Modeling of hematopoiesis using ES and patients-derived iPS cells

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III. Abbreviation

AML	Acute myeloid Leukemia
ABCG2	ATP-binding cassette sub-family G member 2
AFP	Alpha-fetoprotein
AP	Alkaline phosphatase
APEL	albumin polyvinyl alcohol essential lipids
CLL	Chronic Lymphocytic Leukemia
BSA	Bovine serum albumin
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bFGF	Basic fibroblast growth factor
BRACH	T-Brachyury
BMP4	bone morphogenetic protein 4
CAMPT	congenital amegakaryocytic thrombocytopenia
C/EBP	CCAAT-enhancer-binding proteins
c-DNA	Complementary deoxyribonucleic acid
CLP	common lymphoid progenitors
CML	Chronic myeloid Leukemia
CMP	common myeloid progenitors
CN	congenital neutropenia
CyN	cyclic neutropenia
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNMT	DNA-Methyltransferase

EB	embryoid body
ELANE	Neutrophil elastase 2
ESCs	Embryonic stem cells
ER	endoplasmic reticulum
FOXA2	Forkhead box protein A2
FCS	fetal calf serum
G-CSF	Granulocyte-colony stimulating factor
M-CSF	monocyte-colony stimulating factor
G-CSF	Granulocyte-colony stimulating factor
HAX1	Hematopoietic lineage cell-specific protein -associated protein X-1
HD	Healthy donor
hiPSCs	Human induced pluripotent stem cells or induced pluripotent stem cells
HAT	histone acetylase transferase
HAX1	HCLS1 associated protein X-1
HCLS1	Hematopoietic-specific lyn-substrate 1
HDAC	histone deacetylase
HSCs	hematopoietic stem cells
JAK	Janus kinase
KLF4	Kruppel-like factor 4
KSR	Knockout serum replacement
LEF-1	Lymphoid enhancer-binding factor 1
LT-HSC	long-term hematopoietic stem cells

MPP	Multipotent progenitor cells
mRNA	messenger RNA
MYH6	Myosin heavy chain α isoform
NBT	Nitro blue tetrazolium
NE	neutrophil elastase
NEAA	Non-essential amino acids
NTM	Na+ Tris Mg2+
NA	nicotinamide
NAD^+	nicotinamide adenine dinucleotide
NAMPT	Nicotinamide phosphoribosyltransferase
NK	natural killer
OCT3/4	Octamer-binding transcription factor
OSKM	Plasmid containing OCT3/4, SOX2, KLF4, c-MYC
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P/S	Penicillin/Streptavidin
PAX6	Paired box protein 6
PRE	Posttranscriptional regulatory element
RT	Room temperature
RNA	Ribonucleic acid
SCN	Severe congenital neutropenia
SOX2	sex determining region Y -box 2
SDS	Sodium dodecyl sulfate

STAT	Signal Transducer and Activator of Transcription
ST-HSC	short-term hematopoietic stem cells
TUB III	Tubulin beta-3 chain
TPO	Thrombopoietin
VEGF	Vascular endothelial cell growth factor

IV. Abstract

In vitro differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) into hematopoietic cells may help to find new strategies for the treatment of incurable hematopoiesis disorders and to improve (hematopoietic stem cells) HSCs transplantation. In this study we mainly focus on the mechanisms of hematopoietic differentiation of ESCs and iPSCs at early and late stages, using our knowledge from iPSCs of cyclic neutropenia (CyN) and congenital neutropenia (CN) patients.

We found that TPO and bone morphogenetic protein 4 (BMP4) signaling pathway are directly connected. This could elevate early hematopoiesis by TPO/c-mpl signaling. TPO-mediated binding of the HIF-1α transcription factor to the BMP4 gene promoter mediates early hematopoiesis and activation of BMP4 target genes in ESCs.

We also used an embryoid body (EB)-based protocol of granulocytic differentiation of human iPSCs to evaluate the *in vitro* myeloid differentiation of iPSCs derived from one CyN patient harboring a sporadic heterozygous ELANE mutation (p.W241L), and one CN patient with an inherited familial ELANE mutation (p.C151Y) in comparison with myeloid differentiation of iPSCs derived from a healthy donor.

CyN is a hematologic disorder show cycles at approximately 21-day intervals in blood cell counts particularly granulocytic neutrophils, monocytes, platelets, and reticulocyte numbers. The majority of CyN patients (approximately 90%) show inherited mutations in the ELANE gene and CN patients also have inherited ELANE mutations. It is unclear how a mutation in the same gene causes CN or CyN. In addition, the pathomechanism of cycling in hematopoiesis pathway downstream of ELANE mutations is still unclear.

Using the embryoid body (EB)-based protocol of granulocytic differentiation of hiPSCs, we found that the combination of IL-3 with G-CSF is the best

condition to improve our neutrophil maturation protocol for further experiments applicable for CN patients derived from iPSCs. We detected diminished absolute numbers of $CD15^{+}CD16^{+}$ that were derived from iPSCs of a CyN patient, as compared to that of a healthy individual on day 28 of culture. We also found a remarkable reduction of absolute numbers of myeloid and granulocytic cells that were generated from iPSCs of a CN patient, as compared to cells of healthy individuals on day 28 of culture.

1 Introduction

1.1 Hematopoiesis

Formation and maturation of blood cells from hematopoietic stem cells (HSCs) is a well-organized process of hematopoiesis (Doulatov, Notta et al. 2012). Developmental studies of hematopoiesis can help to understand the mechanisms of inherited blood cell disorders or blood cancer as well as to identify the role of HSCs in aging and oncogenesis (Jagannathan-Bogdan and Zon 2013). HSCs are infrequent cells homing in the bone marrow (BM) to generate mature blood cell lineages (Orkin and Zon 2008). Using monoclonal antibodies against specific surface markers of HSCs they can be detected by FACS and separated into committed progenitors (Orkin and Zon 2008).

Vertebrate blood cells are developed in a 2-wave program (Fig.1) involving the primitive wave and definitive wave. Erythrocytes and macrophages will develop in the primitive wave in the early embryonic development (Palis and Yoder 2001). in a definitive wave of hematopoiesis, multipotent HSCs will form all blood cell lineages (Orkin and Zon 2008).

It has been demonstrated that all hematopoietic lineages have been derived from a common precursor in embryonic stem cells (ESCs) based on hemangioblast theory (Kennedy and Eberhart 1997).

In vertebrates, definitive HSCs will form in the aorta gonad mesonephros (AGM) region in the embryo, after that they will migrate to the fetal liver and then to the bone marrow (Cumano and Godin 2007).

Further studies using *in vitro* differentiation of mouse ESCs proved the theory of the existence of hemangioblasts in vertebrates (Choi, Kennedy et al. 1998).

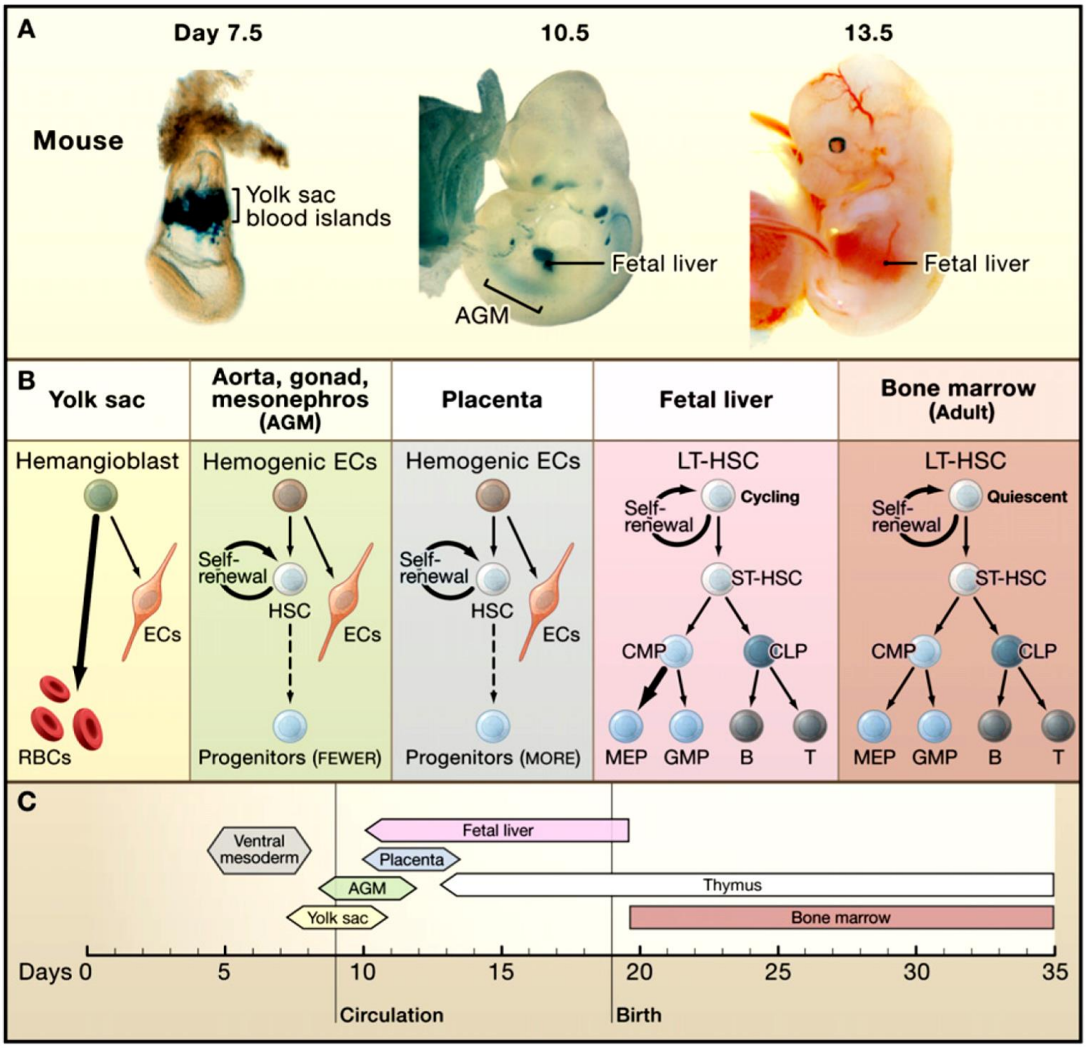

Figure 1. Developmental Regulation of Hematopoiesis in the Mouse

a, Hematopoiesis occurs first in the yolk sac (YS) blood islands and later at the aorta-gonad mesonephros (AGM) region, placenta, and fetal liver (FL). **b,** Hematopoiesis in each location and the production of specific blood lineages in each region.

