funktionsfähige reife Neutrophile erzeugte. Daher könnte ein CRISPR/Cas9-basierter *ELANE* Knockout bei CN-*ELANE* Patienten eine wirksame, sichere, virus- und DNA-freie, universelle Therapieoption sein, insbesondere bei Patienten, die nicht auf G-CSF ansprechen oder hohe G-CSF Dosierungen für ihre Therapie benötigen. Auch solche Patienten, bei denen die Knochenmarktransplantation versagt hat, könnten von dieser neuen Therapie profitieren.

Im zweiten Teil der Arbeit entwickelten wir Strategien zur Verbesserung der Effizienz der sicheren Gen-Editierung in primären menschlichen Zellen. In diesem Zusammenhang

haben wir eine Methode entwickelt, um CRISPR/Cas9 RNP mittels Fluoreszenz zu markieren, so dass die gewünschte Zellpopulation anschließend durch FACS angereichert werden kann. Diesen Ansatz testeten wir dann an Genen, deren Transkripte selten sind und deren Expression Kontext-abhängig ist. Auf diese Weise gelang es uns GADD45B (growth arrest and DNA-damage inducible β), in schwer zu transfizierenden Leukämie-Zelllinien und primären humanen iPSCs, zu deletieren. Durch die Verwendung angereicherter GADD45B Knockout Zellen konnten wir nachfolgend funktionelle Untersuchungen durchführen.

Zuletzt konzentrierten wir uns auf die Analyse der Signaltransduktion, welche die hämatopoetische Differenzierung und leukämogene Transformation hämatopoetischer Stammzellen steuert. Wir untersuchten in diesem Kontext die Rolle der NAMPT-vermittelten Deacetylierung des Transkriptionsfaktors LMO2 in der Hämatopoese und der Leukämogenese. Wir konnten zeigen, dass die Deacetylierung des LMO2 Proteins für die Bildung von Blutzellen sowie die Proliferation und das Überleben von T-ALL Blasten *in vitro* und *in vivo* entscheidend ist. Unsere Untersuchungen zeigen, dass NAMPT und SIRT2 eine wesentliche Rolle bei der Deacetylierung und damit der Aktivierung von LMO2 spielen. Darüber hinaus könnten wir zeigen, dass die Hemmung der NAMPT/SIRT2-vermittelten LMO2 Deacetylierung, durch die selektiven Inhibitoren, das Wachstum von T-ALL Zellen reduziert und deren Apoptose beschleunigt, *in vitro* und *in vivo*. Basierend darauf könnte die Inhibition der NAMPT/SIRT2-vermittelten LMO2 Deacetylierung in Zukunft ein therapeutischer Ansatz zur Behandlung der T-ALL sein.

LIST OF PUBLICATIONS

All relevant publications are listed below. The author contribution is highlighted in bold.

A. **M. Nasri**, M. Ritter*, P. Mir*, B. Dannenmann*, N. Aghaallaei, D. Amend, V. Makaryan, Y. Xu, B. Fletcher, R. Bernhard, I. Steiert, K. Hähnel, J. Berger, I. Koch, B. Sailer, K. Hipp C. Zeidler, M. Klimiankou, B. Bajoghli, D. C. Dale, K. Welte, and J. Skokowa, *'CRISPR/Cas9 mediated ELANE knockout enables neutrophilic maturation of primary hematopoietic stem and progenitor cells and induced pluripotent stem cells of severe congenital neutropenia patients'*. *Haematologica*, vol. 105, no. 3, p. 598-609, June 2019.

B. **M. Nasri***, P. Mir**, B. Dannenmann, D. Amend, T. Skroblyn, Y. Xu, K. Schulze-Osthoff, M. Klimiankou, K. Welte and J. Skokowa, *'Fluorescent labeling of CRISPR/Cas9 RNP for gene knockout in HSPCs and iPSCs reveals an essential role for GADD45β in stress response'*. *Blood Advances*, vol. 3, no. 1, p. 63-71, January 2019. (*equal contribution)

C. T. Morishima , AC. Krahl* , **M. Nasri*** , Y. Xu , N. Aghaallaei , B. Findik , M. Klimiankou , M. Ritter , M.D. Hartmann , C.J. Gloeckner , S. Stefanczyk , C. Lindner , B. Oswald , R. Bernhard , K. Hähnel , U. Hermanutz-Klein , M. Ebinger , R. Handgretinger , N. Casadei , K. Welte , M. Andre , P. Müller , B. Bajoghli , J. Skokowa *'LMO2 activation by deacetylation is indispensable for hematopoiesis and T-ALL leukemogenesis'. Blood*, vol 134, Issue 14, p. 1159-1175 October 2019. (*equal contribution)

D. B. Dannenmann*, **M. Nasri***, K. Welte and J. Skokowa, *'CRISPR/Cas9 genome editing of human-induced pluripotent stem cells followed by granulocytic differentiation'*, RNA Interference and CRISPR Technologies, *Methods in Molecular Biology*, vol. 2115, p. 455-469, February 2020. (*equal contribution)

E. A. Witte, A.K. Rohlfing, B. Dannenmann, V. Dicenta, **M. Nasri**, Kyra Kolb, J. Sudmann, T. Castor, D. Rath, O. Borst, J. Skokowa, M. Gawaz, '*The chemokine CXCL14 mediates platelet function and migration via direct interaction with CXCR4'. Cardiovascular Research,* April 2020.

F. B. H. Alvarez, J. Skokowa, M. Coles, P. Mir, M. Nasri, L. Weidmann, K. W. Rogers, K. Welte, A. Lupas, P. Müller, M. ElGamacy. *'Design of novel granulopoietic proteins by topological rescaffolding',* Accepted manuscript, PLOS Biology.

G. J. Skokowa, B. H. Alvarez, M. Coles, P. Mir, M. Nasri, A.-Ch. Krahl, K. Welte, A. Lupas, P. Müller, M. ElGamacy, '*A topological refactoring design strategy yields highly stable granulopoietic proteins*', manuscript in revision.

H. Y. Xu, M. Nasri, B. Dannenmann, P. Mir, A. Zahabi, K. Welte, T. Morishima, J. Skokowa, '*NAMPT/SIRT2–mediated inhibition of the p53-p21 signaling pathway is indispensable for maintenance and hematopoietic differentiation of human iPS cells* ', manuscript in revision.

PERSONAL CONTRIBUTION

The following section indicates the personal contribution to each of the above listed publications.

A. First author, involved in the initial observations (together with J. Skokowa), designing the CRISPR/Cas9 strategy and validation in the THP-1 cell line, performing gene editing in iPSCs and HSPCs (together with M. Ritter, P. Mir, and B. Dannenmann), performing colony-forming unit (CFU) assays and granulocytic liquid culture differentiation of HSPCs (together with P. Mir, M. Ritter, and D. Amend), evaluation of neutrophil functions *in vitro* (together with M. Ritter). I also analyzed & interpreted the data and was involved in the writing of the manuscript.

B. Co-first author, the main investigator (together with P. Mir). Involved in designing the study, performing experiments, analyzing & interpreting the data, and preparing the manuscript.

C. Co-second author (together with A.C. Krahl). Designed the CRISPR/Cas9-based LMO2 KO strategy, performed the gene-editing experiments and analyzed the results. Performed data analysis and interpretation of RNA-sequencing results starting from RNA-sequencing reads till the publication-ready figures and description/discussion of the results.

D. Co-first author (together with B. Dannenmann), role in structural design of the book chapter (with B. Dannenmann and J. Skokowa), wrote protocol sections for sgRNA designing and validation, CRISPR/Cas9 RNP nucleofection of iPSCs and clonal isolation of single-cell derived gene-edited clones, estimation of CRISPR/Cas9 gene-editing efficiency. Main role in preparing figures and figure legends (together with B. Dannenmann).

E. Fifth author. Advisory role in designing of the CRISPR/Cas9 gene-editing strategy, performing the gene editing in iPSCs and isolation of confirmed gene-edited single-cell derived iPSC clone.

F. Fifth author. Designing the CRISPR/Cas9 strategy, performing the gene-editing and isolation of confirmed knockout single-cell derived cell clones. Analyzing the gene-editing results, preparation of the figure, description/discussion of the results.

G. Fifth author. Designing the CRISPR/Cas9 strategy, performing the gene-editing and isolation of confirmed knockout single-cell derived cell clones. Analyzing the gene-editing results, preparation of the figure, description/discussion of the results.

H. Second author. Designing the molecular cloning strategy, cloning the recombinant DNAs and sgRNA constructs into related plasmids and validation of the plasmids, testing CRISPR/Cas9 KO efficiency.

