Thesis

Engineering of mesenchymal stromal cells to improve culture conditions for primary leukemia

submitted by

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in partial fulfillment of the requirements for the degree of

Doktor der gesamten Heilkunde
(Dr. med. univ.)

at the

Medical University of Graz

executed at the **University departments of Internal Medicine**
Institute of Hematology

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Graz, 30.01.2024

Declaration of Academic Integrity

I hereby confirm that the present diploma thesis is the result of my own independent scholarly work. I also confirm that in all cases, where material from the work of others (in books, articles, essays, dissertations, and on the internet) is acknowledged, quotations and paraphrases are clearly indicated. No material other than that cited in the reference list has been used. I have read and understood the Medical University's regulations and procedures concerning plagiarism.

Graz, 30.01.2024 *Thomas Brauchart m.p.*

Acknowledgement

As this thesis would never be possible without the help and support from a lot of people, I would like to recognize the invaluable assistance that you all provided during my study.

First of all, I would like to express my gratitude to my supervisors Dr. Andreas Reinisch and Dr. Heinz Sill for their useful comments, remarks and engagement through the learning process of this diploma thesis, helping me whenever it was necessary and guiding me through each stage of the process.

Furthermore, I would like to acknowledge the members of my working group, whose contribution was a big help. I want to thank Lisa Auinger, Erdem Özkaya, Tommaso Sconocchia, Angelika Schlacher and especially Johannes Foßelteder not only for their professional input and knowledge, but also for the funny conversations and the entertainment between long incubation times.

Finally, I like to pay my special regards to my family Ingrid, Dieter, Iris and Philipp and my partner Johanna Windisch, who have assisted me throughout the entire process, both by helping me putting pieces together and keeping me going on. Without them this thesis would not have been possible. Thank you.

Zusammenfassung

Einleitung

Die Knochenmarks–Nische besitzt ein einzigartiges Milieu mit einer Vielzahl an unterschiedlichen Zelltypen. Eine dieser Zellen ist die, für die Hämatopoese zuständige, hämatopoetische Stammzelle (HSC), welche in dieser Nische, die notwendigen Bedingungen für ihre Proliferation und Differenzierung vorfindet. Eine weitere Stammzelle, die mesenchymale Stammzelle (MSC), ist nicht nur eine Vorläuferzelle für multiple Zelltypen, sondern spielt auch eine wichtige Rolle in der Entwicklung der HSC. Erste Versuche, HSCs ex vivo zu kultivieren, führten aufgrund ihrer komplexen Millieuanforderungen der Notwendigkeit verschiedener Zell-Zell Interaktionen zu ernüchternden Ergebnissen.(1) Ko-Kultivierungsexperimente versuchten, besagte Problematiken zu überkommen, die Zugabe der Zytokine erfolgte jedoch extern, was sich als teuer und unphysiologisch herausstellte.(2)

Methoden

Wir modifizierten genetisch humane mesenchymalen Stammzelltypen (MSC-hTERT und HS27a) um die Zytokine FLT3L, SCF, TPO und IL-6 über zu exprimieren. Fluoreszenzmarker beinhaltende cDNA-Konstrukte (FLT3L_SCF_BFP und TPO_IL-6_BFP) wurden mittels CRISPR/Cas9-vermittelter homologer Rekombination (HDR) in sogenannte „safe harbor“ Sequenzen (*HBB* oder *AAVS1*) eingebracht, wobei rekombinante adenoassoziierte Viren (rAAVs) als „template DNA“ Vektoren verwendet wurden. Nach einer, mittels in-out PCR, durchgeführten knock-in Kontrolle wurden bei erfolgreicher Einbringung, die Zytokinspiegel durch einen ELISA-Test gemessen. Anschließend wurden humane mesenchymale Stammzellen, welche Zytokine exprimierten, entweder mit Zytokin-abhängigen akuten myeloischen Leukämiezelllinien (AML) oder aus Nabelschnurblut stammenden hämatopoetischen Stammzellen kultiviert. Das Zellwachstum der getesteten Zelllinien wurde mittels Durchflusszytometrie quantifiziert. Weiters wurde die $CD34^+$ Teilpopulation und die koloniebildende Eigenschaften bestimmt.

Ergebnisse

Insgesamt konnten wir 8 Klone (vier für TPO_IL-6_BFP, einen für FLT3L_SCF_BFP und drei, welche beide Konstrukte beinhalteten) für die MSC-hTERT und 14 Klone

für die HS27a Zelllinie (sechs für TPO_IL-6_BFP, fünf für FLT3L_SCF_BFP und vier, beide Konstrukte beinhaltete) konstruieren. Vier MSC-hTERT Klone und neun HS27a Klone produzierten signifikante Mengen der getesteten Zytokine ($p<0,05$).

Die Kokulturen mit Zytokine exprimierenden Zellen führte zu einer signifikanten Expansion von Leukämiezelllinien im Vergleich zu nicht modifizierten Wildtyp-Zellen (MSC-KI:MSC-WT: $p<0,001$) oder Monokultur-Bedingungen (MSC-KI:neg. ctrl: $p<0,001$). Auch nicht modifizierte Wildtyp-Stromazellen verstärkten das Zellwachstum im Vergleich zu Monokultur-Bedingungen (MSC-WT:neg. ctrl.: $p<0,001$). Die Expansion von HSPCs, evaluiert durch die Gesamtzahl der CD34 positiven Zellen, war in Kokulturen mit Zytokin-exprimierenden Stromazellen signifikant erhöht (MSC-KI:neg. ctrl: $p<0,001$), während der Prozentsatz der $CD34^+$ Zellen ($p = 0,051$) und die Gesamtzahl der Kolonien nicht signifikant von den ausgeschiedenen Zytokinen beeinflusst wurden.

Conclusio

Durch die Nutzung des CRISPR/Cas9-Systems zur präzisen Konstruktion von Zytokin exprimierenden Stromazellen sollte diese Studie die lang bestehenden Herausforderungen der in vitro Kultivierung von AML und HSPCs überkommen. Die erfolgreiche Einführung des Expressionskonstrukts in MSCs führte zu ausreichender Produktion der humanen Zytokine FLT3L, SCF, TPO und IL-6.

Kokulturexperimente mit Konstrukt–beinhaltenden MSCs zeigten deutliche Vorteile in Bezug auf Zellzahlen und Expansionsrate und verstärken somit die Annahme, dass MSCs eine entscheidende Rolle in der Knochenmarknische spielen und die wichtige Rolle dieser essenziellen Zytokine hervorheben.

Unter Berücksichtigung der mit der Studie einhergehenden Limitationen, einschließlich nicht physiologisch aktivierter Zytokingene und der hohen Heterogenität der kleinen Probenmenge, ermöglicht diese Arbeit die Bedeutung der MSCs in der HSC–Entwicklung hervorzuheben. Die CRISPR/Cas9 vermittelte Einbringung der Zytokine bietet vielversprechende Möglichkeiten für zukünftige Studien, welche auch erforderlich sind, um genauere Einblicke in die molekularen Mechanismen der MSC-HSC Interaktion zu geben und physiologischere HSC Langzeitkulturen zu ermöglichen.

Abstract

Introduction

The bone marrow niche provides a distinctive microenvironmental area, with its great variety of cell types. Harboring the hematopoietic stem cells (HSC), this niche provides crucial elements and structures for their proliferation and differentiation and therefore, is elemental for hematopoiesis. A second stem cell, the mesenchymal stem cell (MSC), is also located in the bone marrow niche. The MSC is not only the progenitor of multiple different cell types, but also plays a necessary role in the proliferation of the HSC. Ex vivo attempts to expand HSCs, led to disappointing results, due to their high demands of cytokines and cell-cell interaction.(1) Co-culture experiments tried to overcome this obstacle, but still relied on external cytokine substitution.(2)

Methods

In this thesis we engineered human stromal cell lines (MSC-hTERT and HS27a), to overexpress human cytokines FLT3L, SCF, TPO and IL-6. Complementary DNA (cDNA) constructs together with a fluorescent marker (FLT3L_SCF_BFP and TPO_IL-6_BFP) were precisely introduced into safe harbor loci (*HBB* or *AAVS1*) by CRISPR/Cas9-mediated homology-directed repair (HDR) employing recombinant adeno-associated virus (rAAVs) as vectors for DNA template delivery. Precise knock–in was confirmed via an in-out PCR and cytokine levels were measured by an ELISA.

Thereafter, human cytokine expressing stromal cells lines were co-cultured with either cytokine-dependent acute myeloid leukemia (AML) cell lines or cord blood-derived HSCs. Cell Expansion of AML cell lines and HSCs was quantified using flow cytometry. Additionally, HSPC subpopulation and colony-forming potential were evaluated.

Results

We successfully engineered a total of eight single cell-derived clones (four for TPO_IL-6_BFP, one for FLT3L_SCF_BFP and three with an introduction of both constructs) for the MSC-hTERT and 14 single cell-derived clones for the HS27a cell line (six for TPO_IL-6_BFP, five for FLT3L_SCF_BFP and four with an introduction

of both constructs). Out of them four MSC-hTERT clones and nine HS27a clones provided measurable production of the introduced cytokines ($p< 0.05$).

Co-culture with cytokine-expressing cell resulted in significant expansion of leukemia cells lines when compared to non-engineered wild type cells (MSC-KI:MSC-WT: $p<0.001$) or mono-culture conditions (MSC-KI: neg. ctrl: $p<0.001$)

Interestingly, also non-engineered wild type stromal cells supported cell growth, when compared to mono-culture conditions (MSC-WT:neg. ctrl.: $p<0.001$). HSPC expansion, evaluated by total $CD34^+$ cell counts, was significantly increased in co-cultures with cytokines expressing stromal cells samples (MSC-KI:neg. ctrl: $p<0.001$), while the percentage of $CD34^+$ cells ($p=0.051$) and the total number of colonies was not significantly influenced by the secreted cytokines.

Conclusion

By utilizing the CRISPR/Cas9 system to precisely engineer a cytokine-expressing stromal cell, this study aimed to address the long existing challenges of culturing AML and HSPCs in vitro. The successful introduction of expression construct into MSCs resulted in the sufficient production of the human cytokines FLT3L, SCF, TPO, and IL-6.

Co-culture experiments utilizing knocked-in MSCs further let to a substantial expansion and increased survival, supporting the theory of MSCs playing a crucial role in the bone marrow niche and highlight the critical role of these essential cytokines.

While acknowledging certain limitations, including the non-physiological introduction of cytokines, high sample heterogeneity and small sample size, our research underlines the importance of MSCs in HSC maintenance and proliferation. The cytokine introduction via CRISPR/Cas9 holds promise for future advancements in culture settings, urging further studies to investigate the molecular mechanisms underlying MSC-HSC interactions and providing insights for long-term HSC culture possibilities.

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Abbreviation

AAV	adeno–associated viruses
AAVS1	adeno–associated virus integration site 1
AML	acute myeloid leukemia
ANGPT1	angiopoietin 1
BARD1	BRCA1–associated RING domain protein 1
BFP	blue fluorescent protein
BFU–E	erythroid burst–forming units
BIR	break–induced repair
BM	bone marrow
bp	base pair
BRCA1	recombination mediator breast cancer 1
BSA	Bovine serum albumin
Cas	CRISPR–associated
CD	cluster of differentiation
CFU–E	erythroid colony forming unit
CFU–F	colony forming unit fibroblasts
CFU–GEMM	colony forming unit for granulocyte, erythrocyte, monocytes, and macrophages
CFU-G/M/GM	bi- or unipotent granulocyte, monocyte colony forming unit
CFU–S	spleen colony forming unit
c–Kit	receptor tyrosine kinase
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CPP	Cell–penetrating peptides
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CRP	C–reactive protein
crRNA	CRISPR RNA
CXCL 12	CXC–chemokine ligand 12
dHJ	double holiday junction
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSB	double–strand DNA breaks
DSBR	double–strand break repair
ELISA	enzyme–linked immunosorbent assay
EPO	erythropoietin
FACS	fluorescence–activated cell sorting
FBS	fetal bovine serum
FLT3L	Fms–related tyrosine kinase 3 ligand
G-CSF	granulocyte colony–stimulating factor
gp130	glycoprotein 130
HBB	Hemoglobin subunit beta (β–globin)
HDR	homology directed repair
HPGM	hematopoietic growth media
HS(P)C	hematopoietic stem (and progenitor) cells
ICAM–1	intracellular cell adhesion molecules–1
IL-1a; 3; 6	interleukin 1a; 3; 6
ISCT	International Society for Cell & Gene Therapy
ITR	Inverted Terminal Repeat

JAG1	Jagged Canonical Notch Ligand 1
JAK/STAT	Januskinase/ signal transducers and activators of transcription
KI	Knock–in
MAPK	Mitogen–activated protein kinase
MAT	marrow adipose tissue
MPP	multipotent progenitor cell
MRN	MRE11–RAD50–NBS1
(BM–)MSC	(bone marrow) mesenchymal stem/stromal cell
NHEJ	non-homologous end joining
OPN	osteopontin
PAM	protospacer adjacent motif
PBS	Phosphate-buffered saline
(dd)PCR	(digital droplet) polymerase chain reaction
PEI	polyethylenimine
PIKKs	PI3K–like Kinases
rAAV6	recombinant adeno–associated virus serotype 6
RNA	ribonucleic acid
RNP	ribonucleoprotein
RPMI	Roswell Park Memorial Institute
SCF	stem cell factor
SD	standard deviation
SDSA	Synthesis–dependent strand–annealing
SFEM	serum free expansion medium
sgRNA	single guided RNA
ST/LT–HSC	short term/long term hematopoietic stem cells
TALEN	transcription activator–like effector nucleases
TKR	tyrosine kinase receptor
TNFα	tumor necrosis factor α
TPO	thrombopoietin
tracrRNA	trans activating crRNA
VCAM–1	vascular cell adhesion molecules–1
VLA4/5	very late antigen 4 and 5
WT	wild type

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1. Introduction

1.1. Bone marrow niche

The bone marrow (BM) niche is a unique microenvironmental area consisting of a heterogeneous mixture of interacting cell types in an microenvironmental unique area. In adult individuals the BM is predominantly located within the trabecular–rich metaphysis of long bones.(3) It has a diversity of functions, being essential for stem cell maintenance and hematopoiesis by providing the necessary structure and microenvironment. Due to its heterogeneity and the complex formation of distinctive niches, there is still a variety of poorly understood mechanisms and open questions after decades of research.(4)

To better understand the physiology of the BM niche, scientists shifted their focus on analyzing the properties of the many different members. Two types of stem cells are embedded between the trabecula of the spongiosa, namely the hematopoietic stem cell (HSC) and the mesenchymal stromal/stem cell (MSC).(5) While the multipotent HSCs can differentiate into myeloid, lymphoid and erythroid lineages, the MSC is the progenitor cell for adipocytes, chondrocytes, osteocytes, myoblasts and other cell types as well as multiple studies indicate.(6,7)

1.1.1. Hematopoietic stem cells (HSC)

The first experiment that was evidential for the in vivo existence of HSCs occurred in 1963 by detecting noduls of new forming colonies of hematopoietic progenitors on the spleen (spleen colony forming unit – CFU–S) of BM transplanted and earlier lethally radiated mice.(8) In early stages colony forming unit assays (CFU) were used to separate cells due to their ability to form distinctive and mixed colonies, revealing their lineage potential.(9) Therefore, cells were classified into the multilineage colony forming cell for granulocyte, erythrocyte, monocytes, and macrophages (CFU–GEMM) and already more differentiated cells like the erythroid colony forming unit (CFU–E) and erythroid burst–forming units (BFU–E).(10,11) Today the HSC is better known, sustaining all mature blood cell lineages and giving rise to both, the myeloid and lymphoid lineage.(12) The HSC itself can be further divided into long term–HSC (LT–HSC) and short term–HSC (ST–HSC), while the former is very rare, quiescent and has the capacity for a life–long supply for a normal

hematopoiesis, the latter is only able to provide for 6–8 weeks of hematopoiesis and therefore sometimes defined as a so-called hematopoietic stem and progenitor cell (HSPC) with limited self-renewable properties.(13) A variety of surface markers have been discovered from different groups to establish better HSC purification, locating the HSCs in the Lin-CD34+CD38-CD90+ fraction.(14,15) According to the standard model as depicted in figure 1 the ST–HSC then give rise to the multipotent progenitor cell (MPP), that furthermore is able to differentiate into all hematopoietic lineages, however does not possess the ability of self-renewal.(16)