Abbreviations: ECs, endothelial cells; RBCs, red blood cells; LTHSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitors; **c,** developmental time windows for hematopoiesis **(Source from Orkin et al. 2009).**

1.2 Isolation of HSCs

In bone marrow, long-term (LT) HSCs generate short-term (ST) HSCs, which can produce all blood lineages. Multipotent progenitor cells (MPP) generated by ST-HSC can reconstitute all blood components (Doulatov, Notta et al. 2012, Manz and Boettcher 2014) (Fig.2). These progenitors first form common lymphoid progenitors (CLP) that differentiate to B, T, or natural killer (NK) cells and common myeloid progenitors (CMP) reconstituting megakaryocyte, granulocyte/monocyte and erythrocyte precursors (Fey 2007, Cotta 2010). Hematopoiesis also depends on the bone marrow niche consisting of non-hematopoietic cells, such as fibroblasts, osteoblasts, and adipocytes, which are sources of cytokine homing signals for hematopoietic cells and mechanical anchorage factors (Fey 2007).

several specific cell surface markers are required for the purification and detection of HSCs. For example, CD34 antigen was the first marker found in more than 99 % of hHSCs and in on less than 5 % of all mature blood cells (Doulatov, Notta et al. 2012). CD90 was another stem cell marker detected by Baum et al. (Baum, Weissman et al. 1992). It was demonstrated that cells expressing CD90 (Thy1) in combination with CD34 have the highest multi-lineage capacity. More differentiated markers of hematopoietic progenitors are CD45RA and CD38 that are negatively enriched in HSCs (Bhatia, Wang et al. 1997). Based on these observations, it has been proposed that the $CD34^{+}CD38^{-}Thy1^{+}CD45RA^{-}$ cells represent hHSCs.

HSCs activity is restricted to the $CD49f^{+}$ cells, CD49f is expressed on 50% of human $CD90^{+}$ and 25% of $CD90^{-}$ cells (Notta, Doulatov et al. 2011).

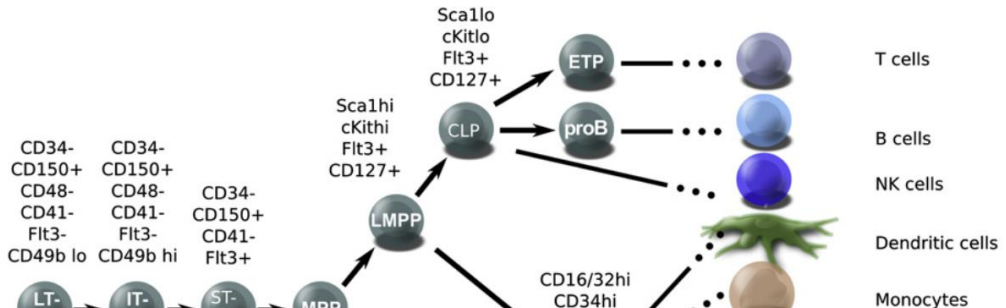

Figure 2. Current Models of Lineage Determination in the Adult Human Hematopoietic Hierarchies

Terminally differentiated cells are shown on the right, and inferred lineage relationships are depicted with arrows. In mice (A), HSCs can be separated into long-term (LT), intermediate-term (IT), and short-term (ST) classes based on the duration of repopulation. In humans (B), HSCs are defined by the expression of CD49f and other markers. In mice, differentiation of HSCs gives rise to transiently engrafting multipotent progenitors (MPPs), and a series of immature lymphoid-biased progenitors (such as LMPPs) that undergo gradual lymphoid specification. In humans, loss of CD49f expressing cells can be identified as MPPs; Both mice and humans have well-defined populations of myelo-erythroid progenitors: CMPs, GMPs, and MEPs.

Lin: cocktail containing cell surface markers for all terminally differentiated populations (B cell; T cell; NK; dendritic cell, monocyte, granulocyte, megakaryocyte, and erythrocyte) **(Source from Sergei Doulatov et al.2012).**

With the application of monoclonal antibodies, cell populations from very early hematopoiesis have been separated based on their surface markers, and theses population can be sorted with a cocktail of different antibodies. However, based on many unknown markers and antibodies, in order to

determine if the isolated cell population remains heterogeneous, more investigations are required (Lu, Neff et al. 2011). Heterogeneity of stem cells plays an important role in cancer, aging or leukemia (Rossi, Jamieson et al. 2008, Beerman, Bhattacharya et al. 2010).

1.3 Cellular and Molecular Mechanisms in hHSCs Regulation

GATA1 and PU.1 are two of the most important transcription factors that regulate primitive erythroid and myeloid hematopoiesis by cross-inhibition (Fig.3). GATA1 is the main regulator of erythrocyte development (Cantor and Orkin 2002). By contrast, myeloid cells, which includes macrophages and granulocytes mainly will regulate by PU.1. In addition, Runx1 is a member of the runt family of transcription factors (RUNX) and that also has a crucial role in definitive hematopoiesis (Wang, Mayo et al. 1996).

There is evidence that osteoblasts are important cells that interact with HSCs in the bone marrow and play an essential role in creating a niche for hematopoiesis. In addition, vascular cells, as well as vascular niche, are also required for HSC regulation (Kiel and Morrison 2006).

There are some essential signaling pathways and cytokines involved in hematopoiesis and formation of hematopoietic progenitors. For example, BMP4 is a morphogenic signaling molecule required for the embryonic ventral mesoderm formation as well as hematopoietic precursor cells formation during embryogenesis (Huber, Zhou et al. 1998, Sadlon, Lewis et al. 2004). In Xenopus embryos, transcription factors responsible for hematopoietic-specification expressions such as GATA-1 and Scl/Tal-1, appear after ectopic expression of BMP4 (Maeno, Mead et al. 1996, Zhang and Evans 1996, Mead, Kelley et al. 1998).

BMP4 is required for the hematopoietic cells development followed by mesoderm induction from pluripotent mouse ESCs (Johansson and Wiles

1995, Nakayama, Lee et al. 2000). Mesoderm induction and blood cell formation in the yolk sac disrupts following BMP4 mutations. This evidence proves the importance role of BMP4 for hematopoietic differentiation (Winnier, Blessing et al. 1995).

BMP signal transduction is elicited through binding of BMP4 to type 1 and type 2 serine-threonine kinase receptors (Sadlon, Lewis et al. 2004), deletion of one of the BMP receptor molecules (BMPR1A) abolishes mesoderm formation (Mishina, Suzuki et al. 1995). Type I receptors are trans-phosphorylated by constitutive activation of kinase domains of the type II receptors (Sadlon, Lewis et al. 2004), this will results in activation of target genes, such as Id1, 2, and 3, and Msx1 and 2 (Mishina, Suzuki et al. 1995, Sadlon, Lewis et al. 2004, Larsson and Karlsson 2005, Zhang, Li et al. 2008).

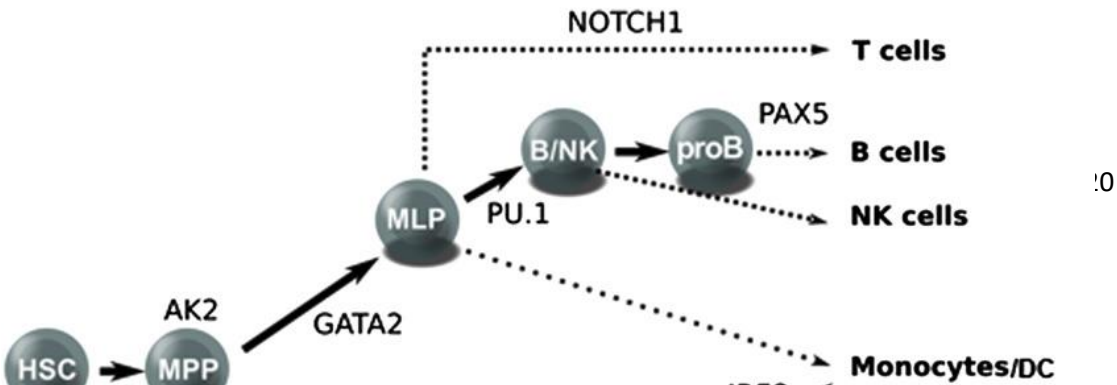

Figure 3. Genes and pathways that control the functions of early progenitors and human HSCs
Important factors on human HSCs regulation including transcription factors and signaling pathways that play an important role for stem cell expansion are shown on the left part of the picture **(Source from Sergei Doulatovet al.2012).**

We have shown that Thrombopoietin (TPO) promotes early hematopoiesis from ES cells (Pramono, Zahabi et al. 2016). Mutations in the TPO-receptor, c-mpl gene leads to a non-responsiveness of hematopoietic cells to TPO which results in the molecular defect of CAMT (congenital amegakaryocytic thrombocytopenia) disease (Ihara, Ishii et al. 1999, Fox, Priestley et al. 2002).