PARTICIPATION IN SCIENTIFIC CONFERENCES

A. **M. Nasri**, B. Dannenmann, P. Mir, M. Ritter, Y. Xu, M. Klimiankou, C. Zeidler, K. Welte, J. Skokowa**,** *'Disease modeling of severe congenital neutropenia using CRISPR/Cas9 gene correction or knockout of ELANE in patients derived Induced pluripotent stem cells'***,** Oral presentation, **ASH Abstract Achievement Award,** Annual meeting of the American Society of Hematology (ASH), Orlando, USA 2019.

B. **M. Nasri**, B. Dannenmann, M. Ritter, P. Mir, D. Amend, Y. Xu, M. Klimiankou, C. Zeidler, L. Kanz, K. Welte, J. Skokowa**,** *'CRISPR/Cas9 mediated gene editing of ELANE enables neutrophilic maturation of primary HSPCs and iPSCs of severe congenital neutropenia patients'*, Oral presentation, Annual Meeting of German Society of Hematology and Oncology (DHGO), Berlin, Germany 2019.

C. **M. Nasri**, B. Dannenmann, M. Ritter, P. Mir, D. Amend, Y. Xu, M. Klimiankou, C. Zeidler, L. Kanz, K. Welte, J. Skokowa**,** *'CRISPR/Cas9 genome editing platform of ELANE mutations in iPSCs and HSPCs of severe congenital neutropenia patients'*, Poster presentation, Annual meeting of German Stem Cell Network (GSCN), Berlin, Germany 2019.

D. **M. Nasri**, M.Ritter. P. Mir, Dannenmann, D. Amend, V. Makaryan, Y. Xu, C. Zeidler, D.C. Dale, M. Klimiankou, K. Welte, J. Skokowa**,** *'CRISPR/Cas9 mediated ELANE knockout enables neutrophilic maturation of HSPCS and IPSCS of severe congenital neutropenia patients'***,** Poster presentation, Annual meeting of The European Hematology Association (EHA), Amsterdam, Netherlands 2019.

E. **M. Nasri**, M. Ritter, B. Dannenmann, P. Mir, Y. Xu, M. Klimiankou, C. Zeidler, L. Kanz, K. Welte, J. Skokowa, *'CRISPR/Cas9 gene-modification platform of ELANE mutations in iPSCs and HSPCs of Severe Congenital Neutropenia patients'*, Oral

presentation, XXXII. Kind-Philipp meeting on pediatric hematology and oncology research, Wilsede, Germany 2019.

F. **M. Nasri**, P. Mir, B. Dannenmann, D. Amend, Y. Xu, A. Solovyeva, S. Stefanczyk, M. Klimiankou, C. Zeidler, L. Kanz, K. Welte, J. Skokowa*, 'A method to fluorescently label the CRISPR/Cas9-gRNA RNP complexes enables enrichment of clinical-grade gene-edited primary hematopoietic stem cells and iPSCs'*, Poster presentation, Annual meeting of the American Society of Hematology (ASH), San Diego, USA 2018.

G. **M. Nasri**, B. Dannenmann, P. Mir, T. Skroblyn, A. Solovjeva, M. Klimiankou, C. Zeidler, L. Kanz, K. Welte, J. Skokowa, ' *Establishment of the safe and efficient CRISPR/Cas9-RNP based gene-correction platform of ELANE mutations in iPSCs of severe congenital neutropenia (CN) patients with no response to G-CSF and high risk to develop leukemia'*, Oral presentation, **ASH Abstract Achievement Award**, Annual meeting of the American Society of Hematology (ASH), Atlanta, USA 2017.

H. **M. Nasri**, P. Mir, S. Stefanczyk, M. Klimiankou, B. Dannenmann, C. Zeidler, L. Kanz, K. Welte, and J. Skokowa, *'GADD45b protein in myeloid differentiation and stress response of human hematopoietic cells'*, Poster presentation, **the Best DGHO Poster Award**, Annual Meeting of German Society of Hematology and Oncology (DGHO), Stuttgart, Germany 2017.

INTRODUCTION

Hematopoiesis with emphasis on granulopoiesis

It is estimated that the human body made out of 3.72 x 10^{13} cells.[1] One of the most regenerative tissues in humans is blood. Approximately one trillion cells (10^{12}) rise daily from human bone marrow (BM) in a healthy condition [2], while hematopoietic stem cells (HSCs) are the only cells that are able to give rise to all blood cell types and by self-renewing keep the hematopoiesis steady and well-regulated. HSCs represent up to 0.05% of cells in bone marrow [3,4] that are responsible for generating more than ten different blood cell lineages (Fig.1) with various functions. Erythrocytes or red blood cells (RBCs) transport oxygen and carbon dioxide to- and from tissues, megakaryocytes generate platelets to clot the blood and heal the wounds, and leukocytes -a specialized range of many different subtypes- are involved in the innate and acquired immunity. Leukocytes categorized into five distinct classes: neutrophils, basophils, eosinophils, monocytes, and lymphocytes.

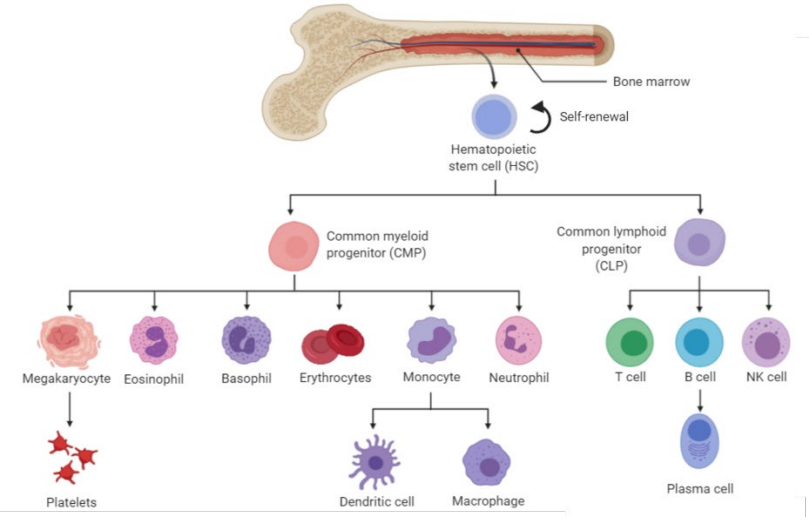

Figure 1. Hematopoiesis, overview. (Adopted and modified from Doulatov, Sergei, et al. "Hematopoiesis: a human perspective." Cell stem cell 10.2 (2012): 120-136.), "Created with Biorender.com."

A substantial proportion of hematopoietic cells is committed to the differentiation into neutrophilic granulocytes, or neutrophils. Neutrophils are the primary cellular defense against fungal and bacterial infection and make 60% of the leukocytes population in the bone marrow. The process of differentiation and maturation of neutrophilic granulocytes takes approximately 6.5 days.[5] Granulopoiesis is a highly elaborated series of orchestrated steps that ultimately lead to the production of neutrophils.[6] Mature neutrophils circulate in the bloodstream for approximately 10 – 24 hours before migrating to tissues and may function for 1 – 2 days in tissue before undergoing apoptosis followed by clearance via macrophages.[6] Although more modern techniques such as deuterium labeling methods *in vivo,* suggest that under homeostatic conditions, human neutrophils may have a circulatory life span up to 5 days.[7] The kinetics of neutrophil production– the amount of cells produced every day - is measured as a rate of turnover of neutrophils in the blood and has been determined by labeling of neutrophils with a radioactive isotope of phosphorus [^{32}P or phosphorus-32]. The neutrophil turnover rate has been described to be about 1.5×10^9 cells/kg per day in a healthy condition, indicating the importance of granulopoiesis and neutrophils as the first line of defense against pathogens.[2,8,9] The human's ability to maintain a balance between neutrophil production and turnover, while adapting to environmental challenges, implies the importance of molecular processes for maintaining neutrophil numbers at any given time.[10] During inflammation or infection, chemoattractant factors such as complement factor C5a, leukotriene LTB4 and CXCL8 stimulate neutrophils to migrate across the bone marrow endothelium that separates the marrow from the circulation and enter peripheral blood. Under homeostatic conditions,[11] G-CSF is the main regulator of granulocyte production and G-CSF receptors are highly expressed on neutrophils surface.[12] Upon activation, neutrophils transcriptional activity increase - in part due to the effect of local G-CSF production -, leading to release of pro-inflammatory/anti-inflammatory cytokines, chemokines, immunoregulatory cytokines, tumor necrosis factor (TNF) superfamily members, and angiogenic factors.[9,13] Neutrophils also perform phagocytosis of foreign microorganisms, and degranulation, which is one of the first steps triggered through neutrophils activation. The components of neutrophil granules are well-known and characterized [14–16]. Besides, activation of the oxidative metabolism takes place in neutrophils intracellularly, leading to the production of reactive

oxygen species (ROS). NADPH oxidase is the main complex responsible for ROS production in neutrophils. These reactive oxygen species are highly effective antimicrobial agents while are also damaging the host tissues. ROS producing neutrophils are rapidly cleared by macrophages. [9] Neutrophils could also form extracellular traps (NETs), extended antimicrobial features that are a result of nuclear swelling and dissolved chromatin, Along with disintegration of granule proteins such as myeloperoxidases (MPO), elastases, pentraxin 3, lactoferrin and MPO9. NET formation is a cell-death dependent process that traps and kills the microbes, amplify the immune responses, and induce coagulation.[9,17] Neutrophils finally undergo apoptosis. Apoptotic neutrophils are not detected in healthy peripheral blood circulation. As neutrophils become senescent, CXCR4 expression increased while CXCR2 decreased. This expression pattern leads to homing of senescent neutrophils to the bone marrow.[18]