The common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP) are descendants of the MPP and possess the ability to either differentiate into mature cells of the myeloid or lymphoid lineage.(17)

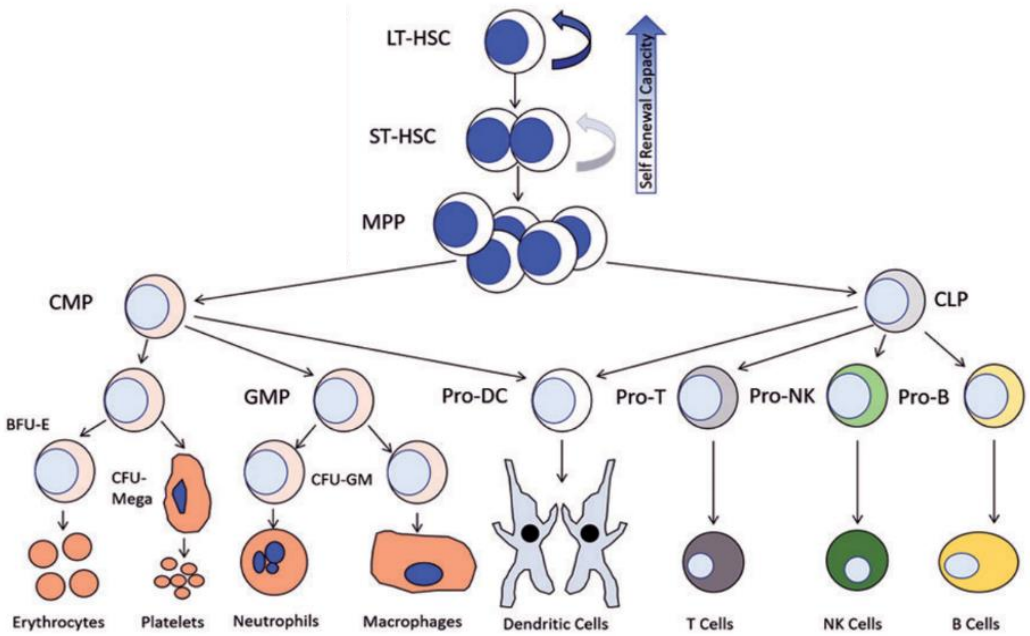

Figure 1 Blood system cell type hierarchy.
While the LT–HSC possesses high self-renewal capacity, the ST–HSC is only active for 6–8 weeks before losing its self-renewal ability. While the ST–HSC differentiates into the MPP, it is losing that ability completely. The MPP is the common progenitor of the CMP and the CLP, that give raise to the variety of cell types of the myeloid and lymphoid lineage.(18)

However as more recent studies indicate, the hierarchical structure of the HSC and their progenitors is not as homogenous and linear as assumed until now.(19) Also mouse data indicates that, in contrast to the former believes, discrete hierarchically organized progenitor populations are passed through while lineage biases are acquired by the individual HSC, proposing newer models with a continuous process

of the differentiation of low–primed undifferentiated HSPC into the respective unilineage restricted descendant.(20)

1.1.2. Mesenchymal stromal/stem cells (MSC)

In contrast to the HSC, the frequency MSCs appear is rare, being around one to two cells per 100.000 in a normal BM puncture sample.(21) First described as fibroblast–like cells, due to their spindle–shaped structure, further experiments revealed their potential as multipotent stromal progenitor cells and with stem cell function, marking them as colony forming unit fibroblasts (CFU–F), being found not only in the BM but also in a variety of other tissue.(6,22,23) Next to the BM derived MSCs, in the search for alternative sources, MSCs could be discovered in adipose tissue, umbilical cord blood, heart, peripheral blood, muscle, lung, intestine, kidney, liver, pancreas, synovium, skin and brain.(24,25) These MSCs are able to self-renew and can differentiate into several cell lineages of mesenchymal originated tissue like bone, cartilage, adipose tissue, tendon and muscle.(26,27) However studies have shown the impact of different source material on the osteogenic and chondrogenic differentiation, as for example cord blood extraction lead to a steady differentiation for adipogenic and osteogenic lineages, while venous blood material resulted in poor osteogenesis.(23) Furthermore, next to the source material other factors have a big impact on the cell development and their secretome as there have to be mentioned donor age, extraction method and culture condition, including the applied media.(28,29)

The full name of the MSC itself is already a delicate business and in 2019 the International Society for Cell & Gene Therapy (ISCT) announced that either mesenchymal stromal cells, mesenchymal stem cells or less frequently used multipotent stem cells can be grouped under the acronym MSC, even though so-called mesenchymal “stem” cells should provide strong evidence for stem cell functionality, when this term is applied.(30) Furthermore, a distinctive separation by surface marker is also still a difficult task, that’s why the typical isolation of MSCs by exploiting their adherence properties is still part of the definition of MSCs.(31) With the introduction of possible markers, a subset of criteria markers has been formulated through a series of studies, even though they still differ from study to study: MSC should express CD105, CD73, and CD90 and there should be no

expression of CD14, CD34, CD45, CD11b, CD19, CD79a and HLA–DR.(32,33) However there is still an ongoing debate about this selecting criteria, due to the fact that plastic–adhering culture conditions and in vitro culturing can lead to the expression changes of different surface marker, for example CD34, that was shown to be positive in human BM samples under different selection criteria and therefore may possess other cell properties.(34)

1.1.3. Osteolineage cells, Adipocytes, cells of the immune system, nerval and vascular cells

First in vitro experiments with osteolineage cells and HSCs seemed to state a clear dependency between these two cell types by an expansion of hematopoietic progenitor cells in the presence of osteolineage cells. These findings are being further backed up by several mouse experiments, where either ablation of osteoblasts led to reduced numbers of all three hematopoietic lineages or parathyroid hormone stimulated overexpansion of osteoblastic cells caused higher HSC levels.(35,36) The cytokines CXC–chemokine ligand 12 (CXCL12) and stem cell factor (SCF) with their respective receptor on HSCs as well as the molecules osteopontin (OPN), thrombopoietin (TPO) and angiopoietin 1 (ANGPT1) at first were thought to be mainly provided by the osteolineage cells and were the reason for an HSC proliferation in the BM.(37) However multiple studies with deletions of the former two resulted in only insignificant effect on the HSCs and other main providers of the other molecules for example hepatocytes for TPO were found.(38,39) This data combined with modern 3D imaging studies indicate no significant association between osteoblast and HSCs. Nevertheless further studies could prove that osteolineage cells strongly influence lineage–committed hematopoietic progenitors, strongly supporting the differentiation of lymphoid precursors.(40)

Not receiving much attention before the last decade, adipocytes are known to be part of the BM tissue, also called marrow adipose tissue (MAT).(41) Even though it seems to be clear that adipocytes have a predominantly suppressive effect on hematopoiesis, caused for example by the secretion of lipocalin, adiponectin and TNFα, there seems to be a difference in the properties of these cell types, depending on the adipocyte progenitors.(42) While a $CD45^{-}CD31^{-}SCA1^{+}CD24^{+}$ MSC population, identified by Ambrosi et al., inhibits HSC engraftment by negative

regulation through dipeptidyl peptidase 4, adipocyte progenitors deriving from Adipoq–CreER–labelled cells enhance regeneration of HSCs by secreting the earlier mentioned SCF, leading to a not fully understood interdependency between adipocytes and HSCs.(43,44)

A variety of other cell types play important roles in the maintenance and proliferation of the HSC (Figure 2), as there have to be mentioned for example the arteriolar and sinusoidal endothelial cells, providing SCF, CXCL12 and Jagged–1, a notch–ligand necessary for HSC self-renewal stimulation.(45) Another big part is the innervation by the autonomous nerval system, regulating cytokines like CXCL12 in a stress related or day/night related pattern. Furthermore, HSC descendants themselves regulate differentiation processes of HSCs as for example studies revealed that megakaryocytes induce quiescence in megakaryocytes primed HSCs, leading to a negative feedback loop.(46)

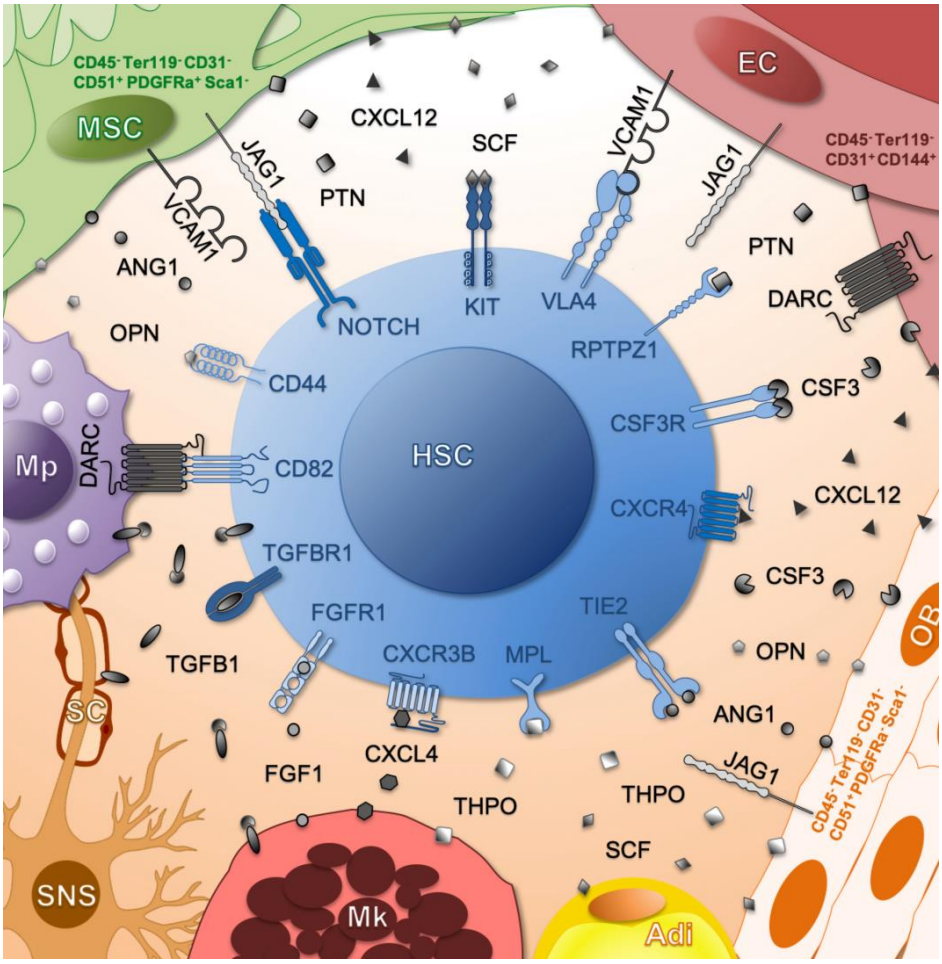

Figure 2 HSC communication in the bone marrow niche.
Multiple cell types interact with HSC to regulate the hematopoiesis. Most noticable members are depicted in this figure as there are the osteolineage cell (OB), mesenchymal stem cell (MSC), enodthelial cell (EC), sympathetic nervous system (SNS), adipocyte (Adi), macrophage (MP) and megakaryocyte(Mk).(45)

1.1.4. Cytokine dependency

Mentioned in the previous chapter, multiple cell types interact with HSC in the BM niche, providing a broad variety of cytokines.(45,47) One of these cytokines is the stem cell factor (SCF), also known as KIT–ligand or soluble steel factor, causing fatal anemia, when deleted in mice experiments.(48) By binding to the KIT–receptor, also present on HSCs, and then activating the JAK/STAT and the MAPK pathway, it promotes proliferation and self-renewal in HSCs.(49) Furthermore, it is known that SCF modulates the adhesion of hematopoietic cells to either fibronectin or VCAM–1, favoring a longer stay in the BM.(48)

Primarily known for its purpose in megakaryocytopoiesis, Thrombopoietin (TPO) is not only produced in liver and kidney cells, but also in BM stromal cells, where it interferes with proplatelet formation.(50) TPO receptor (c–mpl) loss in mice models furthermore revealed not only deficiencies in megakaryocyte maturation and expansion, but also had an impact on non-megakaryocytic lineages, indicating its importance in HSC/HSPC proliferation.(51) Additionally, in vitro studies could support this hypothesis, highlighting the impact of TPO on the proliferation and differentiation of LT–HSCs, in the presence of IL-3 or SCF.(52)

Next to TPO, the Fms–related tyrosine kinase 3 ligand (FLT3-L) is also one of the so-called early–acting cytokines, that promote early progenitor cell proliferation and long-term expansion.(53) It´s binding receptor, the flt3 receptor (Flt3R), is part of the tyrosine kinase receptor (TKR) type III family, including also the c–KIT receptor for the SCF ligand, and promotes HSPC expansion, by proliferation activation and apoptosis inhibition.(54) Next to that, it interacts with other cytokines, like IL-7, and amplify their proliferative effect.(55) Furthermore, it also plays a crucial role in the overexpression of very late antigen 4 and 5 (VLA4/5) on HSCs, leading to a stronger adhesion to vascular and intracellular cell adhesion molecules–1(VCAM–1 and ICAM–1), causing stronger attachment to the BM niche.(56,57)

Interleukin–6 (IL-6), also hepatocyte stimulation factor, plays a key role in inflammation and immune response, for example by activating the expression of acute phase proteins, such as C–reactive protein (CRP) and haptoglobin and decreasing the expression of anti–acute phase proteins, like albumin and transferrin.(58) By binding to the either membrane–bound or soluble IL-6 receptor,

the complex can interact with glycoprotein 130 (gp130), that is found on nearly every cell type, and then initiate multiple pathways, mainly the JAK/STAT and the MAPK pathway.(59,60) Animal experiments were able to provide validation of the importance of IL-6 in the HSPC proliferation and differentiation, by displaying lethal anemias and reduced numbers of hematopoietic progenitor cells in either IL-6 or gp130 deficient models, however not all players in that process are known completely until now and further studies are being conducted.(61,62)

1.1.5. Co-culture

As it became clear that single culture systems are often limited to display in vivo interaction and ecosystems, co-cultures were applied more frequently to overcome this obstacle.(63) With the development of co-culture systems a rise in complexity appeared due to the introduction of new variables. Experiments with five or more different cell types were introduced, however Hatherell et al. could demonstrate that bi–culture models could display the blood–brain barrier better than their compared co-cultures.(64) Combining different cell types is heavily limited due to their necessary environmental culturing conditions, even though a variety of technical systems have been invented to overcome this limitation, for example by partial separation.(65)

Culturing and expanding HSC/HSPCs turned out to be a complicated and expensive task, as a plethora of different cytokines are playing a major role in the differentiation and proliferation of HSCs.(66) For maintenance of their in vivo self-renewal ability and for survival, HSPCs need sufficient concentrations of SCF, IL-3, G-CSF and EPO. Additionally, high concentrations of vitamins and amino acids are contained in the newly developed and used media.(2) Since the HSCs demand is rising and cultivating them is difficult, the expansion of HSCs is well studied and a variety of media, as for example Serum free expansion medium (SFEM) or Hematopoietic growth media (HPGM), have been in use.(67,68) Often applied cytokines are SCF, TPO, IL-6 and FLT3-L, all known to increase the expansion rate of HSCs and are essential for their survival.(2)

As early findings indicated a necessary interaction between BM–MSCs and HSCs, Co-cultures with these two cell types have been gaining attention in the scientific field.(69,70) MSC do not only express CXCL12 and the JAG1 ligand, but also

support the self-renewal properties of LT–HSC, even though they lose this abilities when they differentiate further.(71) Studies revealed that in co-cultures with BM–MSCs and additionally added FLT3-L, SCF and IL-3, HSCs are able to survive and expand. They started to divide into three different compartments: HSCs that were located beneath the MSC layer, on top of it or were non-adherent. By applying FACS analysis and scanning electron microscopy, properties of those three different subgroups have been analyzed and revealed that HSCs beneath the MSC layer preserve their immature $CD34^{+}CD38^{-}$ phenotype better in contrast to the upper layer and the non-adherent comparison group. That data indicates that MSC were able to sustain a niche, similar to in vivo conditions and a differentiation and maturation process occurs while rising from beneath the layer to the top.(72) Nevertheless, even under external cytokine supplementation and MSC interaction, the hematopoietic stem cell $CD34^{+}CD38^{-}$ proportion decreases steadily and until now, it is not fully possible to preserve in vivo properties of HSCs under in vitro conditions. As external cytokine addition is an expensive and unphysiological process, newer designs are evaluated to overcome the current limitations of culturing and expanding HSC in vitro.(73)