It has been found that TPO is an important cytokine for the *in vivo* expansion of HSCs (Lok and Foster 1994, Fox, Priestley et al. 2002). TPO also is a cytokine with lineage-restriction effects on thrombocytopoiesis and megakaryopoiesis (Arai, Takada et al. 1999). However, TPO/c-mpl signaling has an essential role in the maintenance of all hematopoietic lineages in human (King, Germeshausen et al. 2005).

1.4 Granulocytic Differentiation and Maturation

Blood cells are divided into two important branches: lymphoid and myeloid. T, B, and NK cells are in the lymphoid group that is responsible for adaptive and innate immune responses. Granulocytes (neutrophils, basophils eosinophils and mast cells), erythrocytes, megakaryocytes and monocytes are the fully differentiated and short-lived cells in the myeloid lineage (Doulatov, Notta et al. 2012)

Myeloid progenitors express CD123 (IL-3 receptor) and CD135 (FLT3L) in humans (Doulatov, Notta et al. 2012). CMP to GMP transition in myeloid progenitors occurs by the acquisition of CD45RA marker. Single $CD135^{+}CD45RA^{-}$ CMPs can produce all myeloid lineages, but it cannot generate lymphoid, lineages *in vitro* and in vivo after transplantation into mice (Doulatov, Notta et al. 2012).

Neutrophils are continuously generated from CMPs or GMPs in the BM in which a promyelocyte (MB/MP) derived from a myeloblast proliferation into myelocyte and fully differentiate into a segmented granule neutrophil (Fig.4) (Lekstrom-Himes, Dorman et al. 1999).

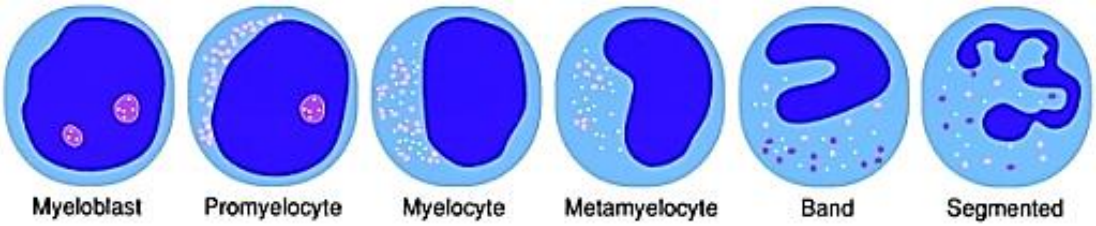

Figure 4. Schema of Granulopoiesis
Granulopoiesis begins when the myeloblast differentiates into a neutrophilic promyelocyte. The promyelocyte can be irreversibly committed to the neutrophilic cell line **(Source from Lekstrom-Himes JA et al. 1999).**

Transcriptional regulators involved in granulopoiesis are mainly core-binding factors (CBFs) and CCAAT/enhancer binding proteins such as C/EBPα (Lenny, Westendorf et al. 1997, Ward, Loeb et al. 2000). C/EBPα expression decreases with myeloid differentiation reaching a peak in myeloblasts (Scott, Civin et al. 1992). Another crucial factor for granulocytic differentiation is the lymphoid enhancer-binding factor 1 (LEF-1) transcription factor, which binds to and activates C/EBPα (Skokowa, Cario et al. 2006).

Neutrophils are key mediators of the innate immunity in the defense against bacterial and fungal microbes (Zhang, Nguyen-Jackson et al. 2010). They ingest and kill invading pathogens and by, releasing inflammatory mediators like chemokines, cytokines and oxygen species, they maintain inflammation. Thus, in neutrophil deficiency conditions that lack the number and function of PMNs can have important consequences, such as increased capability to bacterial infection (Miranda and Johnson 2007). The clinical administration of granulocyte-colony stimulating factor (G-CSF or CSF3) can be used to enhance or restore the levels of differentiated neutrophils in neutropenic patients (Welte, Gabrilove et al. 1996). In addition, administration of G-CSF is widely used for the mobilization of HSCs into the peripheral blood, facilitating the collection of HSCs for transplantation.

1.5 Role of CSFs in myeloid differentiation

Colony-stimulating factors (CSF) play an essential role in hematopoietic cell proliferation, activation and differentiation (Metcalf 2008). There are 3 subtypes of CSF: granulocyte colony stimulating factor (G-CSF or CSF3), granulocyte–macrophage colony stimulating factor (GM-CSF or CSF2) (Horn and Johnson 2010) and macrophage colony-stimulating factor (CSF1 or M-CSF) (Fig.5).

There is evidence that deletion of the GM-CSF gene just reduces neutrophil function without highly affecting their number, while deletion of genes encoding G-CSF, or M-CSF reduces the number of the cells that are stimulated by these cytokines (Metcalf 2008).

G-CSF is involved in both early and late regulation of normal and emergency granulopoiesis (Lieschke, Stanley et al. 1994, Skokowa and Welte 2013). Moreover, G-CSF has an important effect on hematopoietic progenitor cells mobilization from the bone marrow into the peripheral blood, that can be used for HSCs transplantation purposes (Molineux, Pojda et al. 1990, Roberts 2005).

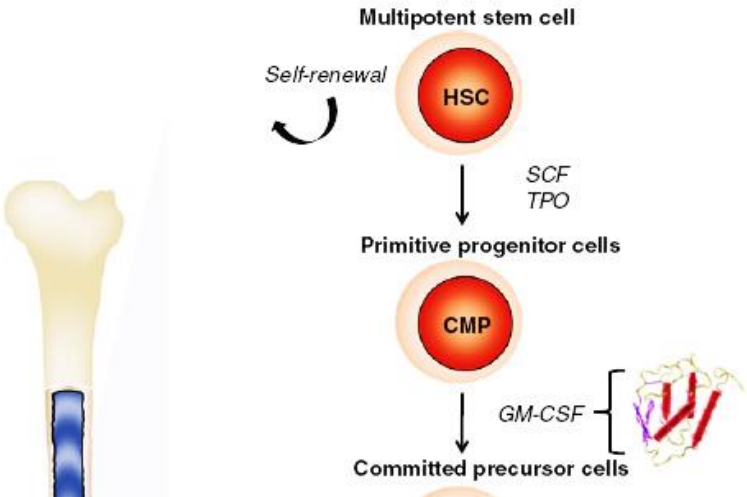

Figure 5. The role of CSFs in myelopoiesis
a haematopoietic stem cell can form the blood cells under either self-renewal or differentiation into a multilineage committed progenitors (common myeloid progenitor). By stimulation of GM–CSF, CMPs can give rise to a further differentiated progenitor and can be committed to the granulocytes and macrophages (GM) linage. GM cells can give rise to uni-lineage committed progenitors for granulocytes maturation by stimulation with G–CSF, IL-5 and SCF or monocytes maturation by stimulation with M–CSF.
SCF: stem cell factor, TPO: thrombopoietin **(Source from Alejandro Francisco-Cruz et al. 2014).**

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disorders

Heterozygous germline mutations in the ELANE gene which encodes neutrophil elastase (NE) (Tidwell, Wechsler et al. 2014) may cause two disorders of hematopoiesis: Cyclic neutropenia (CyN) and congenital neutropenia (CN). However, it is still not clear how mutations in the same gene cause two different diseases. It could be the mislocalization of aberrant NE or the disturbed protein folding caused by ELANE mutations inducing an unfolded protein response in the endoplasmic reticulum (ER) (Tidwell, Wechsler et al. 2014).

CyN is a hematologic disorder in which blood cell counts particularly granulocytic neutrophils, monocytes, platelets, and reticulocytes show cycles at approximately 21 days intervals (Lange 1983) (Fig.6).

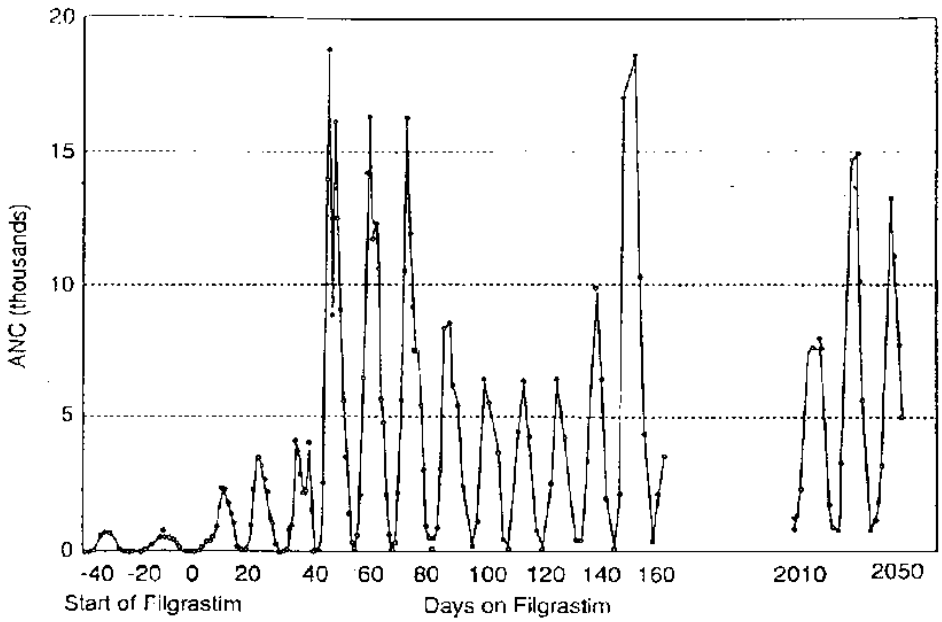


Figure 6. Course of Neutrophils in a Patient with Cyclic Neutropenia (CyN) (Source from Lange RD 1983).