Basophils are the least abundant population of granulocytes, with a prevalence of 0.5% of total leukocytes, and they are mainly responsible for initiating the inflammatory responses by releasing the chemicals histamine, serotonin and heparin. Under normal physiological conditions, their lifespan is a few days, while interleukin 3 (IL-3) promotes the production and survival of basophils *in vitro.*[19] Basophils can produce cytokines such as TGF- α/β , IL-2, IL-4, IL-5, IL-10, IL-12, IL-13 and IL-16. [20] *Youesfi et al.* showed that basophils could also generate extracellular DNA traps - the same as neutrophils- but in contrast to neutrophils, basophils do not die upon releasing their DNA.[21]

In 1879, P. *Ehrlich* reported a subtype of leukocytes that were showing high avidity for the acidic stain eosin; thus, he named this cell subtype "eosinophils".[22] Eosinophils are prominent at sites of parasitic infection and allergic reactions; they are unable to perform phagocytosis. The main component of their granules is major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP). These granules are capable of tissue damage induction as EPO, ECP, and MBP are toxic to tissues like the brain, epithelium, and heart. [20] Monocytes – the largest type of leukocytes– categorized into three major subsets of classical, nonclassical, and intermediate, based on the expression pattern of FcγRIII(CD16) and LPS-receptor CD14.[23] Monocytes are highly plastic cells. They are capable of a wide

range of responses such as clearance of blood from aged RBCs, immunoregulation between innate and adaptive immunity, and even phagocytosis of invading pathogens, but in contrast to neutrophils, they are slower to respond and have higher half-life as they can renew their lysosomes.[24] Activated monocytes can release IL-1, IL-6, TNF, and INF-α/β—cytokines that are involved in the regulation of hematopoiesis.[24] Monocytes divided into two types based on response to pathogenic infection, macrophages, and dendritic cells. Macrophages remain and localized in the tissue, while dendritic cells' primary role is presenting antigens to lymphocytes. Lymphocytes are more common in the lymphatic system rather than blood and categorized into B cells, T cells, and natural killer cells, although they are uniform in appearance.

Inherited bone marrow failure syndromes and severe congenital neutropenia

The inherited bone marrow failure syndromes (IBMFS) are a diverse group of disorders in which qualitative or quantitative abnormalities are affecting one or more hematopoietic lineages. IBMFS is the result of germline mutations that affecting critical cellular pathways such as telomerase biology, DNA repair, or function of structural proteins. Depending on the affected pathway, IBMFS leads to anemia, leukopenia, thrombocytopenia, or pancytopenia; either because of an absence of one or more lineages of hematopoietic progenitors in the bone marrow.[25] The major IBMFS are summarized in **Table 1**.

Table 1. Major bone marrow failure (IBMF) syndromes

IBMFS	Abbrv.	Phenotypic characterization
Congenital Amegakaryocytic thrombocytopenia	CAMT	severe thrombocytopenia
Diamond Blackfan anemia	DBA	red blood cell production failure
Dyskeratosis congenita	DC	a triad of abnormal skin pigmentation, nail dystrophy, and mucosal leukoplakia
Fanconi anemia	FA	physical anomalies, progressive bone marrow failure, and cancer predisposition
Pearson syndrome		a unique combination of refractory sideroblastic anemia, vacuolization of marrow precursors and exocrine pancreatic dysfunction
Severe congenital neutropenia	CN	persistent neutropenia and severe life-threatening infections
Shwachman Diamond syndrome	SDS	a ribosomopathy characterized by neutropenia, associated pancreatic exocrine function insufficiency, and metaphyseal dysostosis
Thrombocytopenia with Absent Radii	TAR	hypomegakaryocytic thrombocytopenia, periodic leukemoid reactions, and eosinophilia

- Data from different sources[25–32]

Severe congenital neutropenia

Severe congenital neutropenia (CN) is an inherited bone marrow failure syndrome characterized mainly by absolute neutrophil counts (ANC) lower than 500 cells/µl in peripheral blood. While the normal ANC in peripheral blood is in the range of 1500 to 8500 cells/µl, the low number of neutrophils makes severe congenital neutropenia patients susceptible to recurrent life-threatening bacterial infections already starting in the first months of life.[28,33] Severe congenital neutropenia is a rare disease with a prevalence of 3 to 8.5 cases per million individuals.[28,34] It is a genetically heterogeneous disease, and a wide range of mutations in different genes such as *ELANE*, *G6PC3*, *GFI1*, and *HAX1* can cause the disorder. Interestingly, the manner of inheritance is different

depending on the gene mutation caused the disease, and it could be autosomal dominant (AD) or autosomal recessive (AR). Table 2 represents the main genes known to be mutated in severe congenital neutropenia patients.[28,35] autosomal dominant *ELANE* mutations have been identified in the majority of severe congenital neutropenia patients (Fig. 2).

Table 2. Severe congenital neutropenia causing genes and pattern of inheritance

Gene mutated	Disease	Inheritance pattern
ELANE	Severe congenital neutropenia	AD
ELANE	Cyclic neutropenia	AD
GFI1	Severe congenital neutropenia	AD
HAX1	Severe congenital neutropenia	AR
JAGN1	Severe congenital neutropenia	AR
G6PC3	Severe congenital neutropenia	AR
CSF3R	Severe congenital neutropenia	AR

The availability of recombinant human granulocyte colony-stimulating factor (rhG-CSF) for clinical use was a breakthrough in the treatment of severe congenital neutropenia.[36] G-CSF therapy induces compensatory granulopoiesis in severe congenital neutropenia patients by activating C/EBPβ dependent emergency granulopoiesis, and increases the neutrophil count up to >1000 cells/μl in peripheral blood which led to declining the frequency and severity of infectious incidents.

While rhG-CSF therapy dramatically improved the quality of life of severe congenital neutropenia patients, about 15 % of patients do not respond to rhG-CSF doses up to 20 μg/kg/day, leaving no therapeutic option except bone marrow transplantation (BMT). Also, approximately 20 % of severe congenital neutropenia patients develop myelodysplasia (MDS) or acute myeloid leukemia (AML). These conditions emphasize the importance of alternative therapeutic options.[28,37]

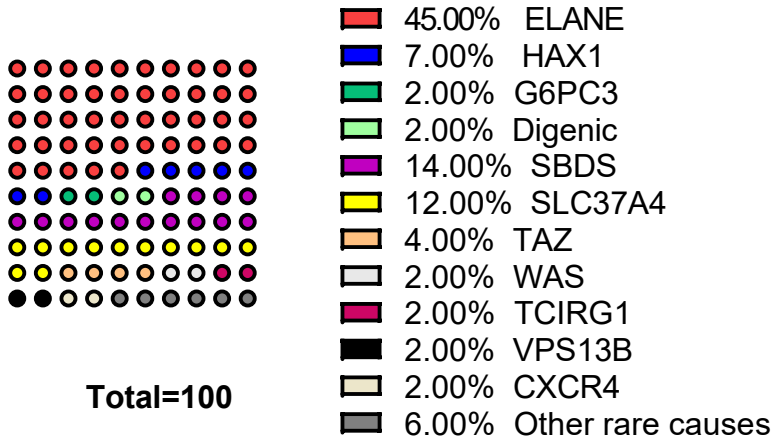

Figure 2. Genes with germline mutations associated with congenital neutropenia (Adopted and modified from "Severe congenital neutropenias" by Skokowa et al., 2017, Nat Rev Dis Prim, copyright 2017 by Macmillan publishers.)