1.2. CRISPR

Since the discovery of the DNA double helix, scientists have tried to find ways to manipulate the DNA sequence. In the early 21st century first breakthroughs appeared by the use of zinc finger–mediated DNA binding (ZNFs) functioning as site specific nucleases and by the application of transcription activator–like effector nucleases (TALENs), that were naturally occurring in plant infecting bacteria.(74,75) Both systems introduce double–strand DNA breaks (DSBs), leaving the DNA repair system in charge to introduce changes at the targeted site.(76)

So-called clustered regularly interspaced palindromic repeats, short CRISPRs, have been first described in 1987 in *E. coli* (77) and while it took nearly 20 years, the origin of the, in between the short direct repeats, embedded spacer sequences could be traced to short fragments of foreign DNA – mostly viral and plasmid origin–, indicating a role in an adaptive defense system of the cell.(78) This theory was supported by the fact that CRISPR associated(Cas) genes were encoding nuclease and helicase domains. In 2012 Doudna and Charpentier (79) finally were able to

depict the mechanism behind the CRISPR/Cas9 system and since then a variety of different Cas enzymes have been discovered and designed offering a broad range of new possibilities in genome editing.(80)

1.2.1. CRISPR/Cas – Members

During the transcription process the earlier mentioned integrated foreign DNA and the CRISPR repeat sequences yield precursor CRISPR transcripts, that are being further processed through endonucleolytic cleavage to short mature CRISPR RNA (crRNA) including the complementary sequence from the foreign DNA on the 3’ end and a short CRISPR repeat sequence on the 5’ end.(81) Additionally to this crRNA, further members are necessary for a functional CRISPR system including the tracrRNA, the PAM region and the Cas protein complex.(82)

The so-called trans activating crRNA, short tracrRNA, is a small non-coding RNA, forming a dual–RNA hybrid structure with the crRNA, by connecting to the CRISPR repeat sequence on the 5’end, while recruiting the Cas protein or Cas protein complex, depending on the CRISPR/Cas class and type.(83) In current scientific approaches single–guide RNA, including properties of both, the crRNA and tracrRNA, has been designed as a more elegant approach and has now mostly replaced the former two in the experimental setting.(84)

For a functional recruitment of the Cas protein another specific strongly conserved sequence, the protospacer adjacent motif (PAM) region is necessary in a close proximity to the target region.(85) As a discriminator for “self” vs “foreign”, its absence makes the target resistant to Cas induced enzymatic cleavage.(86)

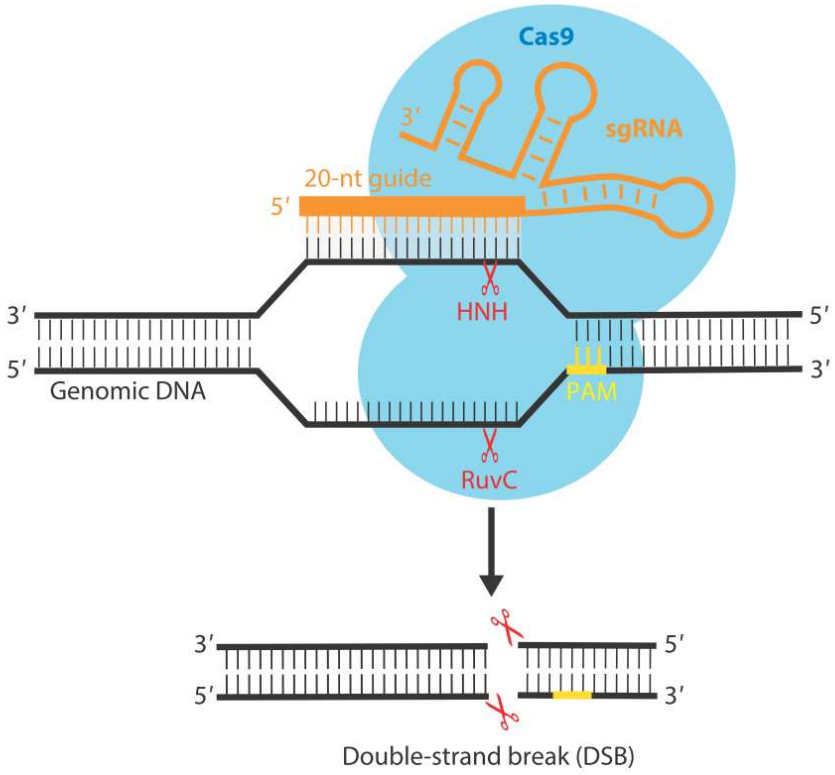

Figure 3 CRISPR/Cas9–mediated introduction of double–strand breaks via sgRNA.
By identifying the user–pre–defined guide RNA sequence the sgRNA directs the Cas9 endonuclease to the target side. Then the HNH–like nuclease domain and the RuvC–like nuclease domain introduce a double–strand break upstream of the PAM region.(82)

While some CRISPR/Cas types use multiple proteins to form a sufficient working enzyme, like the CRISPR/Cas type I and III, the most applied one, the CRISPR type II, is only requiring one Cas protein.(87) The C–terminal domain of this protein detects the around 20bp long target sequence with the help of the sgRNA and introduces blunt–ended double strand breaks 3bp upstream of the PAM region.(82) While the HNH–like nuclease domain cuts the crRNA–complementary DNA strand, the RuvC–like nuclease domain is in charge of the non-target DNA strand cleavage (Figure 3).(88) Depending on the subsequently repair mechanism a template strand can be required, as it is described in the next chapter.

1.2.2. DNA repair mechanisms

Around 10^4 DNA lesions per human cell occur daily under normal circumstances, introduced by mismatched base pairing, endogenous sources (e.g. reactive oxygen or nitrogen species) or exogenous sources (ionizing radiation, mutagenic chemicals etc.).(89,90) Therefore the eukaryotic cell has developed a variety of repair mechanisms to counter this potentially lethal events, each mechanism favoring their

specific lesion set up.(91,92) The most frequently appearing double strand repair mechanism are the default non-homologous end joining (NHEJ) mechanism and the Homology directed repair (HDR).(93) In contrast to the NHEJ, HDR is restricted to the S/G2 phase and since a template strand is being used, less errors occur here.(94)

For the HDR a specific enzyme cascade initiates the repair process, following on the introduction of a DSB including the three PI3K–like Kinases (PIKKs), BRCA1–associated RING domain protein 1 (BARD1) and the MRN complex. (95,96) Multiple sub–pathways of HDR are known for the next steps, as the most common are Classical double–strand break repair (DSBR), Synthesis–dependent strand–annealing (SDSA) and break–induced repair (BIR) pathway.(97) DSBR occurs, when an earlier in the cascade formed ssDNA tail finds a homology sequence on the template strand. After the strand invasion of the homologous strand and the creation of a displacement loop, a so-called double holiday junction (dHJ) is formed. After finished annealing, DNA synthesis and ligation, the two holiday junctions can resolve either in a crossover or non-crossover of the lesioned DNA with the template DNA.(98,99)

1.2.3. Packing CRISPR and the homology template

Next to the problem of the low HDR rate, the delivery of CRISPR/Cas and the template DNA into the target cell, turned out to be a significant obstacle, in terms of efficiency, handling and packing size. Figure 4 highlights the main three approaches in which form the CRISPR/Cas system can be introduced into the cell as they can be either delivered in form of an RNP-complex and a Cas9–sgRNA, or in the pre–form as a Cas9mRNA + sgRNA combination or coded in a plasmid.(100)

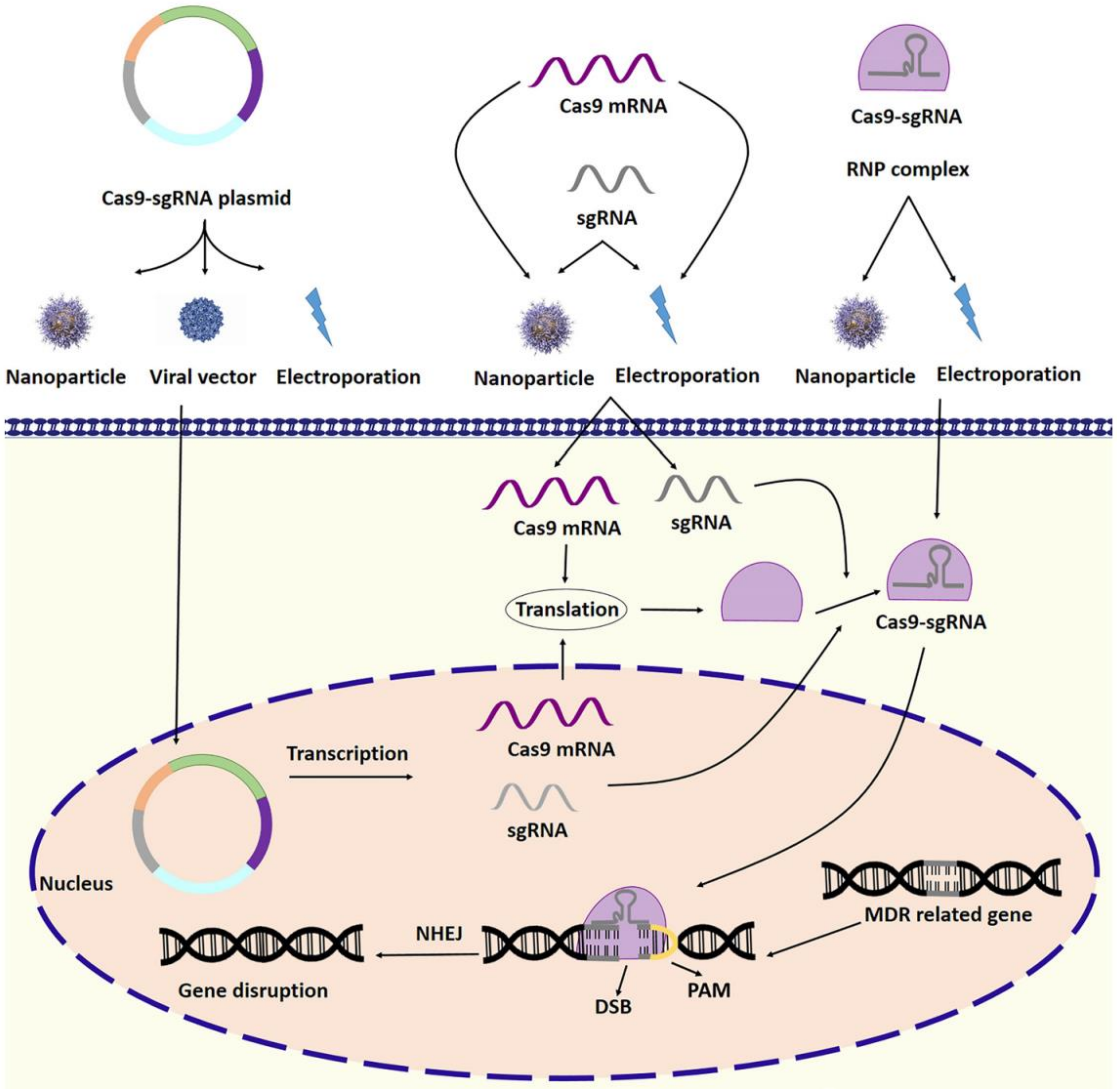

Figure 4 Transport systems for CRISPR/Cas introduction.
The sgRNA and the RNP complex, can be either transported as a finished complex, as RNA (Cas9 mRNA and sgRNA) or coded on a plasmid. A variety of techniques can be applied for the transfer into the cell including physical delivery methods, like electroporation, viral vectors, like adeno–associated viruses or the non-viral vector delivery utilizing various types of nanoparticles. After being fully active in the targeted cell via transcription, translation or direct transport, the sgRNA guides the Cas9 complex to the target site and after a DSB is introduced, repair mechanism like the NHEJ are activated.(100)

For HDR also a template has to be added to the mix either as DNA or as part of the same plasmid or on an additional plasmid. While inserting an RNP complex/sgRNA is an effective and functional method, it has only transient effect until the complex is degraded. On the other hand plasmid delivered CRISPR/Cas has a high stability, however provides only low levels of efficiency.(101) The least used introduction of mRNA/sgRNA offers low off–target effects, but even newer designs have not overcome their short operating time.(102)

As mentioned before different techniques are used to deliver the chosen CRISPR/Cas system into the cell and figure 4 also displays three examples of the common options.(103) There are physical delivery methods, including electroporation, microinjection, membrane deformation and hydrodynamic injection, however these systems are only used in in vitro and ex vivo approaches.(104) Another group of methods pack their CRISPR/Cas system int viral vectors, mostly being represented by systems using adeno–associated viruses (AAVs), but also adenovirus and lentivirus. These techniques have been proven to be simple and by combining multiple plasmids, packing size and efficiency could be improved over the last years.(105) The third and newest set of approaches, the non-viral vector delivery, utilizes nanoparticles, like gold nanoparticles, lipid nanoparticles, Cell–penetrating peptides (CPPs)–mediated or polyethyleneimine–based delivery systems.(106) Modern systems have even incorporated exogenously activated molecules that can be triggered, for instance, by light, in order to reduce off–target effects and enhance safety.(107)

2. Material and methods

2.1. Thawing, culturing and freezing cells

Table 1 gives an overview over the utilized cell lines. After thawing the frozen vial in the water bath (37°C), cells were dropwise added to 10ml of prewarmed cell culture medium (Supplementary table 4). Remaining DMSO was removed by centrifugation (10min, 350g, 4°C) and the cells were cultured in their respective medium. Cells were split (1:2-1:4) 2–3 times per week, depending on their growth kinetics. For storage in liquid nitrogen, $1{,}0x10^6$ – $2{,}0x10^6$ cells were resuspended in 1ml freezing medium (Sup. table 4) containing 10% DMSO and gradually cooled down in a container with 100% isopropyl alcohol (Mr. Frosty system, Thermo Fisher Scientific, Breda, The Netherlands).

Table 1 Utilized cell lines, properties

Cell line	Short description
MSC-hTERT	Mesenchymal stem cell line (bone marrow)(adherent)(108)
HS27a	Mesenchymal stem cell line (bone marrow)(adherent)(109)
UT-7/TPO	AML cell line; TPO depended (suspension)(110)
Cord blood-derived HSPCs	Hematopoietic stem and progenitor cells (suspension)

2.2. Mesenchymal stromal/stem cell engineering

2.2.1. rAAV vector design

All plasmids were designed and cloned by J. Foßelteder, a postdoc in the lab of the thesis supervisor Andreas Reinisch. For all applications a pAAV–MCS2 plasmid (#46954, Addgene, Watertown, MA, USA) with AAV serotype 2 ITRs was use as a backbone. Both vectors (FLT3L+SCF and TPO + IL-6) were cloned to finally contain an SFFV–promoter for constitutive expression of codon optimized cytokine cDNA and a P2A self-cleaving peptide between the cytokines. Downstream of the cytokines expression cassette a BFP fluorescent reporter and a bovine growth hormone (bGH) poly–adenylation signal was coupled via a self-cleaving T2A peptide. Left and right homology arms of 400bp length flanked the expression

cassette and were either homologous to the HBB (FLT3L–SCF) or AAVS1 (TPO–IL-6) locus.

2.2.2. rAAV production and quantification

For transfection $13x10^6$ HEK293FT cells (Life Technologies, Carlsbad, CA, USA)/dish were cultured for 24 hours in 150mm dishes. Per dish standard polyethylenimine (PEI) double transfection with 6µg ITR-containing plasmid and 22µg pDGM6 (#110660, Addgene, Watertown, MA, USA), including AAV6 cap genes, AAV2 rep genes, and adenovirus 5 helper genes, was performed. After an incubation period of 72 hours rAAV6 was purified using the AAVpro® Purification Kit (Takara Bio, Shiga, Japan) according to the manufacturer´s protocol (111). For vector genome quantification viral DNA was extracted by resuspending 5µl virus in 15µl Quick Extract™ DNA extraction solution (Lucigen, Middleton, WI, USA) and performing two heating steps (65°C, 6min; 98°C, 2min) with vortexing before and after each step.