CN patient's neutrophil counts are sustained low and their bone marrow shows a promyelocytic maturation arrest (Fig.7) (Welte, Zeidler et al. 2006). There are also some other gene mutations causing CN, including *HAX1*, *GFI1*, *G6PC3* (Tidwell, Wechsler et al. 2014) and *WASP* (Wiskott-Aldrich syndrome protein) (Skokowa and Welte 2009). Different mutations can lead to defects in the same downstream signaling pathways, resulting in the maturation arrest of promyelocytes in CN patients (Skokowa, Germeshausen et al. 2007, Zeidler, Germeshausen et al. 2009). More than 20% of patients will go on to develop acute myelogenous leukemia (AML) therefore, CN is considered to be a pre-leukemic syndrome (Rosenberg, Alter et al. 2006).

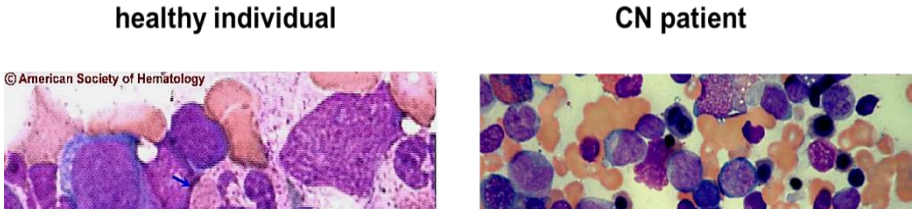


Figure 7. Morphological picture of bone marrow (BM) in healthy individual (left) and congenital neutropenia (CN) patient (right).
Granulocytic progenitors and neutrophils are presented in healthy BM (marked with blue arrows) and absent in CN patient **(Source from Welte, Zeidler et al. 2006).**

The ELANE mutations causing CN sometimes overlap those seen in CyN (Horwitz, Duan et al. 2007) (Fig.8). Heterozygous mutation of ELANE also causes almost all cases of CyN (Horwitz, Benson et al. 1999) and the majority of CN (Dale, Person et al. 2000). Most ELANE mutations are dominant heterozygous single nucleotide mutations (Nayak, Trump et al. 2015).

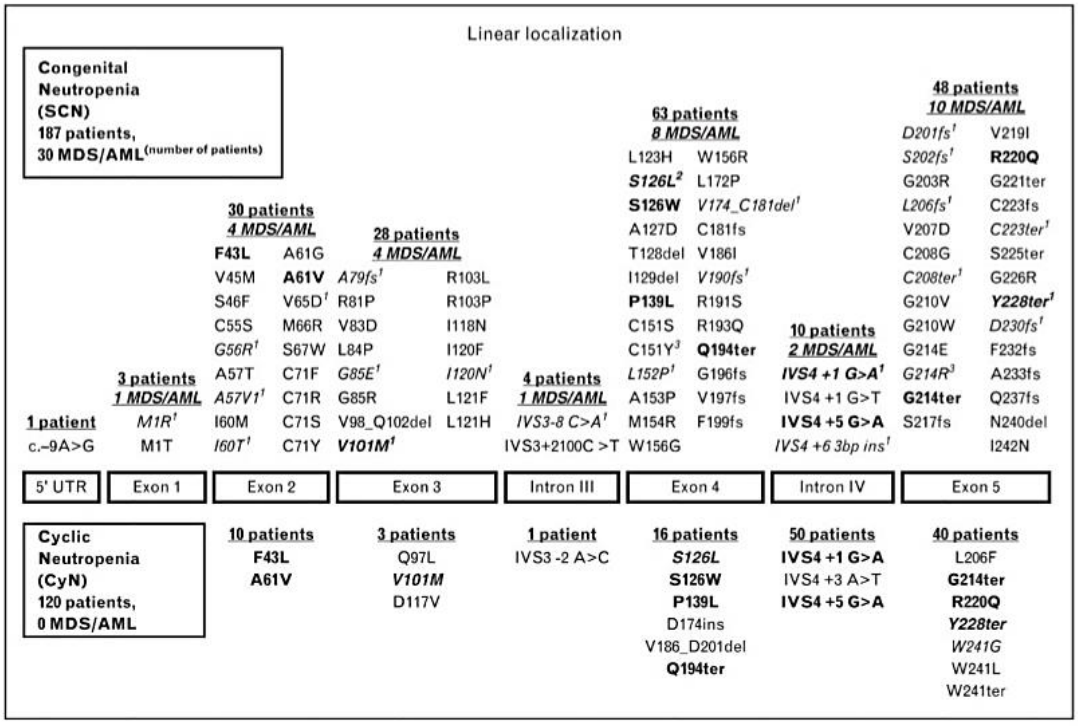


Figure 8. Cyclic and congenital neutropenia ELANE mutations
Mutations of SCN in 307 patients has been shown above the map, and those mutations seen in CyN are below. Shared mutations in both SCN and CyN has been represented in bold.
AML, acute myeloid leukemia; CyN, cyclic neutropenia; MDS, myelodysplasia; SCN, severe congenital neutropenia **(Source from Makaryan V et al. 2015).**

CN patients respond to high doses G-CSF therapy, which increases neutrophil blood counts to above 1000/µl and by that prevents life-threatening bacterial infections (Dale, Cottle et al. 2003). The risk of leukemia development directly correlates with the G-CSF dose required (Rosenberg, Alter et al. 2006), and with the variant of CN (Horwitz, Corey et al. 2013). It has been discussed, that life-long treatment with G-CSF predisposes CN patients to myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) (Freedman and Alter 2002) with a 20% cumulative incidence observed after 10 years (Dale, Cottle et al. 2003, Dale, Bolyard et al. 2006).

1.7 Defective signaling pathways in CN

With the first successful clinical trials of human G-CSF in patients with CN in 1987 (Bonilla, Gillio et al. 1989, Kimura, Toyama et al. 1995), the clinical outlook and quality of life of CN patients was dramatically improved.

Multimerization of G-CSFR occurs by binding to its ligand followed by tyrosine phosphorylation of proteins, and activation of several intracellular

signaling pathways, such as JAK/STAT, phosphatidylinositol-3'-kinase (PI3)/AKT and Ras/Raf/MAPK (mitogen-activated protein kinase) pathways. This results in transcriptional changes that influence proliferation, survival, migration, and differentiation (Touw and van de Geijn 2007).

The canonical Wnt signaling pathway includes the LEF/TCFs (or T-cell factors) family of transcription factors that cooperate with β-catenin to make transcriptional complexes on target genes (van de Wetering, de Lau et al. 2002). It has been shown that LEF-1 may also act independently from ß-catenin (for example, in the TGF-ß and Notch pathways) (Ross and Kadesch 2001, Nawshad and Hay 2003).

It has been shown that LEF-1 mRNA and protein expression was dramatically downregulated or absent in promyelocytic cells of the CN patients compared to healthy individuals (Fig.9) (Skokowa, Cario et al. 2006). Down-regulated LEF-1 expression resulted in decreased LEF-1 target genes, such as cyclin D1, survivin, c-Myc and granulopoietic transcription factor C/EBPa.

It has been shown that C/EBPa is a critical factor in the induction of granulocyte differentiation and regulation between proliferation and differentiation balance of the myeloid precursors (Zhang, Nelson et al. 2002, Ross, Radomska et al. 2004).

LEF-1 dysfunction in CN patients results in C/EBPa downregulation (Skokowa, Cario et al. 2006). In patients with AML disease mutations in C/EBPa gene results in the production of non-functional C/EBPa and disrupted granulocyte differentiation (Skokowa, Cario et al. 2006).

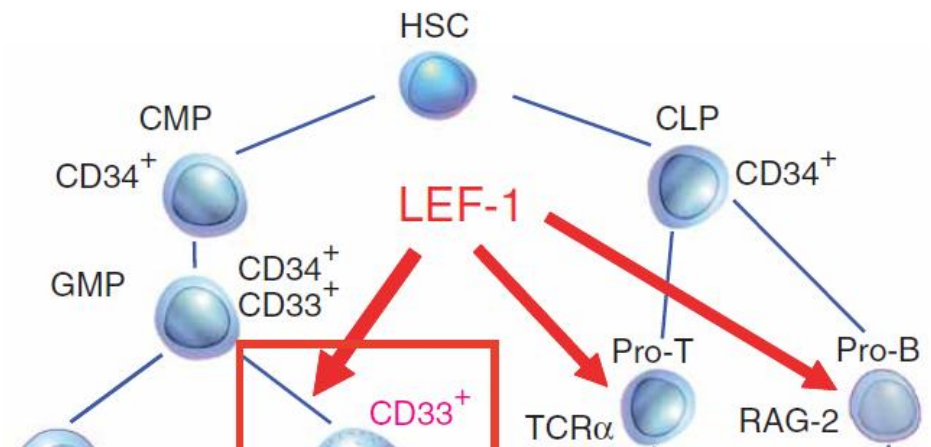


Figure 9. Myelopoietic maturation through the regulation of specific target genes by LEF-1 transcription factor (Source from Skokowa J. et al. 2006).