ELANE is a protein-coding gene located on the short arm of chromosome 19 and encodes neutrophil elastase protein. More than 150 unique pathogenic variants were identified in the ELANE gene that could cause severe congenital neutropenia or cyclic neutropenia. These variants could be categorized into six categories: missense, frameshift, nonsense, splice junction loss, non-coding, and deletion or insertion. Table 3 summarizes these variants.

Table 3. Variants in ELANE with known pathogenicity

Coding impact	Pathogenic	Likely pathogenic	Uncertain	Likely benign	Benign	Total
Synonymous	0%	0%	20.7%	44.8%	34.5%	29
Missense	64.3%	6.3%	22.7%	2.9%	3.9%	207
Nonsense	50%	0%	50%	0%	0%	2
Frameshift	100%	0%	0%	0%	0%	1
Inframe Indel	20%	40%	40%	0%	0%	5
Splice junction loss	40%	0%	60%	0%	0%	5
Non-coding	25%	25%	0%	50%	0%	4%
Total	54.9%	6.3%	23.3%	8.3%	7.1%	253

- *Data source: Total classified variants extracted from UniProt, ClinVar, VarSome and PubMed.*[38]

Neutrophil elastase

Neutrophil elastase (NE) is a myeloid-specific 267 amino acid long monomeric 29 kDa glycoprotein encoded by the ELANE gene (Fig. 4). NE is a serine protease that hydrolyzes, or process many different proteins, including cytokines, chemokines and growth factors, such as elastin, collagen-IV, G-CSF, tumor necrosis factor-alpha (TNF-α), SDF-1α, Interleukin 33 (IL33), interleukin 8 (IL8), IL1-β and tumor suppressor p200 CUX1.[28,35,39–42] More than one-third of known proteolytic enzymes are serine proteases whereas, 2 - 4% of human genome encode proteolytic enzymes.

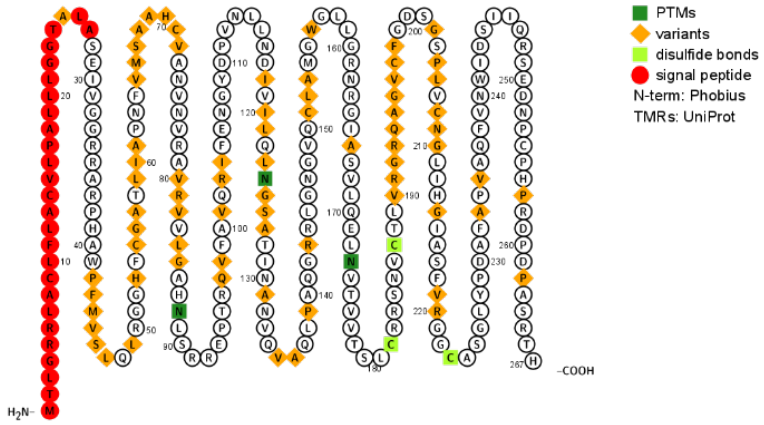

Figure 3. Neutrophil elastase (Data source: U. Omasits et al., Bioinformatics. 2014)[43]

Serine proteases are the highly abundant and functionally diverse group among proteolytic enzymes. Abundance is a key measurement of success in evolution.[44,45]

The mechanism by which *ELANE* mutation leads to congenital neutropenia is not fully understood. However, the current hypothesis proposes that while wild-type neutrophil elastase is detectable in the nucleus, cytoplasmic compartments, and cell surface, mutated neutrophil elastase is going through aberrant localization and induce endoplasmic reticulum (ER) stress along with unfolded protein response (UPR)[46]. The level of ER stress is decreased during neutrophil and macrophage differentiation in normal physiological conditions, and the activities of ATF6 and PERK decrease in normal granulopoiesis[47]. Mutations in *ELANE* increase the level of ER stress and UPR and

interrupt the homeostatic state needed for successful granulopoiesis. This might lead to maturation arrest of granulopoiesis at promyelocyte stage.[28,47]

Induced pluripotent stem cells as a tool to study congenital neutropenia

It is exceptionally rare for a method to become so impactful that it forms the backbone of the next decade experiments. Examples of such experiments that reshaped biomedical research are the generation of induced pluripotent stem cells (iPSCs) and the development of CRISPR/Cas9 technology.[48] Using *Yamanaka* factors, oct3/4, sox2, c-myc and klf4, the somatic cells could be reprogrammed to a pluripotent state, and these cells can indefinitely retain the pluripotency and simultaneously the ability to differentiate into all three germ layers (Fig. 4).

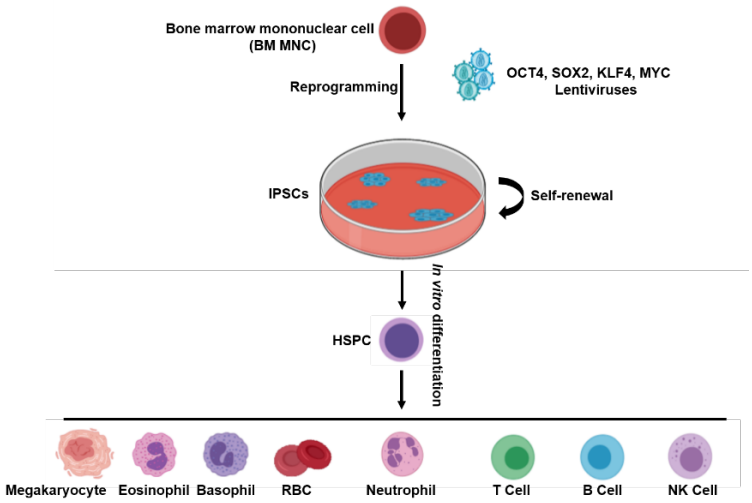

Figure 4. Overview of the implementation of iPSC technology to study hematopoiesis (adopted and modified from Hockemeyer D, Jaenisch R. "Induced Pluripotent Stem Cells Meet Genome Editing. Cell Stem Cell". 2016;18(5):573–586. DOI:10.1016/j.stem.2016.04.013), "Created with Biorender.com."

Without having any ethical issues that accompanied embryonic stem (ES) cell research[49], the iPSC technology makes it possible to generate an indefinite valuable source of different types of patients` cells that might be used as a tool for disease modeling to elucidate the pathways behind the disease with the aim to identify the potential

therapeutic windows. iPSCs also are a valuable tool for the high-throughput patient-specific and cell type-specific drug screening.[50]

As an example, *Dannenmann et al.* used CN patient's derived iPSC to study the myelopoiesis and granulopoiesis in CN patients.[51] Based on these data, hematopoietic stem cells derived from iPSC of CN patients has elevated levels of unfolded protein response and DNA damage.[51]

CRISPR/Cas9 based genome editing of patient-derived induced pluripotent stem cells

Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) protein 9 system provides a robust and efficient genome editing tool for precise manipulation of specific genomic elements, and speed up the understanding of target gene function in biology and diseases.[52] CRISPR/Cas9 consist of a non-specific Cas9 nuclease and a set of programmable sequence-specific CRISPR RNA (crRNA), which can guide Cas9 to a target site and cleave DNA by generating double-strand breaks (DSBs). Cas9 nucleases execute the strand-specific cleavage by using the conserved HNH and RuvC nuclease domains. [53] CRISPR/Cas9 technology could have a wide range of applications such as gene knockout, targeted transcriptional regulation, epigenetic engineering, CRISPR imaging, base editing, and targeted knock-in. [54,55] An important but frequently ignored challenge of iPSC research is the high-level degree of variations between each iPSC lines. The main reason for the variation is mostly unpredictable but could be caused by the different genetic backgrounds and different reprogramming histories of each iPSC line. Therefore, to investigate and study a disease mechanism, comparing patient-derived iPSC to healthy donor iPSC may lead to misleading results that originated mainly from the variations mentioned above. To generate an isogenic model, we established an efficient gene-editing platform for induced pluripotent stem cells (iPSC) of CN patients using CRISPR/Cas9 technology. By using ribonucleoprotein form of CRISPR/Cas9 and single-stranded oligonucleotides strands (ssODNs) as a repair template, the editing approach became much safer compared to viral or plasmid formats. The edited heterogeneous population of iPSCs goes through single cell-derived colony

isolation by seeding iPS cells and incubation for up to 14 days until the emergence of colonies. Single-cell derived clones are characterized by Restriction Fragment Length Polymorphism (RFLP) and Sanger sequencing to select and confirm isogenic gene-edited lines. [50] The role of isogenic iPSC lines in disease modeling studies is indispensable as the variability between isogenic lines are limited to disease-causing mutations only (Fig 5).[56]