A viral DNA dilution series was prepared ($1:4x10^4$-$1:1{,}25x10^8$) and an IDT custom primer-probe assay (IDT, Coralville, Iowa, USA; sequence in sup. table 5) and ddPCR™ Probe Supermix (Bio–Rad, Hercules, CA, USA) were added for the ddPCR mastermix (Sup. table 6). Droplets were generated via the BIORAD QX200 droplet generator and after the conducted PCR (thermocycler program in sup. table 7) the viral genome copy number was measured with the BIORAD QX200 droplet reader (Bio–Rad, Hercules, CA, USA).

2.2.3. CRISPR/Cas9 application

To insert side-specific DSBs in the targeted *AAVS1* and *HBB* locus via CRISPR/Cas9, $2{,}5x10^5$ cells – either MSC-hTERT or HS27a – were harvested and suspended in 20µl nucleofection solution. A preincubated RNP complex, consisting of 3µg Cas9 enzyme and 1,6µg side-specific sgRNA, was added to the suspension and the mix was electroporated by using the 4D–Nucleofector® system (Program: “cm-119”; Lonza group, Basel, Schweiz). The CRISPR/Cas pretreated cells were collected in 380µl culturing media.

2.2.4. Transduction

The cells were transduced by directly adding recombinant AAV6 (5,000 GC/cell) for 6-8h at 37°C. Thereafter, transduction medium was replaced by pre-warmed culturing media.

2.3. Single cell cloning

For single cell cloning, the transduced cells were diluted in culturing media to a final concentration of 7.5 cells/ml. 100µl (0,75 cells/well) were seeded into individual wells of a 96 well plate to allow for seeding of only a single cells per individual well. To confirm deposition of individual cells and exclude cell doublets, all wells were checked via an inverted microscope after 24, 48, 72 hours. Only wells containing a single starting cell in total were chosen for further experiments. Individual cells were expanded by serial passaging and thereafter gene knock–in (KI) was verified by "in-out"-PCR. For each condition 1-5 clones were chosen for further experiments.

2.4. Knock-in confirmation

For confirmation of correct KI "in-out"-PCR was performed. Briefly, $2x10^5$ cells were pelleted after two washing steps in DPBS (centrifugation for 5min, 350rpm,Gibco™, Thermo Fisher Scientific, Breda, The Netherlands). DNA was extracted using 50µl Quick Extract™ DNA extraction solution according to chapter 2.2.2.

For the PCR, a specific set of primer was used (Table 2). Wild type (WT) cell lines were used as a negative control. The exact primer sequences and PCR protocol are outlined in sup. table 8. and sup. table 9.

Table 2 Primer conditions for knock–in confirmation

Condition	Used primer set	Applied on cell lines with the following constructs	Molecular weight [bp]
Unmodified	HBB_in–out_fwd1 HBB_in–out_rev1	WT FLT3L_SCF	1130
KI	HBB_in–out_fwd1 SFFV_in–out_rev1	FLT3L_SCF + TPO_IL-6	760
Unmodified	AAVS1_in–out_fwd1 AAVS1_in–out_rev4	WT TPO_IL-6	1010

KI	AAVS1_in–out_fwd1 SFFV_in–out_rev1	FLT3L_SCF + TPO_IL-6	627

Amplified DNA was separated in 1,5% agarose gel at 100V for 50min. For DNA visualization, Midori green DNA dye (NIPPON Genetics EUROPE GmbH, Düren, Germany, 5µl per 100ml) was added. The gel was analyzed on a ChemiDoc Touch (Bio–Rad Laboratories, Hercules, California, USA) imaging system. For the FLT3L_SCF_BFP construct the amplified product for the unmodified control was 1130bp long and 760bp for the KI. For the TPO_IL-6_BFP it was 1010bp (unmodified) and 627bp (KI).

2.5. ELISA

To quantify cytokine production cell culture media was fully exchanged after the cells reached over 80% confluency. 72 hours after media change, cell supernatant from engineered and WT stromal cell lines was harvested, centrifuged (5min, 350rpm) to remove cellular debris and stored at –20°C until quantification of cytokine concentrations using an ELISA. Prior to ELISA the samples were centrifuged (5min, 350rpm) and diluted for the experiment (dilution factor ranged from 1:50 to 1:500 depending on the analyzed cytokine). Unmodified WT stromal cells served as a control. ELISA kits for TPO (ab219632), IL-6 (ab178013), FLT3L (ab254507) and SCF (ab176109; all Abcam, Cambridge, UK) were applied. The ELISAs were processed according to the manufacturer's protocol (112) and light absorbance was analyzed on a CLARIOstar Plus microplate reader (BMG Labtech, Ortenberg, Germany) using the MARS data analysis software (V6.20.; BMG Labtech, Ortenberg, Germany). Predetermined standards were used to calculate the concentrations within the samples.

2.6. Co-culture

2.6.1. Co-culture with UT-7/TPO

$1{,}0x10^5$ feeder cells – either MSC-hTERT_TPO_IL6 clone D9 or MSC-hTERT_WT – were seeded in 2,5ml culture media (Sup. table 4) per well into five plates (Figure 5).

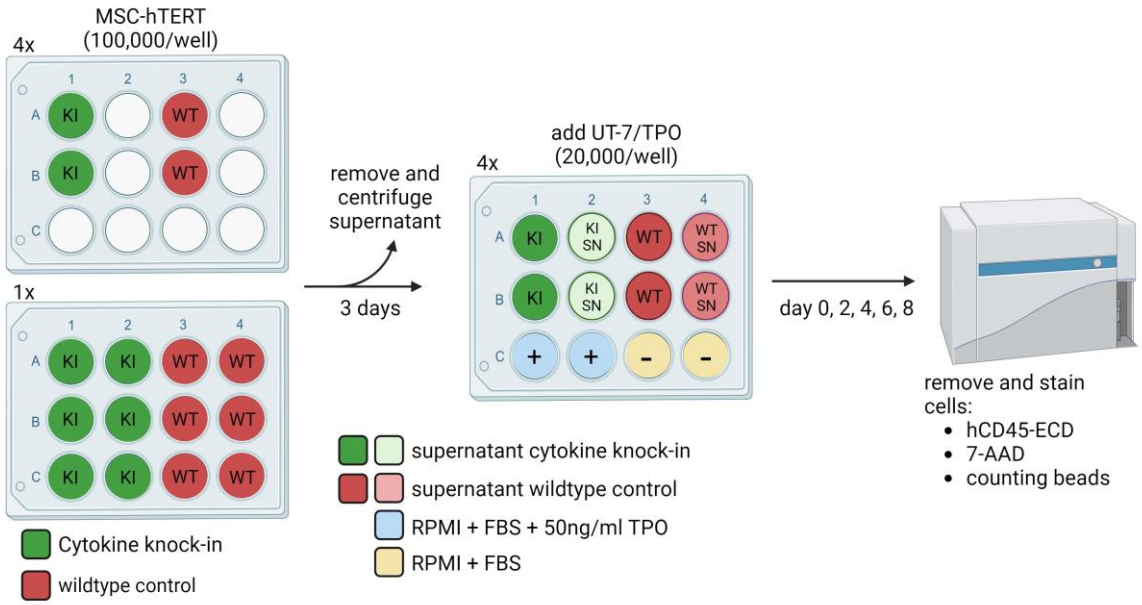

Figure 5 Co-culture with UT-7/TPO.
For three days 1,0x10^5 wild type (WT) or knocked-in (KI) MSC-hTERT cells were preincubated before being co-cultured with 2,0x10^4 UT-7/TPO cells. On day 0,2,4,6 and 8 cells were collected and stained with hCD45– ECD. Afterwards 7–AAD and counting beads were added and the samples were analyzed with the CytoFLEX flow cytometer. On each plate a positive control (+) with additional 50ng/ml TPO and a negative control (–) with only RPMI and FBS were added.

After three days incubation, reaching confluency between 80% and 95%, the supernatant derived from cytokine-expressing or WT (ctrl.) feeder cells was harvested and centrifuged (5min, 1000g) before admixing (1:2) with fresh media. 2,0x10^4 UT-7/TPO were either directly seeded on top of pre-culture confluent feeder cells (cytokine-expressing or WT) or cultured without feeder cells in the respective feeder cell derived supernatant in a final volume of 2,5ml. This resulted in the following conditions: UT-7/TPO + i) MSC-hTERT_TPO_IL-6 feeder cells ii) diluted MSC-hTERT_TPO_IL-6 supernatant, iii) MSC-hTERT_WT feeder cells iiii) diluted MSC-hTERT_WT supernatant (Figure 5).

For the positive and negative control 2,0x10^4 UT-7/TPO cells were seeded in 2,5ml culture media, with or without exogenous recombinant human TPO (50ng/ml).

During the analysis period, media was renewed on day 4 and 6 by replacing 1ml per well with the respective pre–centrifuged and diluted (1:2) supernatant media.

2.6.1.1. Flow cytometry

Flow cytometry was conducted on day 0, 2, 4, 6 and 8. On day 0, 2x10^4 UT-7/TPO cells were suspended in 50 µl staining buffer (PBS + 1% BSA). For non-co-culture conditions, the cell suspension was transferred to the FACS tube on day 2, 4, 6 and

8. For co-culture conditions, the whole supernatant containing UT-7/TPO cells was transferred to a FACS tube. The remaining non-adherent and adherent feeder cells were trypsinized with 400µl TrypLE for 3min at 37°C, and combined with the rest in the FACS tube. Cells were centrifuged (5min, 350rpm) and resuspended in 50µl staining buffer.

For staining 2,0µl of hCD45–ECD was added and the mix was incubated 30min at 4°C. After another washing step (1ml staining buffer, centrifugation: 5min, 350rpm) 2,5µl 7–AAD and 15µl counting beads were admixed and the cells were incubated (no light, 5min, RT). Data were acquired on the CytoFLEX flow cytometer (Beckman Coulter, Brea, USA) and analyzed using FlowJo V10.8.1 software.

2.6.2. Co-culture with $CD34^+$ HSPC

$1,0x10^6$ cord–blood derived HSPCs from four different donors (#10071, #10224, #10248, #10255) were thawed (chapter 2.1.) and cultivated in their belonging media (Sup. table 4) three days prior to the start of the experiment. Additional $1,0x10^5$ feeder cells – either MSC-hTERT_FLT3L_SCF_TPO_IL6 clone C10 or MSC-hTERT_WT– were seeded in 2,5ml culturing media (Sup. table 4) per well into 5 plates and were separated from their supernatant three days later at a confluency between 80–95% (Figure 6).

$2,0x10^4$ HSPCs were seeded in their respective media with or without feeder cells as in the UT-7/TPO co-culture (chapter 2.6.1.) obtaining the following conditions: HSPC + i) MSC-hTERT_FLT3L_SCF_TPO_IL6, ii) diluted MSC-hTERT_FLT3L_SCF_TPO_IL6 supernatant, iii) MSC-hTERT_WT iiii) diluted MSC-hTERT_WT supernatant.

$2,0x10^4$ HSPCs seeded in culturing medium with or without exogenous recombinant human FLT3L, SCF, TPO, IL-6 (all 50 ng/ml) functioned as positive and negative control. The media was renewed, as descripted in chapter 2.6.1., on day 3, 5 and 6.

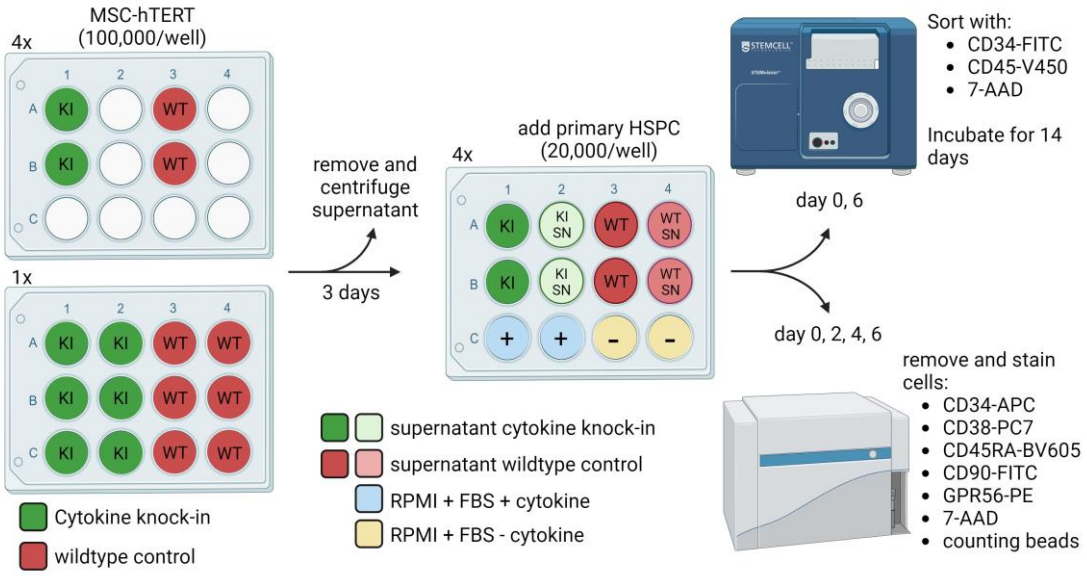

Figure 6 Co-culture with cord blood-derived HSPC.
For three days 1,0x10^5 wild type (WT) or knocked-in (KI) MSC-hTERT cells were preincubated before being co-cultured with 2,0x10^4 cord blood-derived HSPCs deriving from four different donors. On day 0,2,4 and 6 cells were collected and stained for FACS analysis with the CytoFLEX flow cytometer. Additionally on day 8 cells were selected for a MethoCult assay. On each plate a positive control (+) with additional 50ng/ml TPO and a negative control (–) with only RPMI and FBS were added.

2.6.2.1. Flow cytometry

Flow cytometry was performed on day 0, 2, 4 and 6, according to chapter 2.6.1.1. Cells were stained with 2,0µl of CD34–APC, CD38–PC7, CD45RA–BV605, CD90–FITC and GPR56–PE each.

2.6.2.2. Methocult

300 (day 0) or 1200 (day 7), live (7-AAD negative) CD34$^+$, CD45^{dim} HSPCs were FACS-sorted into 100µl IMDM and then mixed with 1ml Methocult^{TM} medium (STEMCELL technologies, Vancouver, Kanada). The mix was seeded into 6–well smart dishes and incubated for 14 days at 37°C.

Colony formation was analyzed with the StemVision^{TM} Software using the STEMvision^{TM} Automated Colony–Forming Unit (CFU) Assay Reader (STEMCELL technologies, Vancouver, Kanada). For each condition triplicates were prepared. A separation into the colony-forming subgroups CFU-GEMM (multilineage colony forming unit for granulocyte, erythrocyte, monocytes, and macrophages), BFU-E (erythroid burst–forming unit) and the CFU-G/M/GM (bi- or unipotent granulocyte, monocyte colony forming unit) was conducted.

2.7. Statistical analysis

An one-way ANOVA test was used for direct group comparisons (n=2). For variables n>2 a two-way ANOVA test was performed. P-values less than 0.05 ($p<0.05$) were considered statistically significant. All data are presented as mean ± SEM.

3. Results

3.1. Generation of human cytokine expressing stroma cell clones

Known from previous studies HSCPs rely heavily on the cytokines SCF, FLT3L, TPO and IL-6 for their survival and expansion. By producing genetically engineered MSCs containing two constructs (FLT3L_SCF_BFP, TPO_IL-6_BFP) the aim is to provide sufficient levels of these cytokines. Single cell-derived clones were generated to further use them in co-culture experiments with AML cell lines and HSPCs. For each construct at least two single cell-derived clones could be generated, leading to total 9 clones for the MSC-hTERT and 15 clones for the H27a cell line as depicted in table 3.

Table 3 MSC-hTERT and HS27a clone selection

Cell line	Construct	Number of clones
MSC-hTERT	FLT3L_SCF_BFP	2 (G2, C12)
	TPO_IL-6_BFP	4 (H6, D9, A4, G11)
	FLT3L_SCF_BFP + TPO_IL6_BFP	3 (G1, C10, C5)
HS27a	FLT3L_SCF_BFP	5 (D3, F3, F12, H6, G7)
	TPO_IL-6_BFP	6 (B9, C6, D8, E7, E9, F7)
	FLT3L_SCF_BFP + TPO_IL6_BFP	4 (D3, E3, G3, G8)

3.2. Detection of successfully introduced genes via “in-out” PCR

The 24 selected single cell-derived clones were validated for either mono- or biallelic insertion analyzing the extracted DNA in an “in-out” PCR. For each “in out” PCR two primer pairs, are applied for each sample in a separated mix. Both forward primers are located on the targeted gene, outside of the KI region. While one primer pair includes a reverse primer targeting the KI sequence, the other reverse primer is located outside of the KI area, only being active in the unmodified samples. The sample can only yield detectable amplified products, when both primers of a primer pair (fwd and rev) are active (Figure 7).