1.8 G-CSF activates NAMPT in CN

Recently, a new Nicotinamide phosphoribosyltransferase (Nampt) /NAD^+/SIRT1-dependent activation of protein deacetylation during G-CSF–triggered myelopoiesis was identified by our group (Skokowa, Lan et al. 2009). NAMPT is an important activator of NAD^+-dependent sirtuin protein deacetylases that could convert nicotinamide to nicotinamide mononucleotide (Skokowa, Lan et al. 2009). Finally, nicotinamide mononucleotide converts into nicotinamide adenine dinucleotide (NAD^+) (Rongvaux, Shea et al. 2002).

Upon activation of neutrophils and monocytes by IL-6 and TNF-α, NAMPT is up-regulated (Jia, Li et al. 2004, Iqbal and Zaidi 2006, Nowell, Richards et al. 2006). The NAD^+-dependent SIRT1 activation resulted in the activation of transcription factors such as C/EBPα and C/EBPβ, which are important in granulopoiesis, and subsequent up-regulation of G-CSF synthesis and G-

CSFR expression. G-CSF, in turn, enhances NAMPT levels, providing an autoregulatory feedback loop (Fig.10) (Skokowa, Lan et al. 2009). In patients with CN, disfunction C/EBP-α and thus abrogated steady-state granulopoiesis leads to C/EBP-β response in emergency granulopoiesis regulation (Fig.9). To activate C/EBPß, high levels of NAMPT and therefore daily treatment with high dose of G-CSF is required in CN patients (Skokowa, Lan et al. 2009).

In vitro inhibition of NAMPT could increase apoptosis and cell death in CLL cells (Gehrke, Bouchard et al. 2014). NAMPT is over-expressed in patients with AML and inhibition of NAMPT leads to apoptosis and cell death of AML blasts (Skokowa, Lan et al. 2009, Thakur, Dittrich et al. 2013). Importantly, specific pharmacological inhibitors of NAMPT have been designed and are now in phase II-III clinical trials (Montecucco, Cea et al. 2013, Tan, Young et al. 2013, Chini, Guerrico et al. 2014).

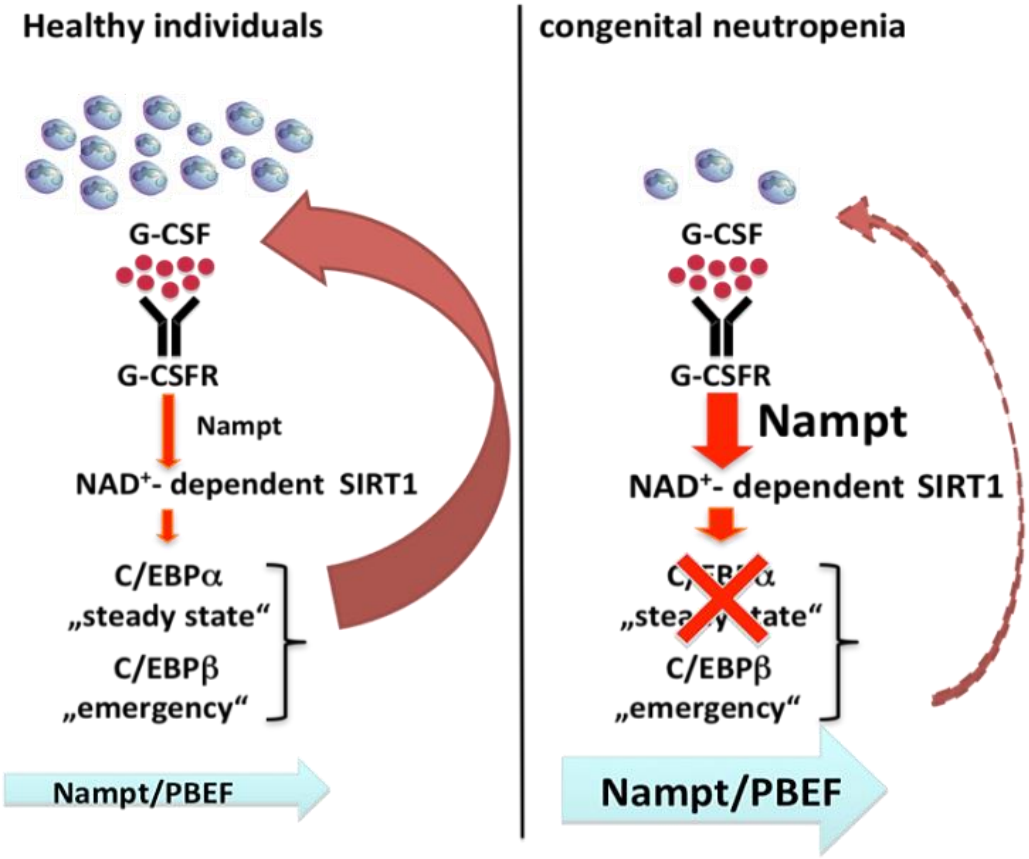


Figure 10. Dose-dependent role of NAMPT in healthy individuals and in congenital neutropenia
G-CSFR activation induces NAMPT synthesis leading to the increase of NAD^+ levels. Activation of NAD^+-dependent SIRT1 results in GCSF and G-CSFR induction of granulopoiesis elevated levels which is C/EBP-α- and C/EBP-β-dependent via an outoregulatory loop (left). In patients with CN, the non-functional C/EBP-α leads to a defective steady-state granulopoiesis. Thus emergency granulopoiesis controlled by C/EBP-β is activated (right). High levels of NAMPT and therefore daily treatment with high doses of G-CSF is required to activate C/EBPß in CN patients (**Source from Skokowa J. et al. 2009).**

1.9 Induced pluripotent stem cells (iPSCs)

Induced Pluripotent Stem Cells (iPSCs) have been generated by reprogramming of adult or mouse embryonic fibroblasts using four defined transcription factors Oct3/4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka 2006). Oct3/4 (Niwa, Miyazaki et al. 2000) and Sox2 are responsible for maintenance of stem cell pluripotency (Avilion, Nicolis et al. 2003). Klf4 is a zinc finger motif-containing transcription factor which is associated with the self- renewal of stem cells (Li, McClintick et al. 2005) and c-Myc is an oncogenic transcription factor, which induces proliferation and cell cycle.

iPSCs are similar to ESCs and they have the stem cell-like expression profile, and the ability to differentiation into three germ layer cells in teratoma formation assays (Takahashi and Yamanaka 2006). iPSCs are a wide source for cell replacement transplantations, disease modeling, regenerative medicine and drug discovery (Wu and Hochedlinger 2011). iPSCs are a model to discover the early and late human development and cellular differentiation (Fig.11) (Lee, Kim et al. 2014). iPSCs have already been

obtained from patients with various diseases, including disorders of hematopoiesis (Giorgetti, Montserrat et al. 2009).

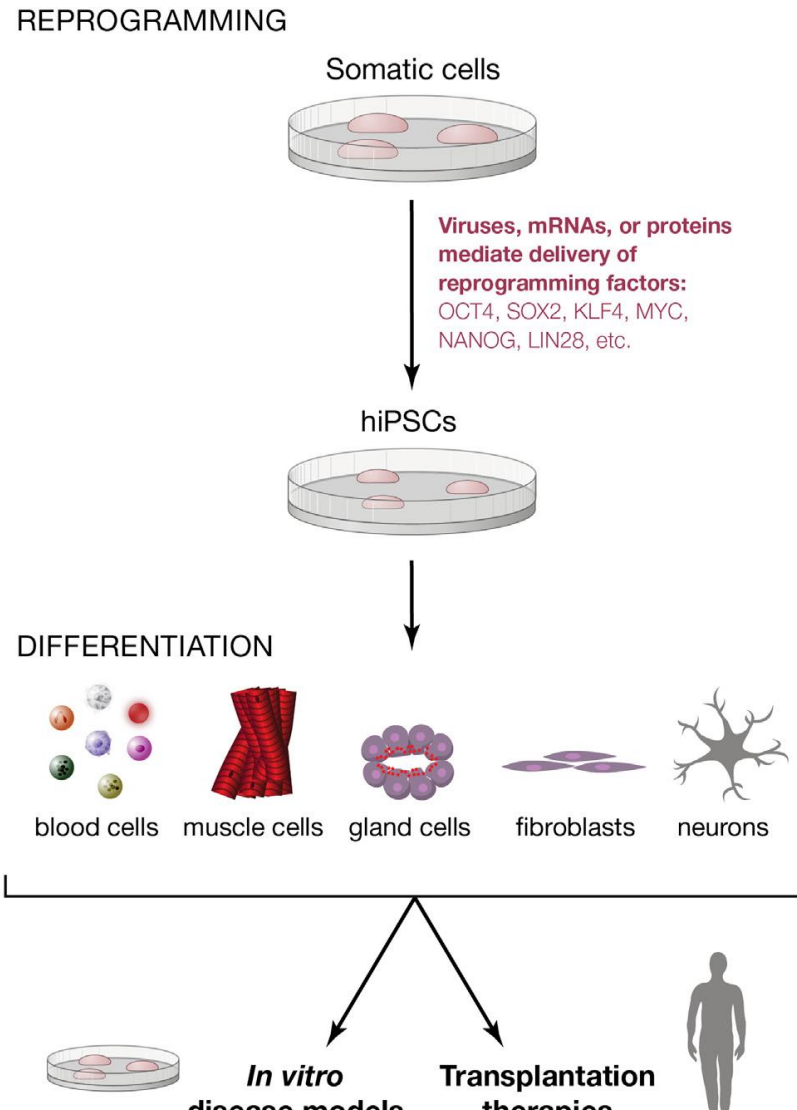


Figure 11. Overview of the iPSCs Technology
patient cells can be reprogrammed into iPSCs using small molecules, microRNAs, and combinations of reprogramming factors. Differentiated cells derived from iPSCs could be used either in transplantation therapies or to model of human diseases **(Source from Hockemeyer D. et al. 2016).**

Non-integrative reprogramming methods for the safety of translational applications are one of the most important issues for reprogramming. For this reason, reprogramming using Sendai virus and DNA-based episomal reprogramming, mRNA or protein expression reprogramming methods are recommended instead of classical lentiviral reprogramming techniques (Diecke, Jung et al. 2014). One of the most authentic safe integration-free reprogramming is a plasmid-mediated method (Yu, Hu et al. 2009).