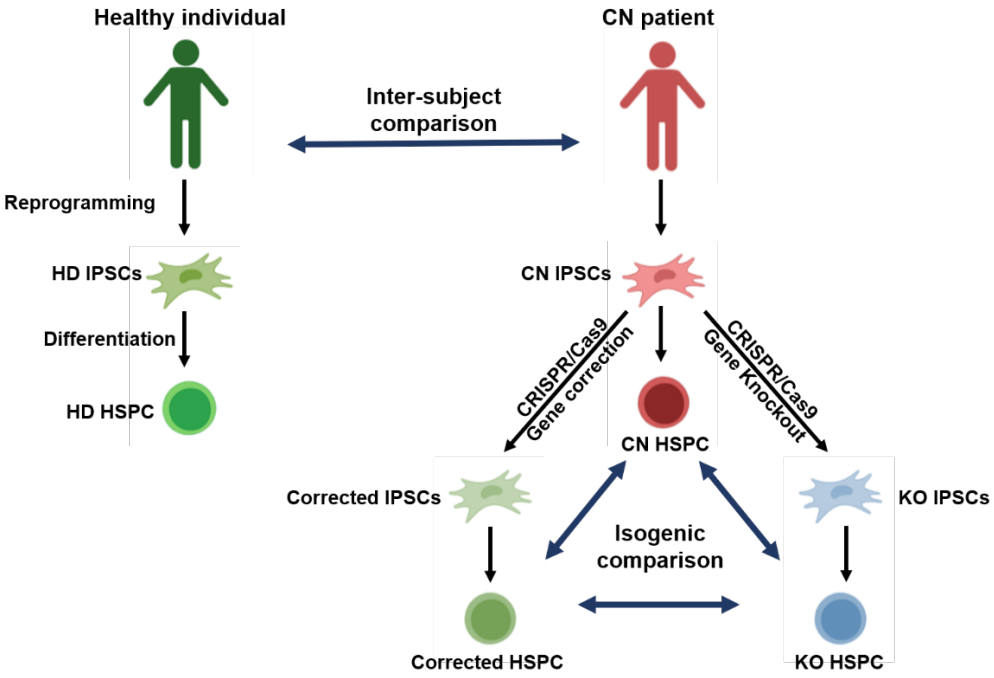

Figure 5. Isogenic iPSC lines in CN studies (Adopted and modified from Ott de Bruin LM, Volpi S, Musunuru K. "Novel Genome-Editing Tools to Model and Correct Primary Immunodeficiencies." Front Immunol. 2015;6:250.), "Created with Biorender.com."

OBJECTIVES OF THE STUDY

In the first part of the Thesis, we aimed to develop a safe and efficient CRISPR/Cas9-based gene therapy approach for *ELANE* related congenital neutropenia that might be applied for other inherited bone marrow failure syndromes.

In the second part, we intended to apply CRISPR/Cas9 gene-editing to investigate the pathomechanism of T-ALL downstream of the hyperactivated LMO2 transcription factor, in order to find valuable therapeutics opportunities for this type of leukemia.

The study focuses on three major parts:

Developing safe and efficient CRISPR/Cas9-based gene therapy approaches for ELANE related congenital neutropenia

CRISPR/Cas9 technology is a promising tool to develop and perform gene therapy for inherited monogenic diseases. However, there are still critical safety concerns that should be addressed, such as the off-target activity of CRISPR/Cas9. Gene therapy using *ex vivo* CRISPR/Cas9 gene-editing of CN patient's hematopoietic stem and progenitor cells (HSPCs) followed by autologous transplantation is a unique alternative to G-CSF therapy for CN. One of the most important goals of this study is to develop an efficient, safe, and virus-free CRISPR/Cas9 based gene-editing of HSPCs of CN patients.

Optimization of the efficiency and safety of CRISPR/Cas9 gene-editing in induced pluripotent- and hematopoietic stem/progenitor cells

CRISPR/Cas9-mediated gene modifications in iPSCs and primary hematopoietic stem cells is challenging. By addressing these challenges, we aimed to establish a safe and efficient gene-editing platform for iPSCs and HSPCs to facilitate the study of the gene of interest’s function in CN.

Translational mechanistic study of T-cell acute lymphoblastic leukaemia (T-ALL) and hematopoiesis

Well-defined, highly regulated transcription factor (TF) networks are vital for retaining hematopoietic tissue homeostasis. Elucidated hematopoietic TF networks could be a valuable map to develop therapeutic strategies in blood disorders such as T-cell acute lymphoblastic leukemia (T-ALL). LMO2 plays a vital role in hematopoiesis and, at the same time, at the onset of T-ALL. We aimed to study the mechanisms leading to LMO2 activation and identify the potential therapeutic target points of hyperactivated LMO2 in T-ALL.

RESULTS & DISCUSSION

Developing safe and efficient CRISPR/Cas9-based gene therapy approaches for *ELANE* related congenital neutropenia

(Covered in the published **Manuscript A**; Nasri. et *al*., *Haematologica*, 2019)

Autosomal dominant *ELANE* mutations are the main reason for severe congenital neutropenia (CN) in approximately 50% of patients that lead to an absence or a very low number of absolute neutrophils in peripheral blood (less than 500 neutrophils per µL) and therefore cause life-threatening severe bacterial infections. Daily treatment of patients with recombinant human granulocyte colony-stimulating factor (rhG-CSF) could increase the absolute neutrophil counts (ANC) and dramatically improve the quality of life and life expectancy of CN patients. However, there is a proven correlation between the therapeutic doses of rhG-CSF for the sufficient ANC, and the possibility of developing blood cancer as approximately 20 % of CN patients emerged myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). Moreover, a group of CN patients does not respond to rhG-CSF therapy at doses up to 50 µg/kg/day. Treatment of choice in CN patients who are rhG-CSF non-responders or who developed MDS/AML is hematopoietic stem cell transplantation (HSCT). HSCT is a complicated procedure related to severe adverse effects, such as acute or chronic graft-versus-host-disease (GvHD), graft failure, or graft rejection. This situation makes the necessity of developing alternative therapies for CN patients inevitable. It is known that mutant neutrophil elastase induces unfolded protein response and the endoplasmic reticulum (ER) stress in hematopoietic stem cells of CN patients. Thus, we hypothesized that knocking out *ELANE* could restore granulopoiesis and correct “maturation arrest” in HSPCs. Of note, *ELANE* has a high level of similarity to other neutrophil serine proteases (NSPs) like PRTN3 and Cathepsin G. Some of the functions of these proteases are redundant.[57] Also, in case of patients with Papillon-Lefèvre-Syndrom (PLS) – the only human disorder known to cause neutrophil serine proteases (NSPs) deficiency, due to loss-of-function mutations in the DPPI gene – while the functionality and the stability of neutrophils elastase is severely diminished,

the PLS patients` neutrophils show no defect in bacteria killing ability. [58,59] The effective bacterial killing ability without neutrophil serine proteases including neutrophil elastase (NE), contradict the necessity of NSPs for killing common bacteria. Therefore, we proposed – for the first time – a CRISPR/Cas9 mediated *ELANE* knockout in HSPCs of CN-*ELANE* patients followed by autologous transplantation of gene-edited cells as a therapeutic option for these patients.

To induce *ELANE* KO, we designed guide RNA (gRNA) targeting the second exon of *ELANE* at the position chr19[+852.969: -852.969]. To validate the on-target cutting efficiency, the THP-1 cell line, an acute monocytic leukemia line expressing a very high basal level of neutrophil elastase, was selected and electroporated with CRISPR/Cas9 RNP complexes. Ninety-six hours post electroporation, the editing efficiency of 77 % was estimated, as analyzed by sanger sequencing of *ELANE* exon 2 using the tracking of indels by decomposition (TIDE) analysis. Western blotting analysis of electroporated THP-1 cells (total gene-edited cell population or pure single-cell derived clones) showed high efficiency of designed gRNA for inhibiting of the neutrophil elastase expression at the protein level. Further, we investigated the *ELANE* knockout strategy in CN-*ELANE* patient-derived iPSCs with *ELANE* mutations p.C151Y (exon 4) or p.A57V (exon 2). Gene-editing was performed by assembling synthetically made guide RNA, and HiFi-Cas9, followed by isolation of single-cell derived pure knockout iPSC clones. The same steps were performed for healthy control iPSCs without obtaining a pure single-cell derived clone, since the knockout efficiency in the total population was 97%, as assessed by the TIDE algorithm. By applying an *in vitro* embryoid body (EB)-based iPSC differentiation protocol that generates mature neutrophils in approximately 30 days, we found the restoration of granulocytic differentiation in CN-*ELANE* iPSC clones after *ELANE* knockout while no significant changes were observed in *ELANE* deficient healthy control iPSC. These results confirmed that the inactivation of *ELANE* restores granulocytic differentiation in CN. The aforementioned iPSC gene-editing platform is a valuable research tool as it can efficiently generate the isogenic equivalent line in approximately 20 days. [50]