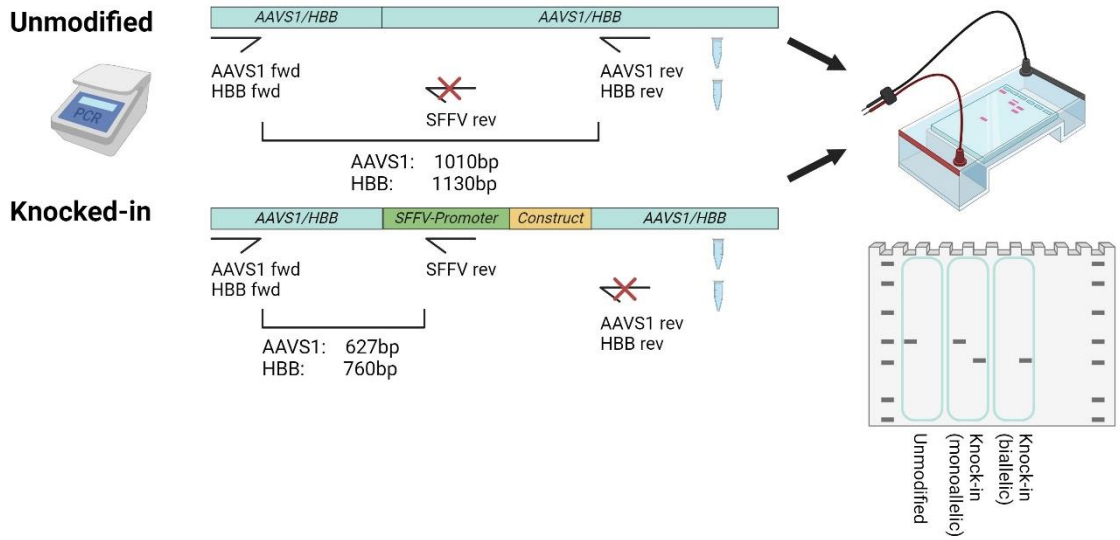

Figure 7 "in-out" PCR.
In the "In-out" PCR two different primer pairs are applied in separated vials for each sample. One primer mix includes a reverse primer targeting the knocked-in sequence (SFFV rev), while the other reverse primer is targeting the gene outside the knocked-in region (AAVS1 rev, HBB rev), only being active in the unmodified samples. Mono- or biallelic knock-ins can be distinguished from unmodified samples in the gel electrophoresis due to different amplification product length.

A successful recombination event could be detected in eight out of nine expanded single cell-derived clones in the modified MSC-hTERT cell line. For the single construct knock-in clones we got one monoallelic knock–in for the FLT3L_SCF_BFP construct and two monoallelic and two biallelic for the TPO_IL-6_BFP construct. For the double construct knock-in clones the following insertions could be achieved: i) clone G1: monoallelic for FLT3L_SCF_BFP and monoallelic for TPO_IL-6_BFP, ii) clone C10: monoallelic for FLT3L_SCf_BFP and biallelic for TPO_IL-6_BFP, iii) clone C5: monoallelic for FLT3L_SCf_BFP and biallelic for TPO_IL-6_BFP (Figure 8a & b).

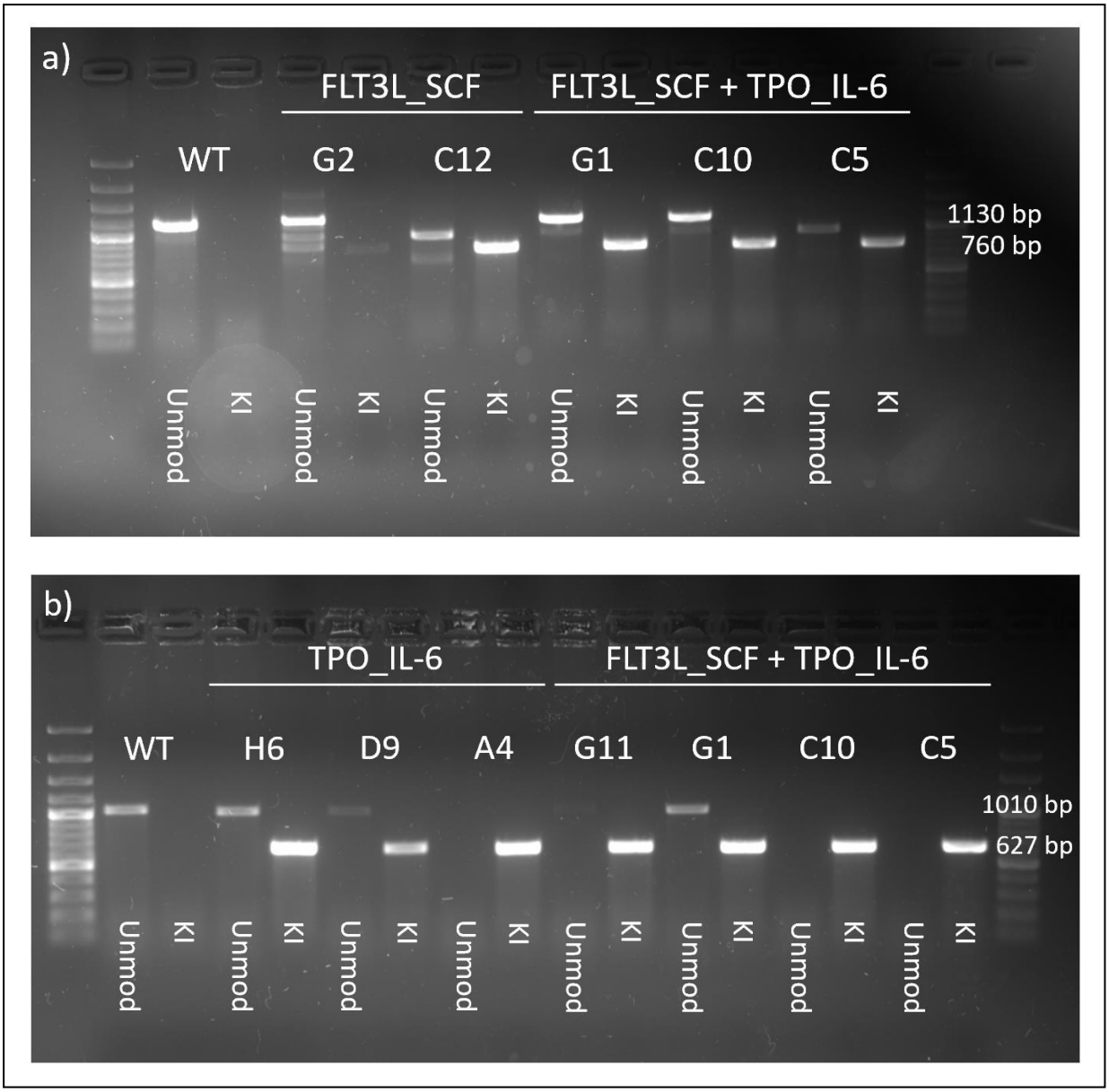

Figure 8 MSC-hTERT Knock–in control with in-out PCR.
a) FLT3L_SCF_BFP single knocked-in or double knocked-in clones were tested for successful insertions. The amplified product for no insertion was 1130 bp long, while the knock–in control was 760bp long. Both the unmodified (unmod) and knock–in (KI) specific primer pairs were applied for each sample. b) The same check for successful knock–in was performed for the TPO_IL-6_BFP construct, with amplified products for no insertion with 1010bp and insertion with 627bp. The wild type (WT) was used as a negative control.

Similar results could be achieved in the HS27a cell line resulting in a successful introduction in 14 out of 15 tested samples. For the single construct KI FLT3L_SCF_BFP we got two biallelic and three monoallelic insertions (Figure 9a) and for the single construct KI TPO_IL-6_BFP four biallelic and two monoallelic knock–in (Figure 9b).

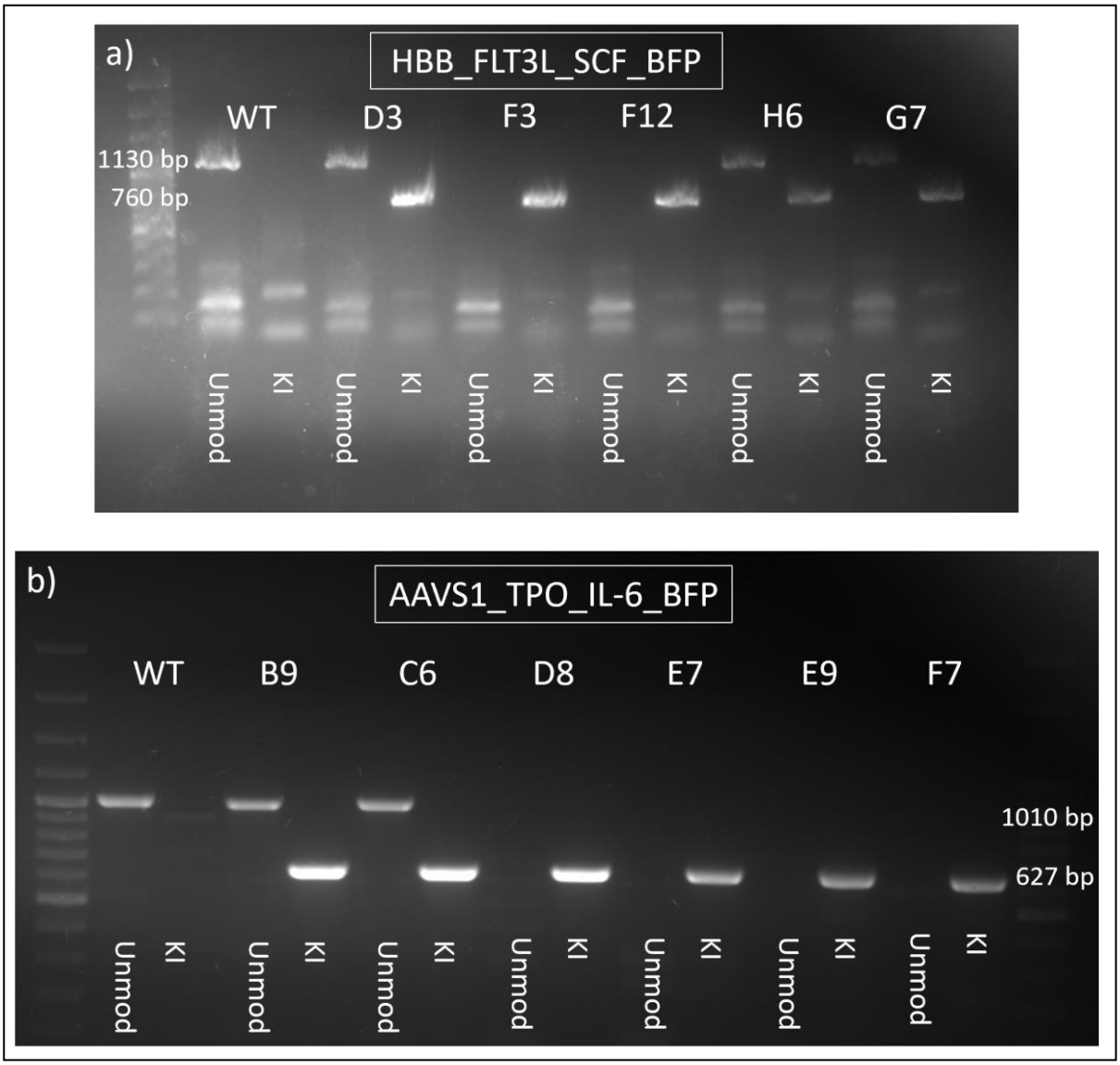

Figure 9 HS27a single–knock–in control with in-out PCR.
a) FLT3L_SCF_BFP single knocked-in clones were tested for successful insertions. The amplified product for no insertion was 1130 bp long, while the knock–in control was 760bp long. For each sample both the unmodified (unmod) and knock–in (KI) specific primer pairs were applied. b) The check for successful knock–in was also performed for the TPO_IL-6_BFP construct, with amplified products for no insertion with 1010bp and insertion 627bp length. The wild type (WT) was used as a negative control.

The double knock–in analysis yielded the following results: i) clone D3 was monoallelic for both constructs; ii) clone E3 and G8 were monoallelic for the FLT3L–SCF_BFP construct and biallelic for the TPO_IL-6_BFP construct; iii) clone G3 had no successful insertion for FLT3L_SCF_BFP and a biallelic insertion for TPO_IL-6_BFP (Figure 10a & b).

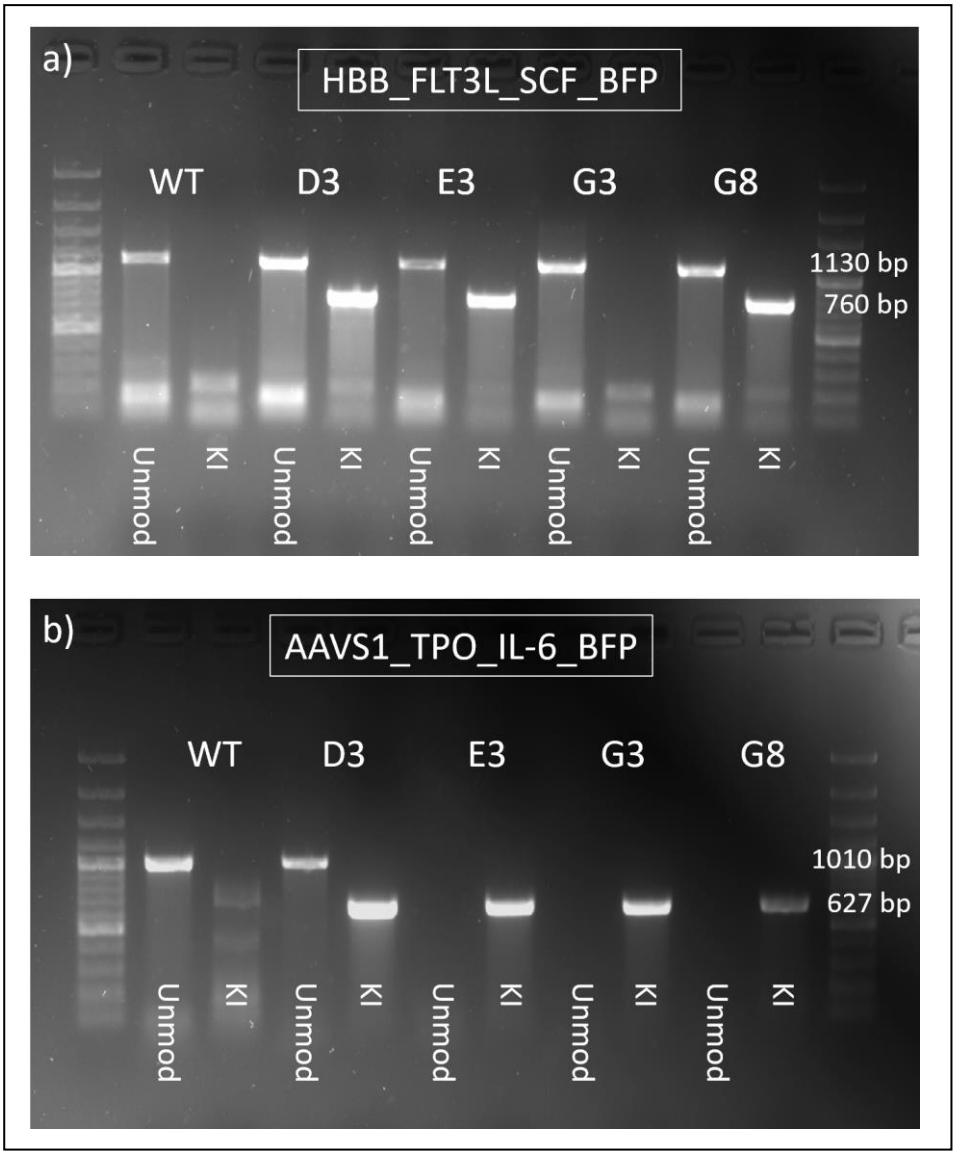

Figure 10 HS27a double knock–in control with in-out PCR.
a) The double knocked-in clones were tested for successful FLT3L_SCF_BFP insertion. The amplified product for no insertion was 1130 bp long, while the knock–in control was 760bp long. For each sample both the unmodified (unmod) and knock–in (KI) specific primer pairs were applied. b) The check for successful knock–in was also performed for the TPO_IL-6_BFP construct, with amplified products for no insertion with 1010bp and insertion 627bp length. The wild type (WT) was used as a negative control.