Plasmids containing EBNA1 gene and OriP DNA sequence from Epstein–Barr virus (EBV) has been one of the most reproducible plasmid systems in a safe iPSCs generation (Chou, Mali et al. 2011). These episomal plasmid features allow the plasmid to replicate inside the cell as circular DNA (episomes) without any integration into the cellular genome as compared to regular plasmids. Safety of this method has been shown by whole genome sequencing of episome-reprogrammed iPSCs. Owing to the epigenetic silencing of foreign vectors, the presence of expressing reprogramming and EBNA1 genes of the episomal vectors is usually transient (Yu, Hu et al. 2009, Chou, Mali et al. 2011, Cheng, Hansen et al. 2012).

Genetic defects in iPSCs can be corrected via targeted editing of the iPSCs genome based on the CRISPR method (Al-Attar, Westra et al. 2011), TALEN and Zinc-finger nucleases (ZFNs) system (Gaj, Gersbach et al. 2013). iPSCs can also spontaneously or directly differentiate into different cell types that might be suitable for stem cell therapy, disease modeling and for drug screening (Fig.12) (Diecke, Jung et al. 2014).

Recent studies using iPSCs generated from CN patients with ELANE mutations demonstrated markedly diminished granulocytic differentiation of these cells *in vitro* (Hiramoto, Ebihara et al. 2013). Interestingly, correction of ELANE mutation in iPSCs from a CN patient using CRISPR/Cas9 technology restored defective granulopoiesis, suggesting the monogenic origin of the ELANE mutation caused congenital neutropenia (Nayak, Trump et al. 2015).

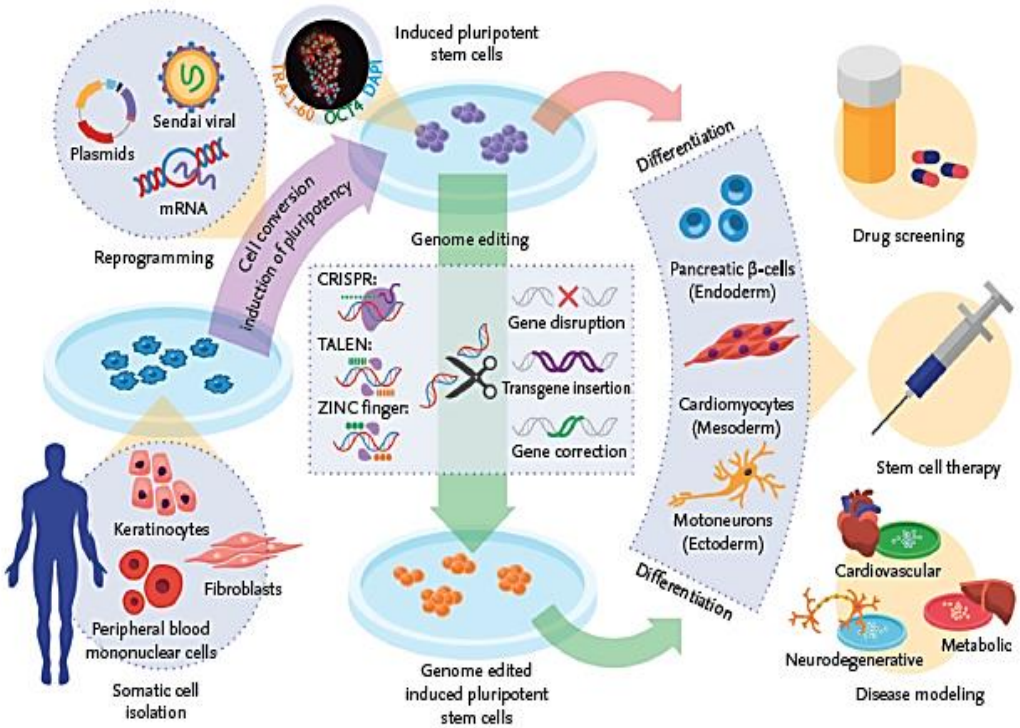


Figure 12. Clinical applications of the patient-specific induced pluripotent stem cells (iPSCs)
Somatic cells are reprogrammed into patient-specific iPSCs using reprogramming factors. Different methods could be used for genome editing of genetic defects in iPSCs. iPSCs with or without genomic modifications could be differentiated into various cells for the reason of disease modeling, stem cell therapy and drug screening **(Source from Dieck S. et al. 2014).**

1.10 Use of iPSCs for CN and CyN disorders

Transplantation of allogeneic HSCs from healthy donors has been used as a potential source for treatment of congenital disorders. Autologous derived HSCs are a valid allogeneic source for *in vitro* genetic defect correction. Homologous recombination (HR) is currently among the advanced ways to restore a genetic defect because the HSC genome is only modified at the position of the mutation and repair by HR creates an identical phenotype as compared to heterozygous or hemizygous cells represent (Fig.13) (van Bekkum and Mikkers 2012).

Due to the requirement of a selection process to identify the corrected cells by HR, this method considered as an inefficient and complicated procedure (van Bekkum and Mikkers 2012). In addition, the cells that have undergone proper HR modifications must be fully expanded to reach the required number for successful transplantation (van Bekkum and Mikkers 2012).

Genetic modifications by viral vectors then HSCs differentiation from iPSCs due to the limited source of endogenous HSCs and contamination with T cells (GVHD) and a lack of HLA-matched engraftment are desired for treatment of congenital disorders (van Bekkum and Mikkers 2012).

Autologous iPSCs are always available and genetic corrections by a viral vector or using homologous recombination is possible, moreover the concerns regarding long-term repopulating of iPSCs derived from HSCs that have not been generated safely have been minimized by episome-reprogrammed iPSCs methods (van Bekkum and Mikkers 2012).

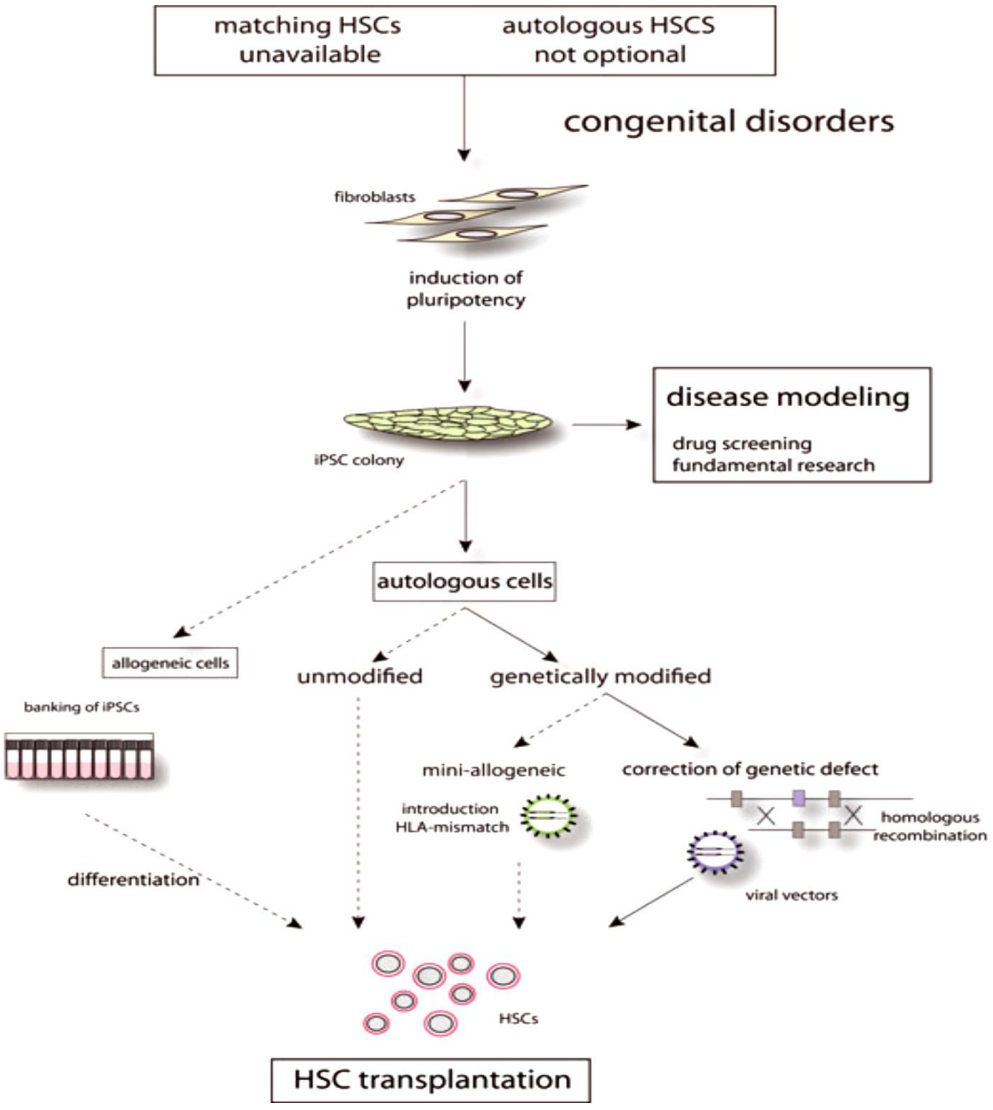

matching HSCs unavailable
autologous HSCS not optional
congenital disorders
fibroblasts
induction of pluripotency
iPSC colony
disease modeling
drug screening
fundamental research
autologous cells
allogeneic cells
unmodified
genetically modified
banking of iPSCs
mini-allogeneic
correction of genetic defect
introduction HLA-mismatch
homologous recombination
viral vectors
differentiation
HSCs
HSC transplantation