To further investigate the *ELANE* knockout strategy as a therapeutic option for CN-*ELANE* patients, we performed CRISPR/Cas9 RNP-mediated gene-editing in primary bone marrow $CD34^+$ HSPCs of four CN patients harboring *ELANE* mutations p.A57V, p.I120F, p.S126L, p.C223AfsX17 and three healthy controls followed by an *in vitro* liquid culture granulocyte differentiation method that generates mature neutrophils within 14 days. The gene-editing efficiency of *ELANE* at the genomic level was between 27 % to 94 %, as assessed by the TIDE algorithm. We could also show a markedly decrease of neutrophil elastase expression at the protein level by western blot of lysates isolated from CRISPR/Cas9 *ELANE* gene-edited HSPCs. Knockout of *ELANE* led to a significantly higher level of granulocytic differentiation, as assessed by flow cytometry analysis of $CD15^+CD11b^+CD45^+$ granulocytic cells. These results were also confirmed by morphological assessment of cytospin preparations of mature granulocytes derived on day 14 of the *in vitro* liquid culture granulocytic differentiation. Simultaneously, *ELANE* knockout showed no defects in granulocytic differentiation of healthy control HSPCs. We also performed scanning- and transmission electron microscopy (SEM and TEM, respectively) of *ELANE* knockout neutrophils and observed no significant changes in morphology or intracellular structures upon gene-editing. These results confirmed that knockout of *ELANE* in the patient's hematopoietic stem and progenitor cells could restore defective granulopoiesis.

To evaluate the activity and functionality of neutrophils differentiated from *ELANE* knockout HSPCs of CN patients *in vitro*, we assessed reactive oxygen species (ROS) production and phagocytosis ability of neutrophils after gene-editing. First, we measured H2O2 levels in N-formyl methionyl-leucyl-phenylalanine (fMLP) treated healthy control neutrophils generated from HSPCs with or without *ELANE* knockout. We detected no significant difference in H2O2 levels upon *ELANE* knockout. Simultaneously, neutrophils, incubated with fluorescein-conjugated Staphylococcus aureus BioParticles for two hours and engulfment percentage assessed by flow cytometry. In parallel, we also measured the kinetics of phagocytosis in neutrophils by IncuCyte ZOOM live cell analysis system that performed microscopy every 20 minutes for 8 hours. Our results showed that knockout of *ELANE* did not interrupt ROS production or phagocytosis by gene-edited neutrophils.

To assess the functions and behavior of *ELANE* knockout neutrophils in an *in vivo* system, we fluorescently labeled neutrophils differentiated from healthy control HSPCs – with or without *ELANE* knockout – and transplanted them into the duct of Cuvier - a wide circulation channel on the yolk sac connecting the heart to the trunk vasculature- of zebrafish embryos. In parallel, Alexa-594-conjugated *Staphylococcus aureus* BioParticles were injected in the tail of zebrafish embryos close to the caudal vein. Live imaging confocal microscopy tracked the migration of *ELANE* knockout neutrophils to the site of infection, so we could confirm unaffected chemotaxis activity of neutrophils upon knockout of *ELANE*. Simultaneously, we recorded the engulfment of *Staphylococcus aureus* BioParticles by *ELANE* knockout neutrophils. These results showed that the *in vivo* functions of *ELANE* knockout neutrophils are not affected.

We further evaluated the mRNA expression levels of the unfolded protein response (UPR) gene, BiP, and an anti-apoptotic factor, Bcl-xl in mature neutrophils differentiated from gene-edited CN patient's HSPCs or patient-derived iPSCs by quantitative real-time PCR. We found that BiP and Bcl-xl expression levels were downregulated upon *ELANE* knockout.

In this research project, we developed – as the first published report – an *in vitro* CRISPR/Cas9 based strategy for gene therapy of severe congenital neutropenia (CN) associated with autosomal dominant *ELANE* mutations, which represents nearly 50 % of confirmed CN cases. This CRISPR/Cas9 based gene-editing strategy can be performed *ex vivo* in HSPC of CN patients, followed by autologous transplantation as a therapeutic approach. However, numerous important issues should be further investigated and addressed for clinical application. Our approach solely uses the RNP form of CRISPR/Cas9 to enhance the editing efficiency and simultaneously to markedly reduce the possibility of CRISPR/Cas9 off-target activity. We analyzed the genomic sites with the highest off-target probability, where our CRISPR/Cas9 RNP may cut DNA not specifically due to a mis-alignment of gRNA with genome. Sanger sequencing did find any off-target activity; however, for clinical purposes, a comprehensive, unbiased off-target activity analysis should be performed using more advanced techniques, such as CIRCLE-seq[60] or GUIDE-seq[61], to fully validate the safety of our guide-RNA for clinical applications. We

showed that the knockout of *ELANE* does not impair neutrophil function. However, we still need to confirm the multi-lineage reconstitution of hematopoietic cells after transplantation, so more comprehensive *in vivo* studies such as xenograft transplantation of *ELANE* knockout HSPCs in irradiated transplant recipient mice or non-human primates is needed. The *ELANE* knockout strategy could be further improved by performing the gene-editing on an allele-specific manner to target the mutated disease-causing allele only. Another promising strategy to increase the safety of *ELANE* knockout strategy as a clinical approach, is targeting of the non-coding regulatory region of the ELANE gene with double paired D10A Cas9 nickases. Using this strategy, we may achieve following: First, inhibiting RNA polymerase II that is required for ELANE mRNA transcription, will avoid targeting ELANE cDNA and possible generation of mutated neutrophil elastase, which in long-term may have damaging effect on granulopoiesis and may lead to leukemia; Second, double nicking gene-editing strategy could also enhance gene-editing and reduce the appearance of off-targets up to 1000 fold.[62] The implementation of these two safety factors could enormously increase the safety of gene therapy of CN patients, which is based on the *ELANE* knockout. We are currently testing this strategy having auspicious preliminary results.

Taken together, we reported for the first time the establishment of the CRISPR/Cas9 based gene-editing strategy in HSPCs and iPSC from CN patients having *ELANE* mutations. This is an efficient and universal approach for all *ELANE*-CN patients regardless of the position of *ELANE* mutation. This strategy led to markedly improved granulocytic differentiation and formation of fully functional normal mature neutrophils from CN patients HSPCs *in vitro*. Hence, CRISPR/Cas9 based *ELANE* knockout in CN-*ELANE* patients might be a safe, virus- and DNA-free alternative therapeutic option, especially in patients that are G-CSF non-responders or requiring high G-CSF dose for treatment of severe neutropenia.

Optimization of the efficiency and safety of CRISPR/Cas9 gene-editing in induced pluripotent- and hematopoietic stem/progenitor cells

(Covered in the published **Manuscript B**; Nasri M. and Mir P. et al., 2019)

(Covered in the published **Manuscript D**; Dannenmann B. and Nasri M. et al., 2020)

To further improve the efficiency of safe gene-editing in primary human cells, we aimed to develop strategies of sorting gene-edited cells by fluorescence activated cell sorting (FACS) and enrich the population of gene-edited cells as there are challenges regarding the application of CRISPR/Cas9 technology in stem cells and primary cells with several limitations for clinical applications. The CRISPR/Cas9 system can be delivered in different forms like viral vectors, plasmids, or ribonucleoprotein (RNP). Comparing the delivery formats, RNP is the safest form of CRISPR/Cas9 due to very low off-target activity, in addition to the fact that viral vectors and even plasmids could randomly integrate into the genome and make the editing outcome unpredictable, especially in case of the gene therapy approach. Gene-editing in primary hematopoietic stem cells and iPSCs could be challenging, and the gene-modification efficiency is dependent on the cell type, cell cycle stage, activation of DNA-repair pathways, chromatin dynamics at the gRNA-targeted gene locus, and the delivery method. RNP complexes that are formed by assembling Cas9 nuclease and synthesized guide RNA (gRNA) outperform other formats in safety issues, but due to a lack of any selection marker, the enrichment of the gene-edited cells is not possible. There have been reported strategies to address this issue, such as tagging of Cas9 protein with GFP.[63] However, Cas9-GFP fusion proteins could form aggregates or GFP tagging might influence the appropriate intracellular Cas9 protein localization and on-/off-target activity of RNP complexes. We showed that Cas9-GFP/gRNA RNP complex has lower efficiency in gene-editing compared to the RNP complex assembled with un-tagged Cas9. To be able to enrich the gene-edited cell population by fluorescent activated cell sorting (FACS), we established an efficient method for chemical fluorescent labeling and delivery of CRISPR/Cas9–gRNA RNP in primary human HSPCs and iPSCs (Fig. 6). The labeling dye composed of fluorophore

label, the linker which facilitate the electrostatic interaction with nucleic acids of gRNA and the reactive alkylation group that covalently bind to the reactive heteroatom within the nucleic acids of gRNA. As a proof of principle, we select growth arrest and DNA-damage-inducible β (*GADD45B*) gene, a gene with low-abundance transcripts that functions as stress sensors regulating cell cycle, survival, and apoptosis in response to various stress stimuli. *GADD45B* is a member of the myeloid differentiation primary response genes (also called MyD118) that belongs to a GADD45 protein family.[64]