3.3. Successfully engineered human stromal cell lines produce significantly elevated cytokine levels

After confirmation of successful genetic KI, we analyzed the cytokine production and by quantifying their abundance in the culture supernatant by ELISA a total of 8 individual single cell-derived clones for the MSC-hTERT (2 FLT3L_SCF_BFP; 3 TPO_IL-6_BFP; 3 FLT3L_SCF_BFP + TPO_IL-6_BFP) and 9 for the HS27a cell line (3 FLT3L_SCF_BFP; 3 TPO_IL-6_BFP; 3 FLT3L_SCF_BFP + TPO_IL-6_BFP) were tested.

Compared to unmodified WT cells, we detected significantly higher concentrations of FLT3L (4 out of 4 clones, single KI C12 and double KI clones C5, C10: $p<0.01$; double KI G1: $p<0.05$) and SCF (4 out of 4 clones; single KI C12, G1 and double KI clones C5, C10: $p<0.05$) in FLT3L_SCF_BFP knock-in MSC-hTERT clones (Figure 11).

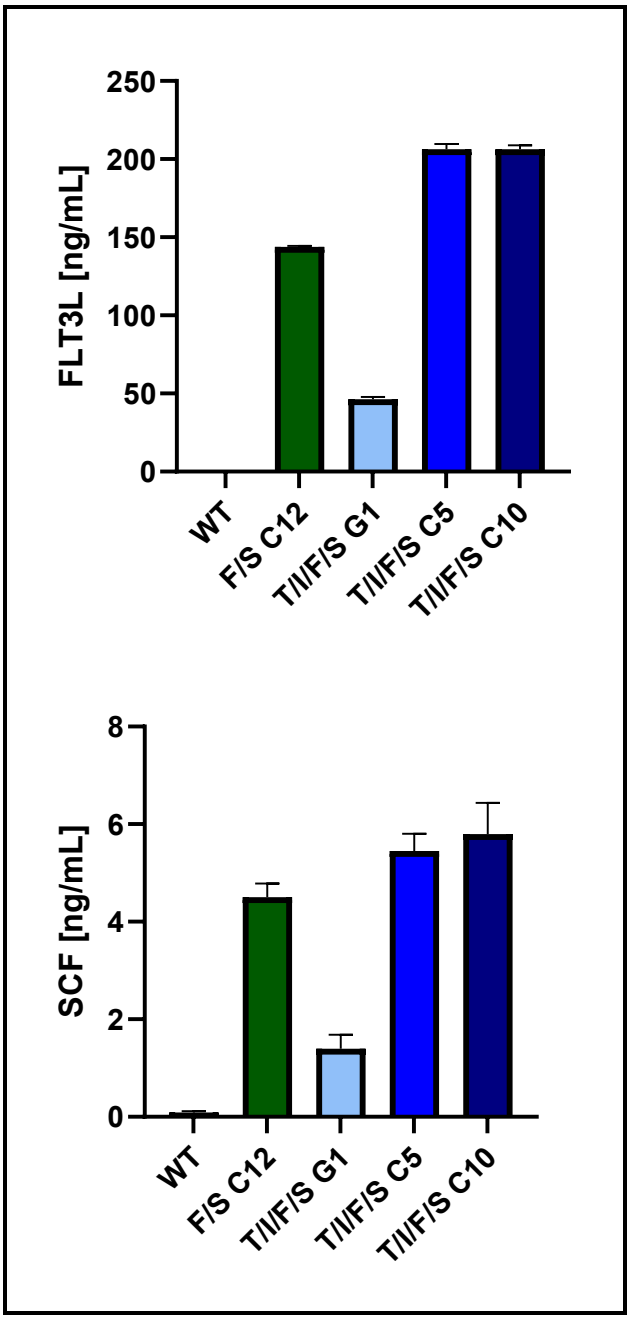

Figure 11 ELISA analysis of the FLT3L_SCF_BFP construct clones in MSC-hTERT.
The single KI clones (F/S=FLT3L/SCF) and double KI clones (T/I/F/S=TPO/IL-6/FLT3L/SCF) were tested for their cytokine production of FLT3L and SCF in an ELISA. The wild type (WT) functioned as a control. Significance was calculated by a one-way ANOVA test. Data represent mean ± SEM.

A similar increase in cytokine secretion could be detected for the generated single cell-derived clones with KI of the TPO_IL-6_BFP construct. We observed a significant increased production of TPO (4 out of 4 clones; single KI G11: $p<0.05$; single KI D9 and double KI C5, C10: $p<0.01$) and IL-6 (6 out of 6 clones; single KI A4, D9, G11 and double KI G1: $p<0.05$; double KI C5, C10: $p<0.01$) in comparison to the WT cells (Figure 12).

Interestingly, when comparing bi- and monoallelic states, we did not find an influence of allelic states on cytokine production (TPO: $p=0.778$; IL-6: $p=0.48$).

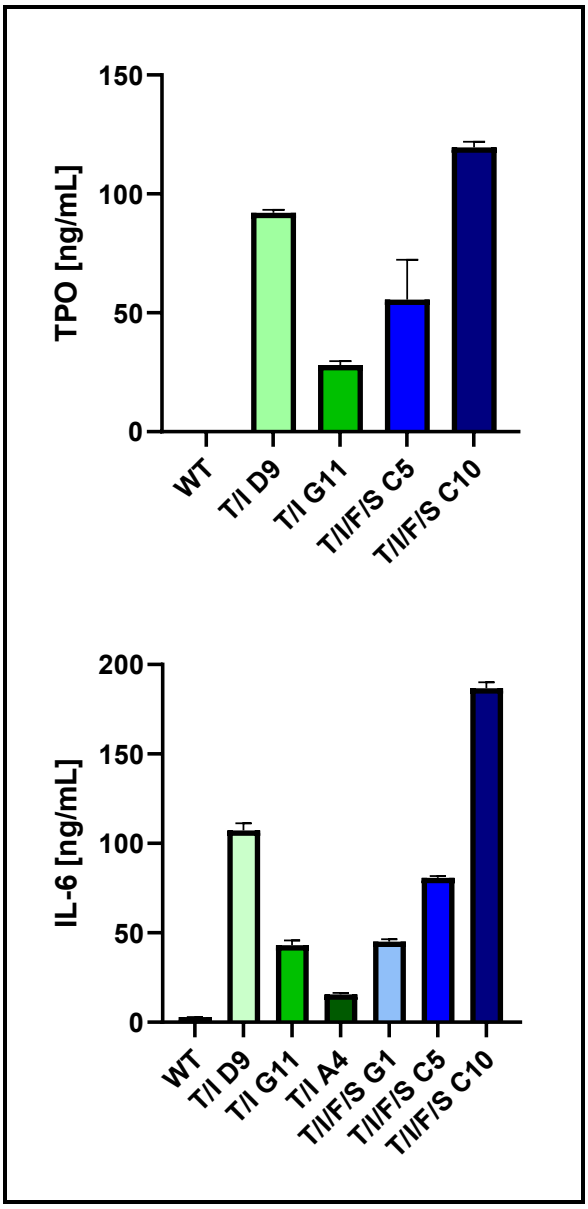

Figure 12 ELISA analysis of the TPO_IL-6_BFP construct clones in MSC-hTERT.
The single KI clones (T/I = TPO/IL-6) and double KI clones (T/I/F/S=TPO/IL-6/FLT3L/SCF) were tested for their cytokine production of IL-6 and TPO in an ELISA. The wild type (WT) functioned as a control. Significance was calculated by a one-way ANOVA test. Data represent mean ± SEM.

The quantification of the cytokine production in the HS27a cell line yielded also significant enhancement for FLT3L (5 out of 6 clones; single KI D3 and double KI D3, G8: $p<0.001$; single KI F3: $p<0.01$; single KIF12: $p<0.05$) and SCF expression (4 out of 6 clones; single KI D3, F3 and double KI D3, G8: $p<0.05$)(Figure 13).

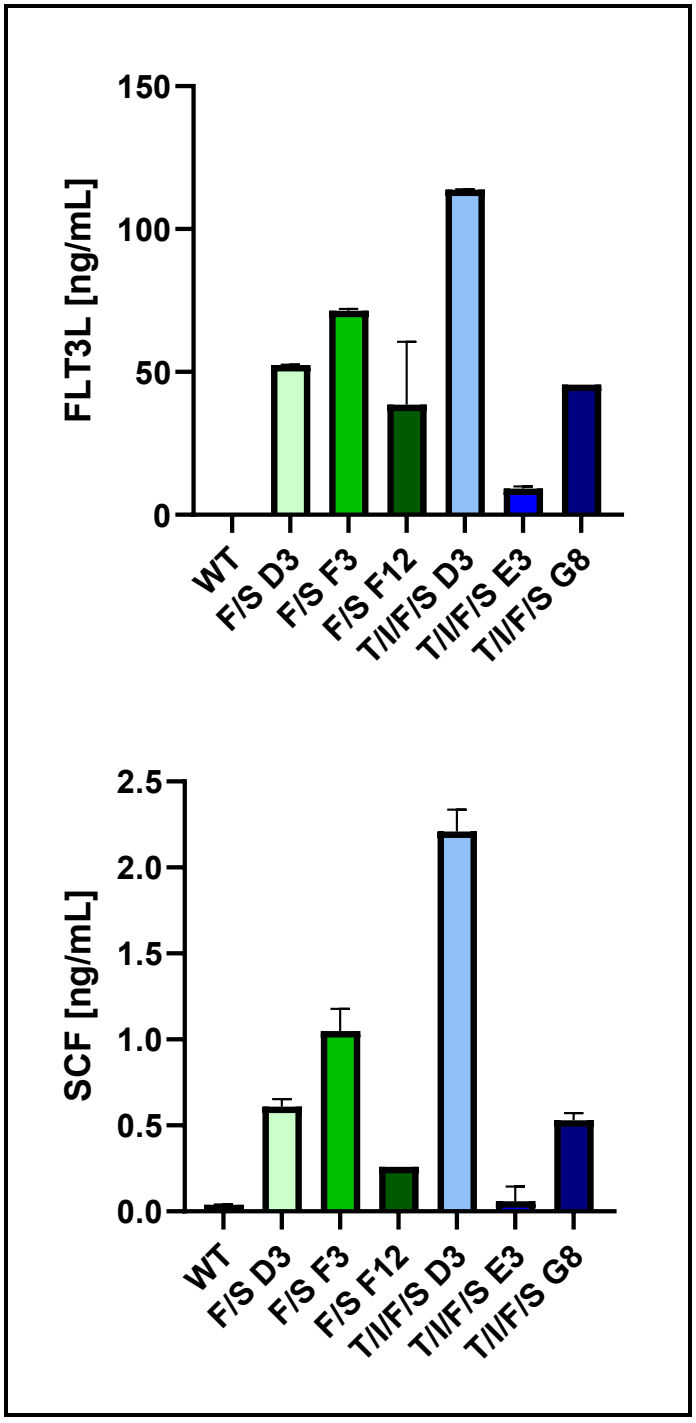

Figure 13 ELISA analysis of the FLT3L_SCF_BFP construct clones in HS27a.
The single KI clones (F/S=FLT3L/SCF) and double KI clones (T/I/F/S=TPO/IL-6/FLT3L/SCF) were tested for their cytokine production of FLT3L and SCF in an ELISA. The wild type (WT) functioned as a control. Significance was calculated by a one-way ANOVA test. Data represent mean ± SEM.

Significantly higher amounts of TPO (5 out of 6 clones; single KI C6, D8 and double KI D3, E3, G8: $p<0.05$) and of IL-6 (6 out of 6 clones; single KI C6, D8: $p<0.001$; double KI D3, E3, G8: $p<0.01$; single KI E7: $p<0.05$) were produced in the TPO_IL6_BFP construct in comparison to WT (Figure 14).

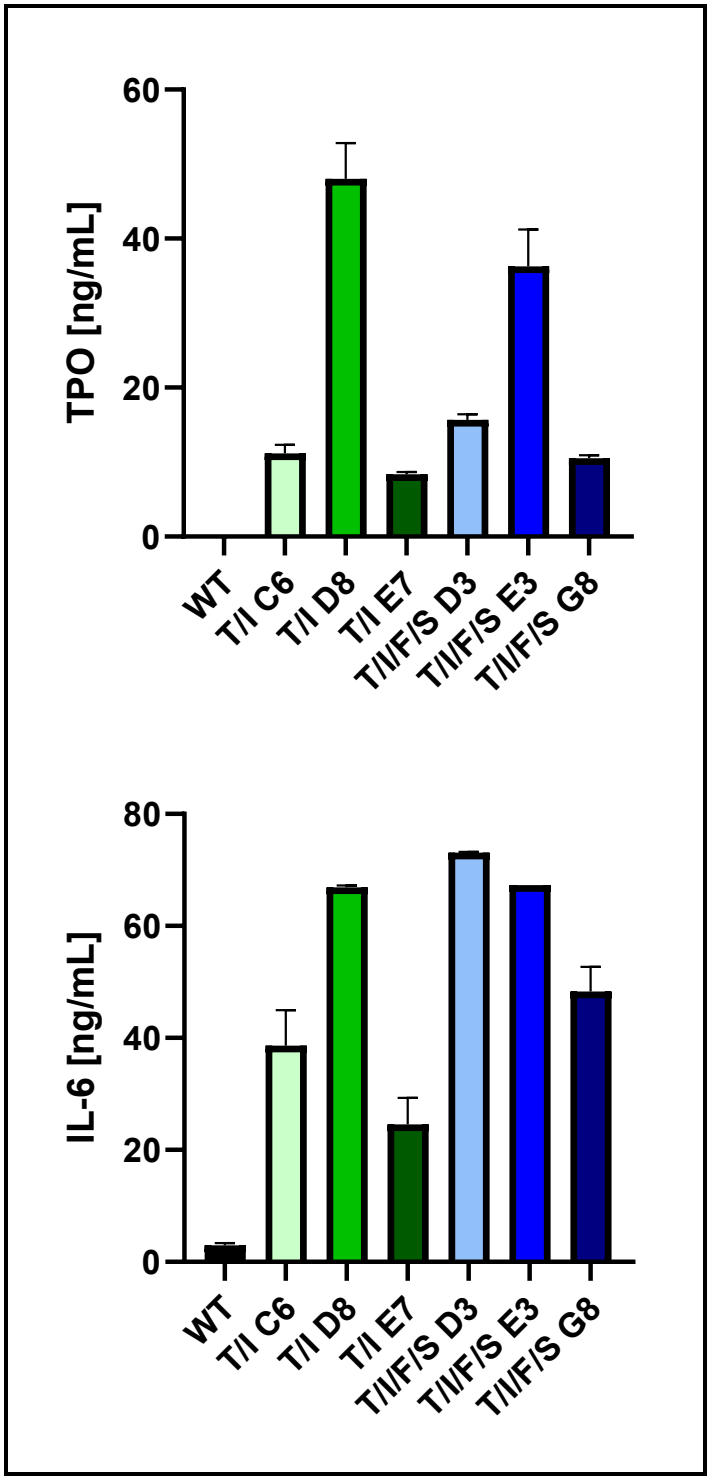

Figure 14 ELISA analysis of the TPO_IL-6_BFP construct clones in HS27a.
The single KI clones (T/I = TPO/IL-6) and double KI clones (T/I/F/S=TPO/IL-6/FLT3L/SCF) were tested for their cytokine production of IL-6 and TPO in an ELISA. The wild type (WT) functioned as a control. Significance was calculated by a one-way ANOVA test. Data represent mean ± SEM.

3.4. Cell confluency does not influence cytokine secretion in engineered human stromal cell lines

To display the effect of the MSC feeder layer confluency on cytokine production, we compared the amount of secreted cytokine at either 50% or 100% confluency. Therefore, MSC-hTERT cells containing the FLT3L_SCF_BFP construct were seeded at a concentration of either $9{,}1x10^3$ or $18{,}2x10^3$ per cm^2 and the corresponding cytokine production (SCF) was evaluated on day 2, 4 and 6 by ELISA.