Figure 13. iPSCs based opportunities for HSC transplantation
Left: Healthy individuals derived iPSCs that can be used as HLA-specific iPSCs banks. genetically modified by gene therapy strategies and mild GVHD due to the autologous sources can be used to treat hematopoietic disorders.
Right: Patient-specific derived iPSCs can be used for disease modeling, drug screening or for the treatment of congenital disorders **(Source from Van Bekkum, D. W. and H. M. M. Mikkers 2012).**

2 Aims

The main aims of this project were:

To evaluate the role of TPO/c-mpl on the early hematopoietic differentiation of mouse ES cells.

To evaluate the *in vitro* myeloid differentiation of iPSCs derived from the CyN patient harboring a sporadic heterozygous ELANE mutation (c.761C>G p.W241L) in comparison to iPSCs derived from a healthy individual and a CN patient harboring an ELANE mutation (p.C151Y).

3 Material and Methods

3.1 Material

3.1.1 Reagents and chemicals

Name	Catalog Number	Company
Sodium Azide, 25 g	58032-256	Sigma-Aldrich
Na Pyruvat 100 ml	511-003	PAA
DMEM/F12 500 ml	D6421-6X	Sigma-Aldrich
FCS 500 ml	50115	Biochrom
Peni/Strepto 100 ml	A2213	Biochrom
L-Glutamine 100 ml	K0283	Biochrom
Trypan Blue Solution 0,4	15250-061	Life Technologies
TeSR1	5850	Stem Cell Technologies
Lenti-X Concentrator	631232	Clontech
Recombinant Human IL-3 50 µg	203-IL-050	R&D Systems
Recombinant Human FGF-basic 100 µg	100-18C	Peptrotech

Corning Matrigel Growth Factor 10 ml	354230	Corning
PBS (12 x 500 ml)	882126	Biozym
2-Mercaptoethanol 100 ml	M6250	Sigma
Stem Pro Accutase Cell Dissociation Reagent	A11105-01	Life Technologies
PCR Nucleotide Mix 10 x 200 µg	11814362001	Roche Lifescience
Light cycler 480 SYBR Green Master Mix	4707516001	Roche
Ethidium bromide solution 0,025 %	HP47.1	Roth
Triton x-100 Detergent Solution	28314	Pierce
0,25% Trypsin-EDTA	25200-056	Gibco
Knockout SR 500 ml	10828028	Life Technologies
Gel Red	NC9594719	Thermo Scientific
Non-Essencial Amino Acids	11140-050	Gibco
Pre-Seperation Filters	130-041-407	Miltenyi
D-MEM F-12	D6421-6X	Sigma
Insulin-Transferrin-Selenium	41400-045	Gibco
Random Hexamer Primer	S0142	Thermo Scientific
B-27 Supplement (50x)	17504-044	Life Technologies
N-2 Supplement (100x)	17502-048	Life Technologies
mTeSR1 500ml	05850	Stemcell Technologies
MethoCult H4435 Enriched Medium 100 ml	04435	Stemcell Technologies
APEL Medium 100 ml	05210	Stemcell Technologies
MACS Rinsing Solution	130-091-222	Miltenyi
Hematoxylin solution 500 ml	T865.2	Roth
Eosin solution 500 ml	X883.2	Roth
DMEM	FG 0435	Biochrome Millipore
FCS	S0115	Biochrome Millipore

DMEM (1x)	41966029	Gibco
L-Glutamine 200 mM	K0283	Biochrome
Fetal Bovine Serum	S0115	Biochrome
DMSO 100 ml	D2650	Sigma
Y-27632 dihydrochloride 10 mg	1254	R&D Systems
Ethylenediaminetetraacetic acid disodium salt dihydrate	E4884-100G	Sigma
BCIP	B8503	Sigma
NBT	N5514-10TAB	Sigma
Polyvinyl alcohol	P8136-250G	Sigma
DMEM high glucose, pyruvate	41966-052	Thermo Fisher
IMDM, no phenol Red	21056023	Thermo Fisher
Ham's F12 nutrient mixture	31765035	Thermo Fisher
PFHM-II Protein Free Hybridoma Medium	12040077	Thermo Fisher
Chemically Defined Lipid Concentrate	11905031	Thermo Fisher
N, N-Dimethylformamide	D4551	Sigma
Recombinant human FGF-8 100 µg	100-25	PeproTech
Recombinant human BMP-4 50 µg	314-BP-050	R&D Systems
Recombinant human VEGF 165 50 µg	293-VE-050	R&D Systems
Recombinant human SCF/c-Kit Ligand 200 µg	255-SC-200	R&D Systems
Recombinant human TPO 200 µg	288-TP-200	R&D Systems
Recombinant human IL-3 50 µg	203-IL-050	R&D Systems
Recombinant human Noggin 25 µg	1967-NG025	R&D Systems
Recombinant human IL-6 10 µg	206-IL-010	R&D Systems
Recombinant human FLT3 Ligand 25 µg	308-FK-025	R&D Systems

3.1.2 Kits

Name	Catalog Number	Company
Human CD34 Cell Nucleofector Kit	VPA-1003	Lonza
P3 Primary Cell 4D-Nucleofector X Kit L	V4XP-3024	Lonza

3.1.3 Antibodies

Name	Catalog Number	Company
Rabbit anti-human SOX1	22572	Abcam
Rabbit anti-human PAX6	SC11357	Santa-Cruz
Rabbit anti-human G-CSFR	Sc-694	Santa-Cruz
Rabbit anti-human C-kit (C-19)	Sc-168	Santa-Cruz
Texas red goat anti-rabbit	2780	Santa-Cruz
Mouse anti-human FLK-1 (A-3)	SC-6251	Santa-Cruz
Mouse anti-human GATA-2 (CG2-96)	SC-267	Santa-Cruz
Mouse anti-human ß Tubulin III	F8660-100	Sigma-Aldrich
Anti-human CD33 MicroBeads	130-045-501	Miltenyi
Anti-human TRA-1-60 Biotin conjugated	13-8863-82	eBioscience affymetrix
Alexa Fluor 488 Phalloidin	A12379	Life Technologies
Alexa Fluor 647 anti-human CD309 (VEGFR2)	359910	Biolegend
Alexa Fluor 488 anti-human CD309 (VEGFR2)	359913	Biolegend

BV510 mouse anti-human CD45 Antibody	304036	Biolegend
BV421 mouse anti-human CD33 antibody	303416	Biolegend
APC-H7 mouse anti-human CD14 clone My P9	560180	BD Biosciences
PE-Cy7 mouse anti-human CD11b	301322	Biolegend
PE mouse anti-human TRA-1-60	12-8863-80	eBioscience
PE mouse anti-human CD34 (AC136)	130-081-002	Miltenyi
PE mouse IgM Isotype Control	555584	BD Biosciences
PE mouse anti-human CD34	550619	BD Biosciences
PE mouse anti-human CD 11b	557321	BD Biosciences
PE mouse Anti-human CD15 Clone HI98	555402	BD Pharmingen
PE mouse anti-human CD43	560199	BD Biosciences
PE mouse anti-human CD177	564239	BD Biosciences
PE-Cy7 mouse anti-human CD34 Clone 8G12	348811	BD Biosciences
FITC mouse anti-human CD 235a	559943	BD Biosciences
FITC mouse anti-human CD41a	557296	BD Biosciences
FITC mouse anti-human TRA-1-85	FAB3195F	R&D Systems
FITC mouse anti-human CD16	555406	BD Biosciences
FITC mouse anti-human CD33 Antibody	555626	BD Biosciences
FITC goat anti-mouse	AP308F	Chemicon
FITC goat anti-rabbit	AP308F	Merck Millipore
FITC Mouse anti-human CD34 clone 581	555821	BD Biosciences
Propidium Iodide	P3566	Life Technologies
7-AAD	559925	BD Biosciences
DAPI	D1306	Life Technologies