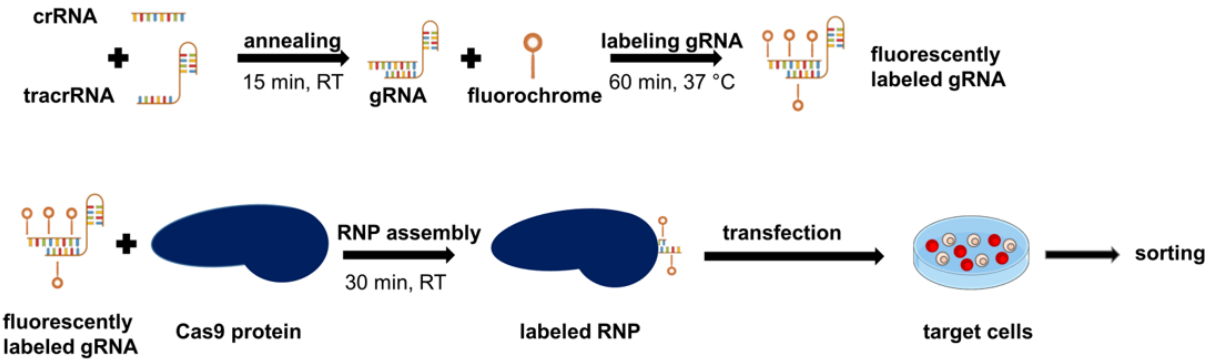

Figure 6. Schematic representation of CRISPR/Cas9–gRNA RNP labeling and cell transfection (Adopted and modified from Nasri M & Mir P et al. "Fluorescent Labeling of CRISPR/Cas9 RNP for Gene Knockout in HSPCs and iPSCs Reveals an Essential Role for GADD45b in Stress Response". Blood Adv. 2019;3(1):63–71.)

We designed a gRNA that knockout the *GADD45B* gene and applied the gRNA labeling strategy using CX-Rhodamine or Fluorescein dye. Covalent labeling was performed by the ratio of 1:1 (v:w) of labeling reagent to duplexed crRNA:tracrRNA, leading to 1 label per 20 to 60 nucleotides. The reaction was incubated at 37°C for 1 hour, followed by ethanol precipitation. We further transfected (HEK293T, Jurkat, iPS cells), or electroporated (healthy control $CD34^+$ HSPCs) cells with labeled CRISPR/Cas9–gRNA RNP followed by enrichment using FACS-based sorting of positive cells. We detected CX-rhodamine or fluorescein signal already 6 to 12 hours after transfection and the fluorescent signal disappeared about 48 hours after transfection. Labeling did not affect the gene-editing efficiency of CRISPR/Cas9 RNP, as assessed by Sanger sequencing and tracking indels by decomposition (TIDE) assay analysis of cells, transfected with

labeled or unlabeled *GADD45B* CRISPR/Cas9 RNP. Sorting of positive cell population by FACS significantly increased the gene-editing efficiency.

Using this method, we were able to show that GADD45B is crucial for DNA damage protection and survival in human leukemia cells and $CD34^+$ HSPCs that are not compensated by other GADD45 proteins.

In this research project, we have developed a new method for fluorescent labeling of CRISPR/Cas9, which facilitates genome editing in primary HSPCs and iPSCs using CRISPR/Cas9–gRNA RNP complexes. Sorting of the labeled cells also allows the removal of untargeted HSPCs that may compete with gene-edited cells. Moreover, the application of CRISPR/Cas9 RNP minimizes the probability of off-target effects, as CRISPR/Cas9 RNP activity is preserved within the cells for less than 72 hours. It is essential to achieve high editing efficiency in hematopoietic cell therapies due to the limited amounts of HSPCs and their restricted cell division capacity. Although we did not observe any inhibitory effects of the labeling on the gene-editing efficiency or any cytotoxicity, it is suggested to perform more detailed safety analysis of labeling dyes such as Cell-titer-Glo viability assay, LDH-Glo cytotoxicity assay and RealTime-Glo annexin V apoptosis assay, to ensure the safety of fluorescent labeling dyes in clinical applications. Another suggestion would be to compare the off-target profiling of labeled CRISPR/Cas9 RNP versus un-labeled one, to ensure that labeling strategy did not affect the off-target rate of the gene-editing. It might be also important to improve the labeling strategy using fluorophore with high signal to noise ratio such as MFP488.

Translational mechanistic study of T-cell acute lymphoblastic leukemia (T-ALL) and hematopoiesis

(Covered in the published **Manuscript C**; Morishima. et *al*., 2019)

In this part of my PhD thesis, I implemented CRISPR/Cas9 mediated gene-editing technology in the analysis of the role of NAMPT-mediated deacetylation of the LIM domain only 2 (LMO2) transcription factor in hematopoiesis and leukemogenesis. Our group has previously identified an essential role of NAMPT-triggered deacetylation of

transcription factors C/EBPα and C/EBPß in the granulopoietic differentiation of HSPCs.[65] The involvement of NAMPT/SIRT2 pathway in the AML leukemogenesis by regulation of the Akt-GSK3ß-ß-catenin signaling was also documented.[66] As a continuation of this study, we analysed the NAMPT/SIRT2-triggered protein deacetylation in early stages of hematopoiesis using iPSCs.

Well-defined regulated transcription factor networks are vital for retaining hematopoietic tissue homeostasis. Hematopoietic transcription factor LMO2 is a member of the TAL1 transcriptional complex and has a crucial function during early hematopoiesis, as LMO2 knockout zebrafish and mice have a complete loss of hematopoiesis.[67] We showed that the NAMPT/SIRT2-mediated deacetylation of LMO2 at the lysine residues K74 and K84 is crucial for the interaction of LMO2 with LDB1 following by subsequent activation of the TAL1 transactivation complex (Fig. 7). We compared the interaction between LDB1 and WT K74/78Q (acetylation-mimic) or K74/78R (deacetylation-mimic) LMO2 expression constructs by co-immunoprecipitation. We observed that the K74/78Q LMO2 mutant has a weaker interaction with LDB1 in comparison with WT and K74/78R LMO2. We also showed that the NAMPT-mediated deacetylation of LMO2 and, therefore, activation of the TAL1 complex. Inhibition of NAMPT or SIRT2 led to a markedly diminished hematopoietic differentiation of iPSCs via regulation of LMO2 deacetylation. Remarkably, patients with T-cell acute lymphoblast leukemia (T-ALL) express higher amounts of LMO2.[68] We proposed inhibition of NAMPT or SIRT2 to hamper LMO2 acetylation as a therapeutical approach aiming to suppress proliferation while inducing apoptosis of T-ALL cells. We confirmed that treatment of LMO2-expressing MOLT4 and MOLT14 cell lines with FK866 - a highly specific noncompetitive inhibitor of NAMPT - or AC93252 - a SIRT2 inhibitor - significantly reduced the proliferation and led to enhanced apoptosis of T-ALL cell lines. We further assessed the effects of SIRT2- or NAMPT inhibition on primary blasts from T-ALL patients and confirmed that treatment of LMO2- and TAL1-expressing primary blasts of T-ALL patients with AC93253 or FK866 also resulted in the significant reduction of cell proliferation. Labeling MOLT-4 cell line or primary T-ALL patient cells with carboxyfluorescein diacetate succinimidyl ester and injecting into zebrafish embryos allowed us to evaluate the effect of FK866 in an *in vivo* system. We found that NAMPT inhibition diminished proliferation of xenotransplanted T-ALL cells *in vivo* in zebrafish.