Interestingly, the level of confluency had no significant influence on the total amount of SCF secreted into the supernatant (Figure 15, $p<0.48$).

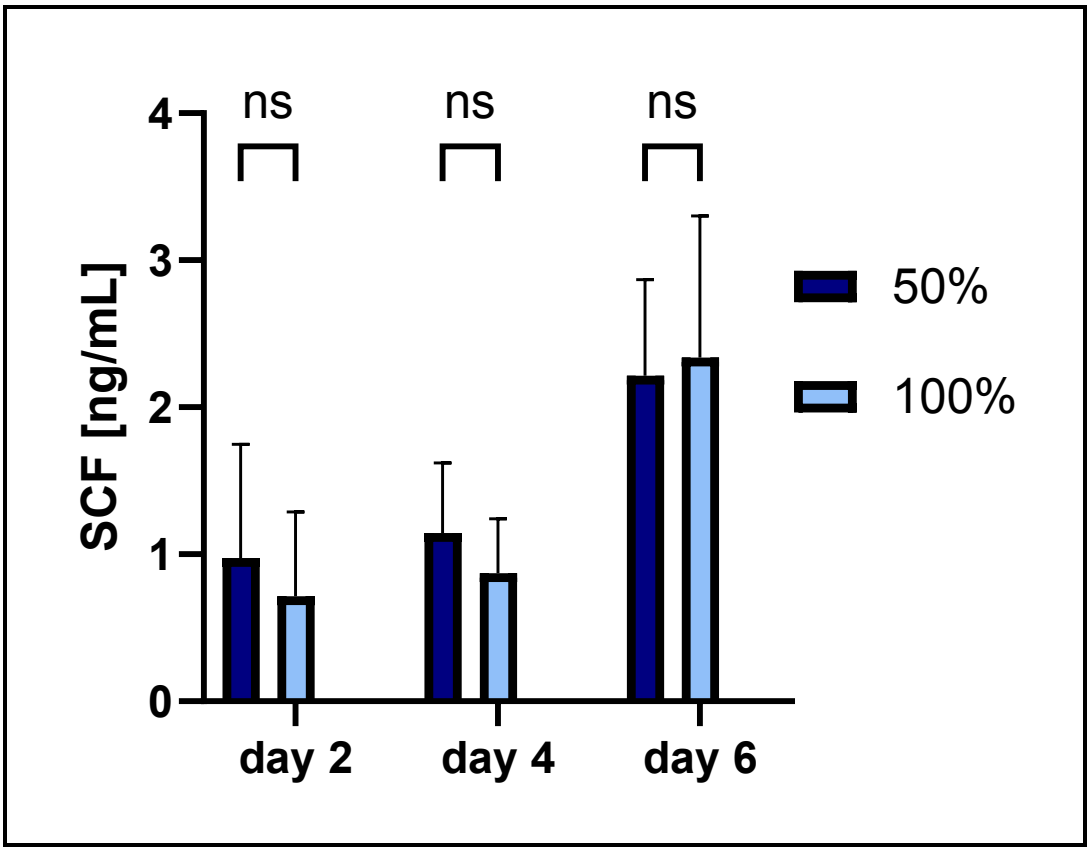

Figure 15 Confluence comparison.
SCF ELISA analysis of FLT3L_SCF_BFP knocked-in MSC-hTERT cells with either 50% or 100% confluence. The supernatant was tested on day 2, 4 and 6. As a negative control MSC-hTERT wild type cells (WT) were used at day 6. Significance was calculated by a one-way ANOVA test. Data represent mean ± SEM.

3.5. Engineered feeder cells support the growth of cytokine dependent cell lines

3.5.1. TPO_IL-6_BFP inheriting MSCs supply adequate levels of TPO for TPO-dependent UT7-TPO megakaryocytic cell line

To test if the secreted cytokines from the engineered feeder cells are indeed functionally comparable to exogenously added recombinant cytokines, we conducted co-culture experiments of MSC-hTERT engineered to express human TPO and IL-6 together with the human TPO-dependent leukemia cell line (UT7/TPO). To evaluate the influence of direct cell to cell contact on cells survival and growth, we also cultured UT7/TPO cells in the presence of the supernatant derived from the same engineered MSC-hTERT feeder cells.

Both conditions, i) co-culture with the cytokine expressing cells (MSC-KI) and ii) culture with the supernatant (KI-SN) derived from the MSC-KI cells, led to a significant proliferation of UT-7/TPO leukemia cells. This effect was less prominent when UT7/TPO were co-cultured with WT feeder cells and almost absent with the respective WT feeder cell supernatant. At day 8, UT-7/TPO cells cultured with the supernatant derived from the MSC-KI showed cell expansion rates similar to the positive control conditions with exogenously added human recombinant TPO (Figure 16).

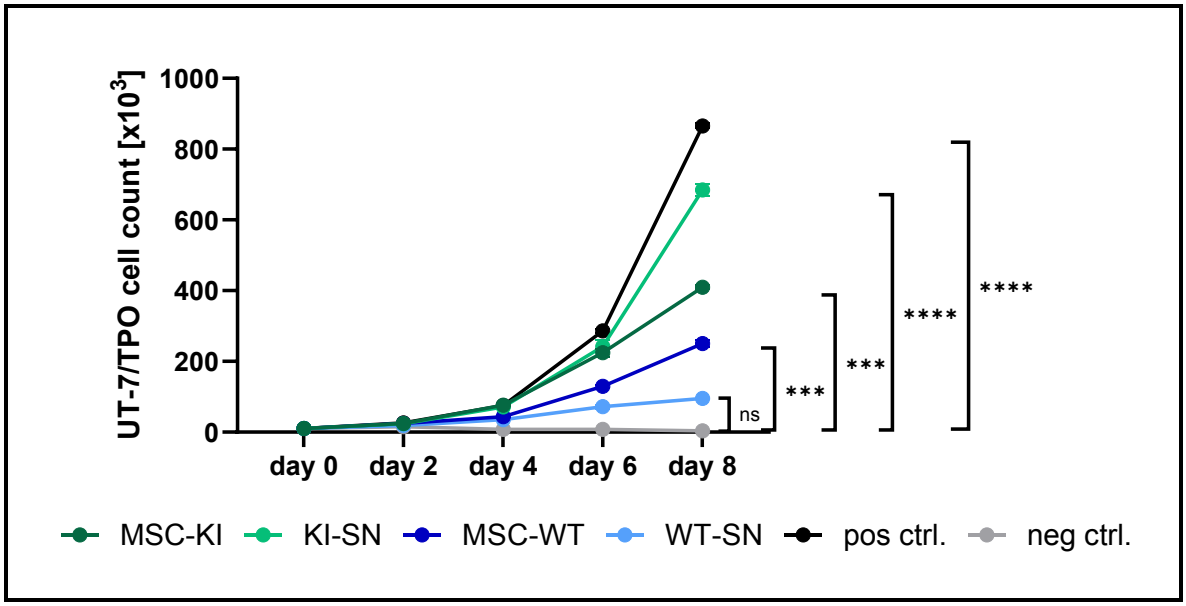

Figure 16 UT-7/TPO Co-Culture with MSC-hTERT.
*UT-7/TPO AML cells were co-cultured for eight days with MSC-hTERT wild type (MSC-WT), TPO_IL-6_BFP construct (MSC-KI) or their collected supernatant (WT–SN; KI–SN). As a positive control TPO was added to RPMI media (pos. ctrl), while none was added to the negative control (neg. ctrl). On day 0, 2, 4, 6 and 8 total cell numbers were counted via FACS analysis. Significance was calculated by a . Significance was calculated by a two-way ANOVA test and marked with ns=not significant, * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001. Data represent mean ± SEM.*

3.5.2. Genetically engineered MSCs provide sufficient cytokine levels for the growth and survival of CD34$^+$ HSPCs

Human HSPCs are highly dependent on the appropriate mix and concentration of several human cytokines.(2) These cytokines include SCF, FLT3L, TPO and IL-6. To investigate, if human HSPC survival and proliferation can be supported by our feeder cells engineered to express those cytokines, we conducted co-culture experiments with human cord blood derived CD34$^+$ HSPCs.

MSC-hTERTs expressing human SCF, FLT3L, TPO and IL-6 not only supported the proliferation and differentiation of human hematopoietic cells upon co-culture, resulting in a significant higher total cell count (TCC) compared with the WT co-culture ($p<0.001$), and with the positive control ($p<0.005$), but also helped to retain expression of the stem cell marker CD34. Co-culture resulted in a significant higher number of CD34$^+$ HSPC cells at the end of the 6 day expansion periode (MSC-KI:MSC-WT: $p<0.001$; MSC-KI:pos.ctrl: $p<0.001$). Also the feeder cell-derived supernatant provided sufficient amounts of cytokines for total cell expansion and conservation of CD34 positive cells, albeit this supportive effect was significantly lower compared to direct co-culture conditions. Both, WT feeder cell co-culture and the respective supernatant outperformed the negative control (MSC-WT:neg. ctrl: $p<0.001$; WT-SN:neg. ctrl,: $p<0.001$). Interestingly, the presence of feeder cells led to a higher total cell number and CD34$^+$ cell number in comparison to the supernatant only (TCC: $p<0.005$; CD34$^+$: $p<0.001$), implicating a particular dependence of HSPCs on direct cell to cell contact in addition on their dependence on specific cytokines (Figure 17, 18).

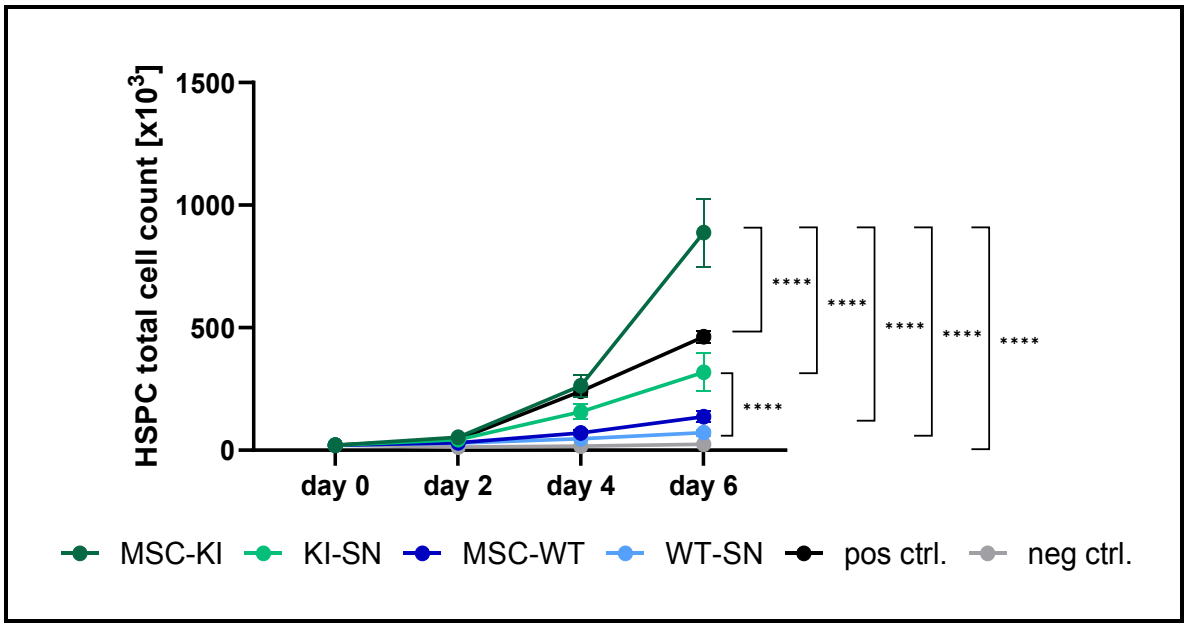

Figure 17 Cord blood-derived HSPC Co-Culture with MSC-hTERT: Total cell count.
*Cord blood-derived HSPCs were co-cultured for six days with MSC-hTERT wild type (MSC-WT), FLT3L_SCF_BFP + TPO_IL-6_BFP double construct (MSC-KI) or their collected supernatant (WT–SN; KI–SN). As a positive control all four cytokines were added to RPMI media (pos. ctrl), while none was added to the negative control (neg. ctrl). On day 0, 2, 4 and 6 total cell numbers were counted via FACS analysis. . Significance was calculated by a two-way ANOVA test and marked with ns=not significant, * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001. Data represent mean ± SEM.*

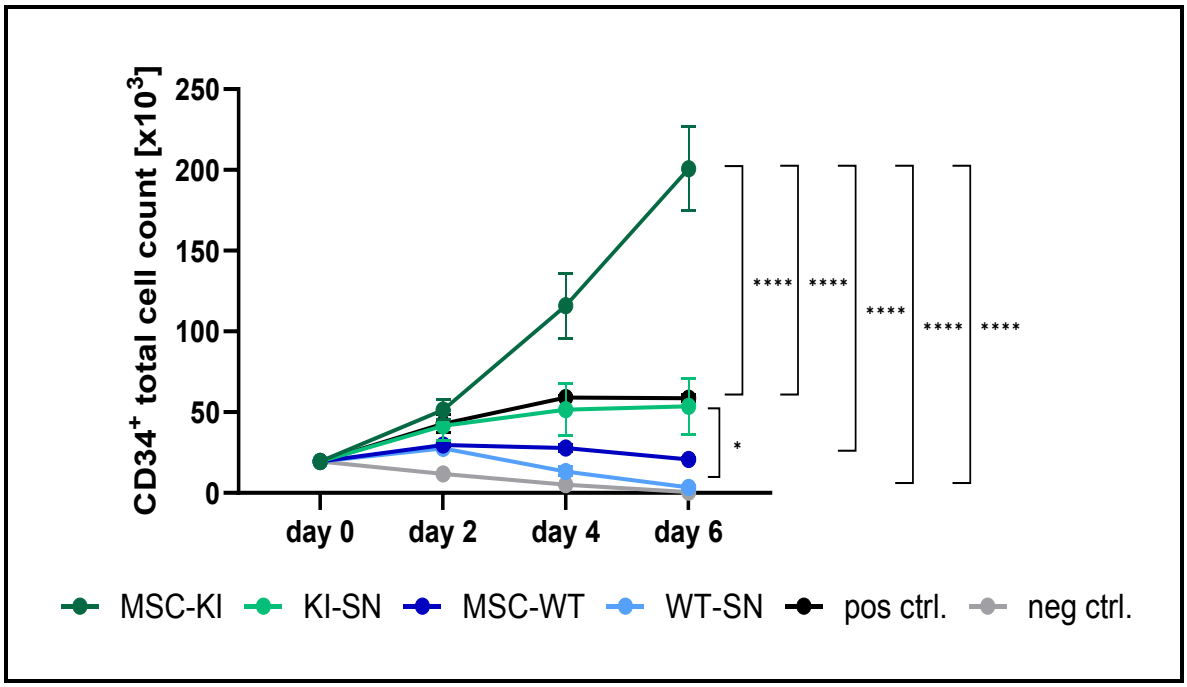

Figure 18 Cord blood-derived HSPC Co-Culture with MSC-hTERT: CD34⁺ cell count.
*Cord blood-derived HSPCs were co-cultured for six days with MSC-hTERT wild type (MSC-WT), FLT3L_SCF_BFP + TPO_IL-6_BFP double construct (MSC-KI) or their collected supernatant (WT–SN; KI–SN). As a positive control all four cytokines were added to RPMI media (pos. ctrl), while none was added to the negative control (neg. ctrl). On day 0, 2, 4 and 6 CD34⁺ cells were counted via FACS analysis. . Significance was calculated by a two-way ANOVA test and marked with ns=not significant, * = p<0.05, ** = p<0.01, *** = p<0.001 and **** = p<0.0001. Data represent mean ± SEM.*

Despite the overall higher numbers of total CD34⁺ HSPCs in the tested samples, the percentage of the CD34 positive fraction appeared to be similar between the different conditions, deteriorating over the 6 day culture period (Figure 19; p=0.051-0.929).

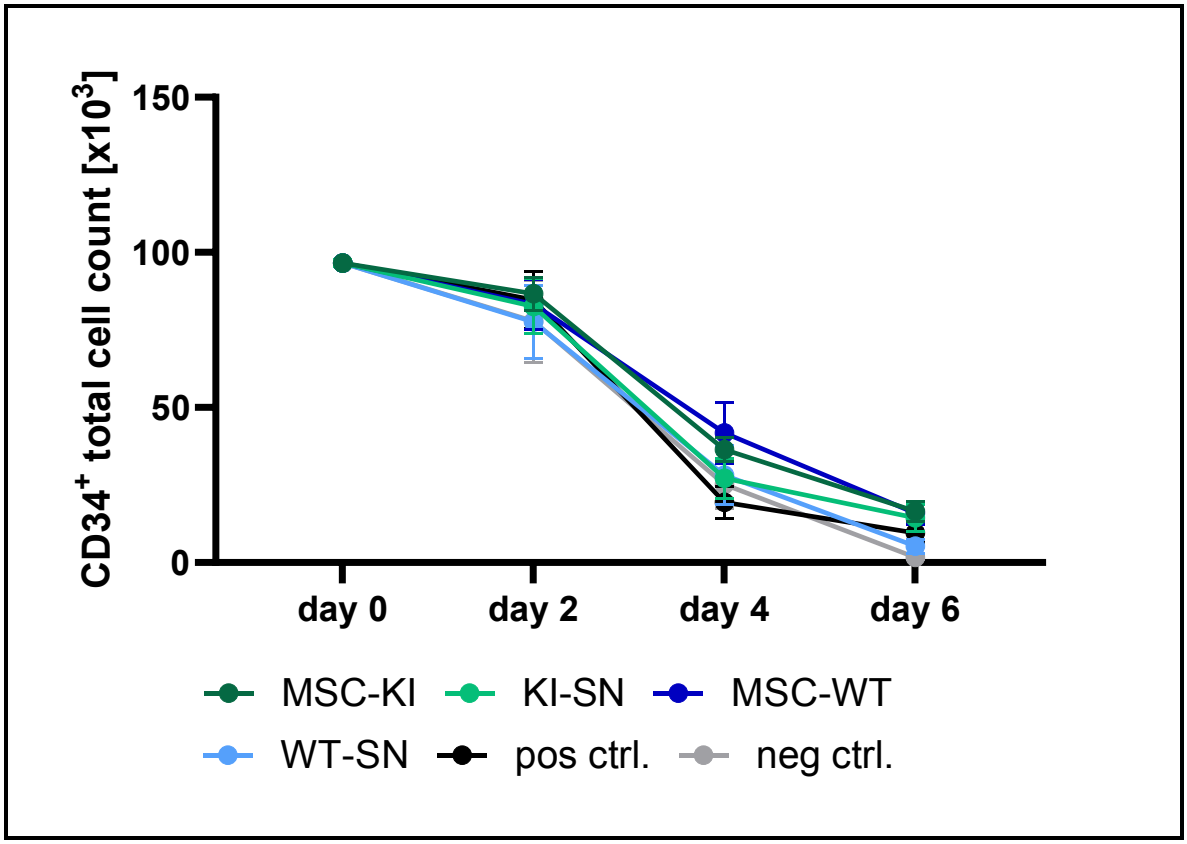

Figure 19 Cord blood-derived HSPC Co-Culture with MSC-hTERT: CD34⁺ percentage.
Cord blood-derived HSPCs were co-cultured for six days with MSC-hTERT wild type (MSC-WT), FLT3L_SCF_BFP + TPO_IL-6_BFP double construct (MSC-KI) or their collected supernatant (WT–SN; KI–SN). As a positive control all four cytokines were added to RPMI media (pos. ctrl), while none was added to the negative control (neg. ctrl). On day 0, 2, 4 and 6 CD34⁺ percentage was calculated via FACS analysis. Significance was calculated by a two-way ANOVA test. Data represent mean ± SEM.

This observation was also in line with our findings from the methylcellulose colony-assay (MethoCult™). After the six day expansion period colony forming capacity of expanded cells was strongly reduced compared to day 0. Nevertheless, co-culture with cytokine-expressing feeder cells or culture with the respective cell-derived supernatant helped in retain colony-forming capacity similar to control cultures with exogenously added cytokines. WT feeder cells also assisted the preservation of the colony formation. However, this effect got lost when the supernatant from WT feeder cells was used, indicating the necessity of a cell-to-cell contact (Figure 20).

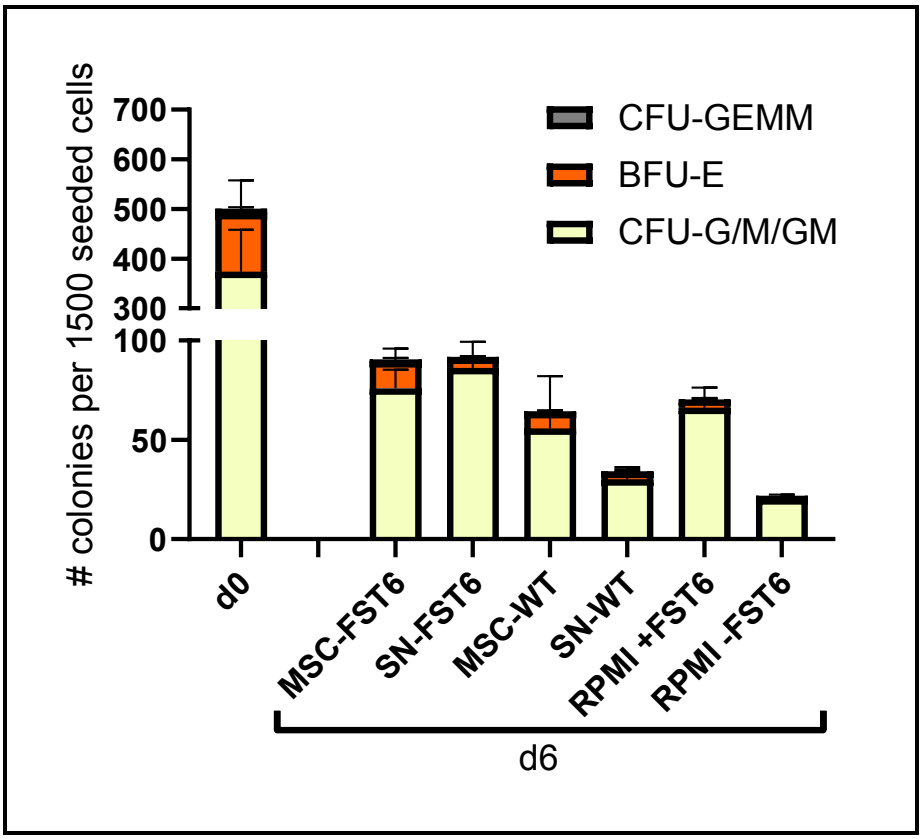

Figure 20 Cord blood-derived HSPC Co-Culture with MSC-hTERT: MethoCult.
Cord blood-derived HSPCs were co-cultured for six days with MSC-hTERT wild type (MSC-WT), FLT3L_SCF_BFP + TPO_IL-6_BFP double construct (MSC-KI) or their collected supernatant (WT–SN; KI–SN). As a positive control all four cytokines were added to RPMI media (pos. ctrl), while none was added to the negative control (neg. ctrl). On day 0 and 6 1500 cells were seeded for each condition and analyzed after 14 days in a Colony–Forming Unit (CFU) Assay Reader. Significance was calculated by a one-way ANOVA test. Data represent mean ± SEM.

4. Discussion

Until today, preserving properties of hematopoietic stem cells and their progenitors under in vitro conditions and promoting their expansion is limited by their reduced ex-vivo growth potential. Additionally, upon culture, HSPCs lose their self-renewal ability and show a tendency to differentiate into more mature hematopoietic cells.(1) Current in vitro expansion protocols rely on the supplementation of exogenous recombinant cytokines, necessary for HSPC proliferation, survival, and stem cell retention. So far these approaches had only limited success. Since cytokines supplementation is very cost intensive (66), finding novel and cheaper ex-vivo culturing conditions would be a great leap toward expanding clinical HSPC applications. In this thesis, we provided a novel, alternative approach by utilizing CRISPR/Cas9-engineered cytokine expressing stromal cell to overcome the named above obstacles.

Crucial for the survival and expansion of HSPCs are the cytokines SCF, FLT3L, TPO and IL-6. These cytokines are normally provided by multiple different cell types in the BM niche and recombinantly produced versions of these cytokines are added exogenously in attempts to expand HSPCs in vitro.(45) By integrating two separate DNA constructs, carrying the genetic information for the expression of the human cytokines FLT3L and SCF (construct 1) and TPO and IL-6 (construct 2) into human MSC cell lines MSC-hTERT and HS27a, we were able to create single cell-derived knock-in clones secreting sufficient amount of human cytokines. Through quantification by ELISA, we could confirm the concentrations reached in the harvested cell culture supernatants were comparable to the concentration usually detected after adding exogenous recombinant cytokines to the HSPCs expansion media.(2)

Although MSCs have been described as the main sources of SCF (46) our data could not confirm this observation. Unmodified, WT mesenchymal stromal/stem cells did not secret SCF, nor any other analyzed cytokine (FLT3L, TPO, IL-6) in sufficient quantity.

Interestingly, our ELISA-based cytokine quantification revealed that the number of alleles modified by homology-derived recombination (HDR) and therefor carrying the correct knock-in construct did not significantly influence the amount of cytokines

secreted. Mono-allelically or bi-allelically engineered cells secreted comparable amount of cytokines (TPO: p=0.778; IL-6: p=0.48). Since constitutive expression of the constructs should be driven by a strong SFFV promotor genetic reasons, like haploinsufficiency or allele-restricted expression are very unlikely to account for this unexpected finding.(113) As cytokines need to be correctly folded in the ER and are post-translational modified before secreted via the Golgi apparatus, an already maxed-out capacity of the stromal cells to fulfill this extra workload of folding, modification and secretion could be the reason for similar cytokine levels in mono- or bi-allelically engineered cells, despite different gene dosage present.(114,115)

Previous literature stated that confluency of BM derived human and murine MSCs impacts their chondrogenic and osteogenic differentiation potential as well as their glucose metabolism.(116,117) Here we also addressed if confluency impacts overall cytokine secretion capacity. Interestingly, we could not see an influence of feeder cell confluency on the production and secretion of our introduced cytokines ($p = 0.48$). This might be explained by the idea that the exogenously inserted genes are driven by their own promotor and are therefore not influenced by cell-endogenous expression regulatory mechanisms that might negatively impacted by the level of confluency.

We assessed the impact of our engineered cytokine-expressing MSCs in co-culture experiments with a cytokine dependent AML cell line and cord blood-derived HSPCs, leaving us with three main findings:

In both experiments, engineered MSCs and their respective supernatant supported the survival and expansion of the hematopoietic cells. These results are consistent with the previous literature(45), highlighting the importance of the knocked-in cytokines in the proliferation and expansion of the tested cells and confirming a sufficient cytokine concentration reached.

While the percentage of HSPCs remaining CD34 positive, decreased similarly over time under all conditions ($p=0.051$), total $CD34^+$ cell count was significantly increased (MSC-KI:MSC-WT: $p<0.001$; MSC-KI:pos.ctrl: $p<0.001$) when HSPCs were co-culture with engineered cytokine-expressing feeder cells. This indicated that the engineered feeder cells help in conserving stem cells properties, as reflected here by the expression of the cell surface maker CD34. Furthermore,

colony-forming potential evaluated by gold-standard methylcellulose assays indicated an improved preservation of stem and progenitor cell properties.

Our data supports the existing theory, that MSCs are a crucial part of the structure of the BM niche and provide support for HSPC expansion and survival. Interestingly, this support was even noticeable if the MSCs were present in their non-edited WT form, not over-expressing high amounts of human SCF, FLT3L, IL6 and TPO (MSC-WT:neg. ctrl: $p<0.001$). These findings are consistent with the research stating that MSCs interact with the HSPCs through adhesive mechanism and also possess multiple other paracrine functions, that lead to proliferation as well as maintenance of the hematopoietic stem cell.(72)

In UT-7/TPO co-culture experiment, when comparing the co-culture condition with its respective supernatant, cell expansion seemed to be higher when only cytokine-containing supernatant was present. Since this observation was only made at later stages of the expansion period (day 6),we conclude that this might be mainly caused by a competition for the limited nutrients in the cell culture media through the remaining feeder cells. The reduced availability nutrients therefore might have resulted in the observed decelerated expansion in the later phases of culture.

A significant limitation of this thesis is the non-physiological introduction and production of cytokines, since we introduced the cytokine expression cassettes into genetic loci, where they are normally not situated. Furthermore, in our engineered cells promoter activation and transcription frequency is different to physiological conditions. An alternative way to activate IL-6 and SCF secretion was presented from Caplan et al. (118). Instead of a non-physiological introduction, they utilized IL-1a to activate IL-1 and SCF secretion. However this approach is currently limited to these two cytokines.

While the beneficial effect of cytokine-expression feeder cells was observed in each donor tested, we still observed a strong heterogeneity between the individually tested donors. The relatively small sample size further limits a generalizability the findings and therefore our system need be verified in bigger cohort.

In conclusion this thesis provided additional evidence supporting the importance of mesenchymal stem cells in supporting the survival and proliferation of hematopoietic stem and progenitor cells, and the retention of their stem cells potential. The results

of our ELISAs indicated successful expression of the human cytokines FLT3L, SCF, TPO and IL-6 upon CRISPR/Cas9-driven knock-in. Furthermore, this thesis demonstrates that feeder cells engineered to express human cytokines offer a cheap and easily applicable system for expanding cytokine-dependent cell lines and human HSPCs reaching similar rates or even exceeding the expansion achieved with externally added recombinant cytokines.

Finally, additional studies are necessary to gain a deeper understanding of the molecular mechanism and interaction between MSCs and HSCs, to offer long-term culture possibilities for HSCs in the future. The incorporation of CRISPR/Cas9 editing systems seems to be one of the key elements to further improve culture settings and provide more physiological culture conditions in upcoming experiments.

5. Literature

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Supplementary

Table 4 Media composition

Medium	Components
RPMI – medium (culturing media)	- RPMI 1640 + L–Glutamine (Sigma Aldrich, Zwijndrecht, The Netherlands) - 10% FBS Good Forte (heat inactivated) (Pan Biotech, Aidenbach, Germany) - 1% Antibiotic/Antimycotic (Gibco™, Thermo Fisher Scientific, Breda, The Netherlands)
α–MEM – medium (culturing media)	- alpha–MEM (Sigma Aldrich) - 10% FBS Good Forte (heat inactivated) (Pan Biotech) - 1% Antibiotic/Antimycotic (Gibco™) - 1% GlutaMax™ (Gibco™)
Freezing medium	- Respective culturing medium - 10% DMSO

Table 5 Primer-Probe Assay

Primer	For: 5'-GGAACCCCTAGTGATGGAGTT-3' Rev: 5'-CGGCCTCAGTGAGCGA-3'
Probe	5'-CACTCCCTCTCTGCGCGCTCG-3' 5' dye: FAM Internal quencher: ZEN 3' quencher: IBFQ
Primer to probe ratio:	3.6

Table 6 Mastermix for ddPCR

Reagents	**Volume [µL]**
2X supermix for probes	12.5
Primer/probe assay	1.25
H_2O	6.25
DNA	5
total	25

Table 7 ddPCR thermocycler program

Step	**Temperature [°C]**	**Time**	**Cycles [n]**
Enzyme activation	95	10 min	1
Denaturation	94	30 sec	42
Annealing	60	1 min	
Elongation	72	30 sec	
Enzyme deactivation	98	10 min	1
Hold	4	infinite	-

Table 8 Primer sequences

Primer	Sequence
HBB_in–out_fwd1 (#151)	AAGGAGAAGATATGCTTAGAACCG
HBB_in–out_rev1 (#152)	ACGATCCTGAGACTTCCACAC
SFFV_in–out_rev1 (#153)	CATGTACCCGCCCTTGATCT
AAVS1_in–out_fwd1 (#174)	CTTAGGATGGCCTTCTCCGAC
AAVS1_in–out_rev4 (#179)	AGGGAGTTTTCCACACGGAC

Table 9 PCR amplification

Reagent	Volume [µl]
DreamTaq PCR Master Mix (2x) (Thermo Fisher Scientific, Breda, The Netherlands)	10
Primer fwd	1
Primer rev	1
nano–filtrated water	7
QE DNA	1