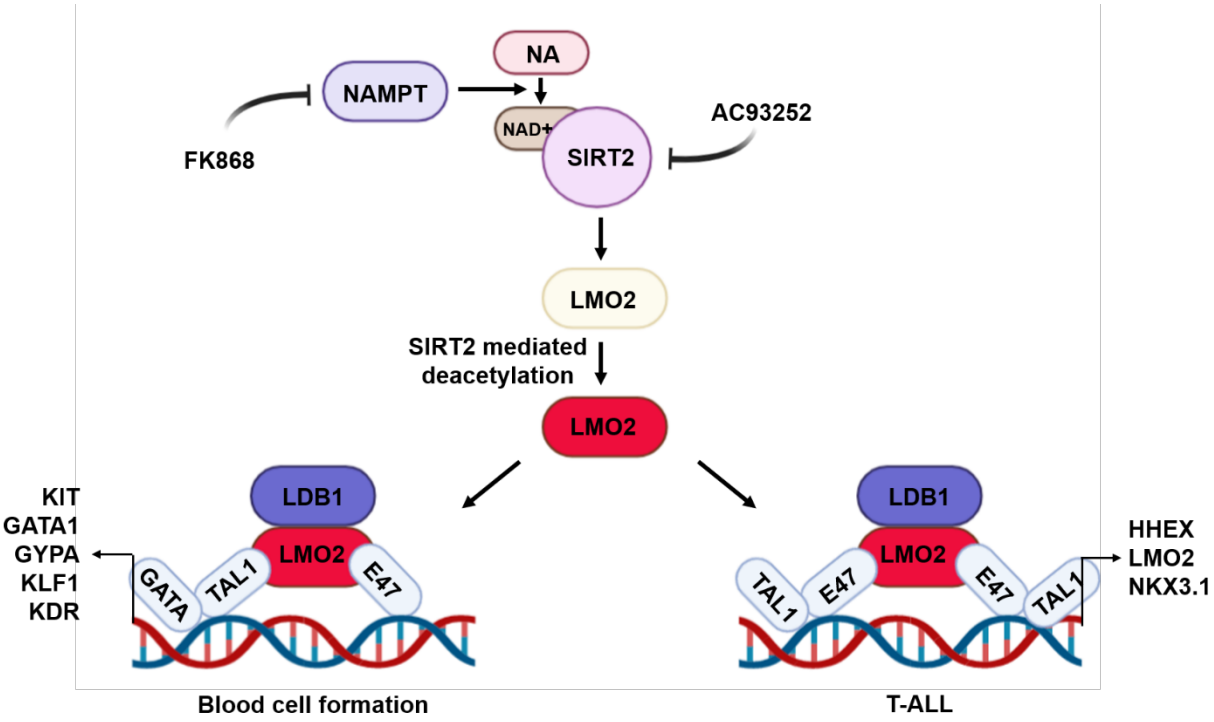

Figure 7. LMO2 deacetylation is essential for hematopoiesis and T-ALL leukemogenesis (Adopted and modified from Morishima. T. et al. "LMO2 activation by deacetylation is indispensable for hematopoiesis and T-ALL leukemogenesis." Blood 134.14 (2019): 1159-1175.)

The same experiment was performed in immunodeficient nonobese diabetic severe combined immunodeficiency IL2Rγc−/− (NSG) mice, and the results also confirmed that NAMPT inhibition reduced T-ALL cell proliferation *in vivo* via hampering deacetylation of LMO2. We designed a CRISPR/Cas9 strategy to perform specific knockout of SIRT2 and observed the reduction of TAL1 complex activity using the GYPA reporter system in SIRT2-KO HEK293FT cells. We also knocked out LMO2 in the MOLT-4 cell line using LMO2-specific CRISPR/Cas9-sgRNA RNP gene-editing. MOLT-4 is a hyper-tetraploid T lymphoblast cell type, established from the peripheral blood of a 19-year-old man with acute lymphoblastic leukemia (ALL). Interestingly, we found markedly diminished viability, proliferation, and elevated apoptosis of MOLT4 cells upon LMO2 knockout. We further performed transcriptomic profiling to identify the set of genes and pathways regulated by NAMPT-deacetylated LMO2 in T-ALL cells. Our analysis revealed 69 differentially expressed genes (DEGs) regulated in wild-type LMO2 but not in K74/78 LMO2 expressing MOLT4 cells after two days of treatment with FK866 or DMSO. Among these

genes, there were leukemia-associated genes such as HOXD13, SOX7, SOX12, CYP1B1, PRDM1, RASL11B, TRAF1, IGFBP4, and CYP2E1. Pathway overrepresentation enrichment analysis also showed upregulation of cytochrome P450, tryptophan degradation, biological oxidation, direct p53 effectors, and tumor necrosis factor α (TNF-α) signaling pathways as a result of LMO2 deacetylation.

In summary, we demonstrated that the NAMPT/SIRT2-triggered deacetylation of LMO2 at K74/78 is essential for its activation ant Tal1 complex formation during early hematopoiesis and T-ALL leukemogenesis *in vitro* and *in vivo*. These results suggest that T-ALL leukemia cells could be clinically targeted through specific inhibition of NAMPT-mediated LMO2 deacetylation. Also, modulation of NAMPT/SIRT2/LMO2 signaling might be used for the improvement of the *in vitro* generation of hematopoietic cells from iPSCs.

Taken together, we demonstrated a novel posttranslational mechanism - NAMPT/SIRT2-mediated lysine deacetylation - which is involved in regulating the developmentally important protein LMO2. The findings in the current study have valuable translational significance, suggesting the possibility of clinically targeting T-ALL leukemia cells through specific inhibition of NAMPT-mediated LMO2 deacetylation. One suggestion as the next step for this project is a high-throughput screening of drug libraries to find small molecules that specifically inhibit TAL1 complex formation. We have established a Bioluminescence Resonance Energy Transfer (BRET) based system to study an interaction between the TAL1 complex members such as LMO2:LDB1 or LMO2:E47. This assay is based on a binary split luciferase system, that, upon structural complementation, generates a luminescent signal quantifiable up to attomolar level. At the same time, it does not disturb the native structure of the protein interactions and screening different types of small-molecules libraries. We performed computational modeling of the interaction modes between LMO2 other proteins (e.g., LDB1 and E47) within the TAL1 complex and DNA. We identified two druggable sites in LMO2 that could be hypothetically used to inhibit these interactions. Based on this model, we designed 25 lead compounds. We are currently analyzing the effects of designed lead compounds on the LMO2:LDB1 and LMO2:E47 protein interactions. The potential lead compound/s will be used as a starting point for rational drug design.

OUTLOOK

ELANE mutations are the main disease-causing reason for CN. Developing an efficient and safe universal gene therapy approach will be beneficial for the majority of CN patients. Here, we showed that CRISPR/Cas9 RNP based knockout of *ELANE* might be a safe, efficient, and universal strategy for all CN-*ELANE* patients, particularly for the patients that do not respond to G-CSF therapy or failed the bone-marrow transplantation. It is essential to evaluate the safety of this approach by performing a high-throughput off-target analysis method, such as Circle-seq sequencing to validate the off-target activity profile of the selected sgRNA. It is also necessary to perform *in vivo* studies to show that *ELANE* knockout HSPCs can generate all blood cell lineages long-term. Additionally, the functions of gene-edited *ELANE*-KO hematopoietic stem cells and mature granulocytes should be profoundly investigated. It would also be important

to make this strategy even safer by targeting non-coding parts of the genome, such as *ELANE* promoter, to reduce the risk of the formation of unwanted mutated *ELANE* proteins. We are currently working on this approach having exciting preliminary data.

We also demonstrated an efficient, fast and easy-to-perform fluorescent-labeling of CRISPR/Cas9 ribonucleoprotein (RNP) complexes that enables sorting of edited HSPCs and iPSCs by FACS and leads to enrichment of the population of gene-edited cells. An alternative labeling approach of gRNA would be *in vitro* transcription of guide RNA using fluorescent nucleotides. Besides the enrichment of gene-edited cells, the labeling strategy could also be used to optimize CRISPR/Cas9 RNP transfection/electroporation conditions in different cell lines and study different kinetics of the CRISPR/Cas9 gene-editing. The labeling strategy, if used with dead-Cas9 (dCas9), can also be applied for the CRISPR/Cas9-mediated fluorescent *in situ* hybridization.

Lastly, we showed that inhibition of the NAMPT-mediated deacetylation of LMO2 could be a promising therapeutic approach for T-ALL. Identification of the specific inhibitors of the LMO2 interaction with other members of the TAL1 complex will lead to the establishment of novel targeted therapy of T-ALL.

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APPENDIX I: LIST OF ACCEPTED PUBLICATIONS

Nasri M. et *al*., 2019, *Haematologica*

Nasri M. & Mir P. et *al.*, 2019*, Blood Advances*

Morishima T. et *al.*, 2019, *Blood*

Dannenmann B. & Nasri M. et *al.*, 2020, *Methods in Molecular Biology*