3.1.4 Real time PCR primers

GAPDH	CTGGGCTACACTGAGCACC
	AAGTGGTCGTTGAGGGCAATG
PRE	GAGGAGTTGTGGCCCGTTGT
	TGACAGGTGGTGGCAATGCC
NANOG	CAGGTGTTTGAGGGTAGCTC
	CGGTTCATCATGGTACAGTC
SOX2	TTCACATGTCCCAGCACTACCAGA
	TCACATGTGTGAGAGGGGCAGTGTGC
ABCG2	TACCTGTATAGTGTACTTCAT
	GGTCATGAGAAGTGTTGCTA
DNMT	ATAAGTCGAAGGTGCGTCGT
	GGCAACATCTGAAGCCATTT
PAX6	ACCCATTATCCAGATGTGTTTGCCCGAG
	ATGGTGAAGCTGGGCATAGGCGGCAG
TUB III	TAGACCCCAGCGGCAACTAT
	GTTCCAGGTTCCAAGTCCACC
MYH6	GCCCTTTGACATTCGCACTG
	GGTTTCAGCAATGACCTTGCC
BRACH	CTGGGTACTCCCAATGGGG

	GGTTGGAGAATTGTTCCGATGA
FOXA2	TGGGAGCGGTGAAGATGGAAGGGCAC
	TCATGCCAGCGCCCACGTACGACGAC
AFP	GAATGCTGCAAACTGACCACGCTGGAAC
	TGGCATTCAAGAGGGTTTTCAGTCTGGA
NAMPT	AGGGCTTTGTCATTCCCAGA
	GCCAGCAGTCTCTTGGGAAG
SIRT1	CAACTTGTACGACGAAGAC
	TCATCACCGAACAGAAGG
SIRT2	GGCAGTTCAAGCCAACCATC
	AGCTTAGCGGGTATTCGTGC

3.1.5 Softwares

Blast http://www.ncbi.nlm.nih.gov

flowJo http://www.flowjo.com

3.2 Methods

3.2.1 Transfection of HEK 293T cells for OSKM virus production

HEK-293T cells were split 1 day before transfection with a confluency of 3× 10^6 cells/T75 flasks in 10 ml of HEK medium as shown below. On the day of, reprogramming medium was replaced with 10 ml of TFM medium (T293T medium containing 2% of 1 M HEPES) supplemented with 25 mM chloroquine (25 µM final concentration) and cells were incubated until transfection (Tiscornia, Singer et al. 2006).

HEK-293T medium	**plasmid mixture**
DMEM high Glu medium 4,5 g/l	20 µg OSKM
1% L-Glut	10 µg GagPol
1% P/S	5 µg Rsv-Rev
1% Natrium Pyruvate	6 µg VSVG
10% FBS heat inactivated	2.5 M CaCl2 50 µl
0,1 mM ß-Mercaptoethanol	Water up to 500 µl

Plasmid mixture containing 10 µg of OSKM lentiviral vector (Schambach, Bohne et al. 2006, Warlich, Kuehle et al. 2011) (Fig. 14 a,b), 15 µg of packaging plasmid (10 µg GagPol, 5 µg Rsv-Rev) and 6 µg of VSVG envelope plasmid were mixed with 50 µl of 2.5 M CaCl2 and refilled with water up to 0,5 ml. 0,5 ml of 2x HBS buffer (50 mM HEPES, 1.5 mM Na2HPO4,280 mM NaCl, pH 6,95-7.0) was added to the plasmid mixture while bubbling with pasture pipette and the final cocktail was added into the cell culture flask after 20 min incubation at room temperature.

Supernatant was collected after 24, 36 and 42 hours and filtered (0,45-micrometer pore size). Virus particles were concentrated by lenti-X concentrator according to manufacturer protocol.

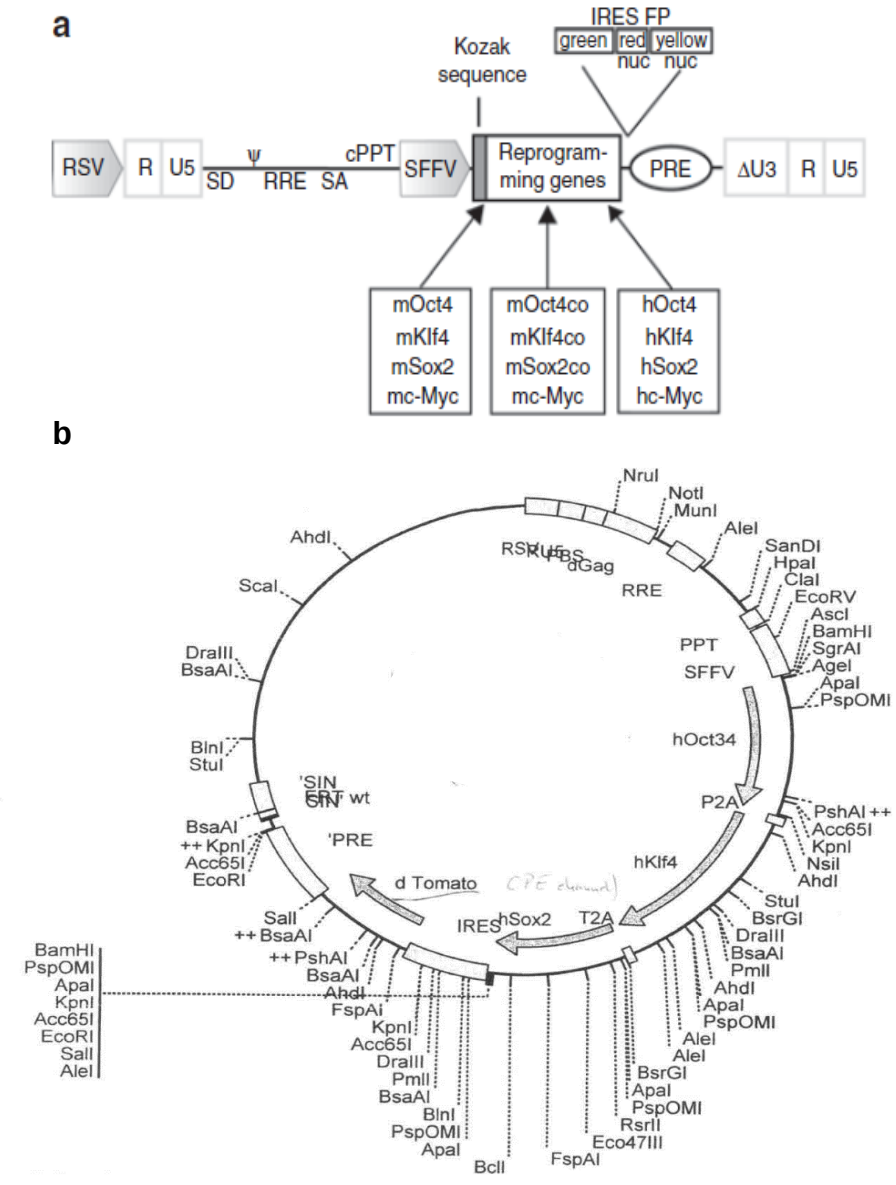


Figure 14. OSKM virus structure
a, Schema of OSKM virus; **b,** OSKM vector map **(Source from Warlich et al. 2011).**

3.2.2 Differentiation of mouse ES cells into hematopoietic lineage

The maintenance of mouse ES cells carried out by using of medium containing DMEM basal medium supplemented with 10% fetal calf serum (FCS) and LIF (1,000 U/mL, Chemicon) on inactivated MEF feeder cells (Pramono, Zahabi et al. 2016).

Hematopoietic differentiation of mouse ES cells (E14) was induced by trypsinization and seeding of the cells into bacterial dishes (2 x 10^5 cells per 3cm dish) in IMDM basal medium supplemented with 10% FCS for generation of embryoid bodies (EBs) from the ES cells. Thrombopoietin (TPO) cytokine was added in 10 ng/mL concentration to the EBs medium and non-treated cytokine group considered as a control group (Pramono, Zahabi et al. 2016).

3.2.3 Reprogramming of $CD34^+$ cells and fibroblasts in iPSCs using lentivirus-based transduction of OSKM plasmids

Plates were coated with Retronectin (Takara) one day before transduction (overnight at 4°C). On the day of reprogramming, Retronectin was aspirated and virus at MOI between 2 to 4 was added to the plates followed by centrifugation for 2h at 2000g, +4°. Supernatant was aspirated and 5x10^5 of $CD34^+$ cells were plated in $CD34^+$ expansion medium centrifuged for 20 min (1200 rpm 32°C). Plates were incubated in a wet chamber at 37°C and $CD34^+$ expansion medium was added every day till day 4-5 of reprogramming. After the appearance of positive transduction signal, cells were split in 3 cm SNL coated plates in $CD34^+$ expansion medium. The iPSCs medium was gradually replaced every day until the appearance of the first iPS colonies (Fig.15).

Fibroblasts were thawed and cultured for 1 week in fibroblast cell culture medium. Fibroblasts were plated in a 6-well plate (5×10^4 cells/ well) one day before reprogramming. OSKM virus at an MOI 2-4 was added to the cells

(Arai, Takada et al. 1999). The addition of the protamine sulfate (4ng/ml) and 50 µg/ml of vitamin C improved the reprogramming procedure. Fibroblast medium was changed the next day. After the appearance of the transduction signal, cells were split (density of 1:3 or 1:6) and transferred on SNL feeders culture. The medium was gradually switched to HESCs medium. Colonies of iPSCs appeared on day 18-30 of culture (depends on the fibroblast quality – doubling time).

In both fibroblasts and $CD34^+$ reprogramming, 4-5 iPSCs colonies were selected for maintenance, characterization and differentiation analysis.

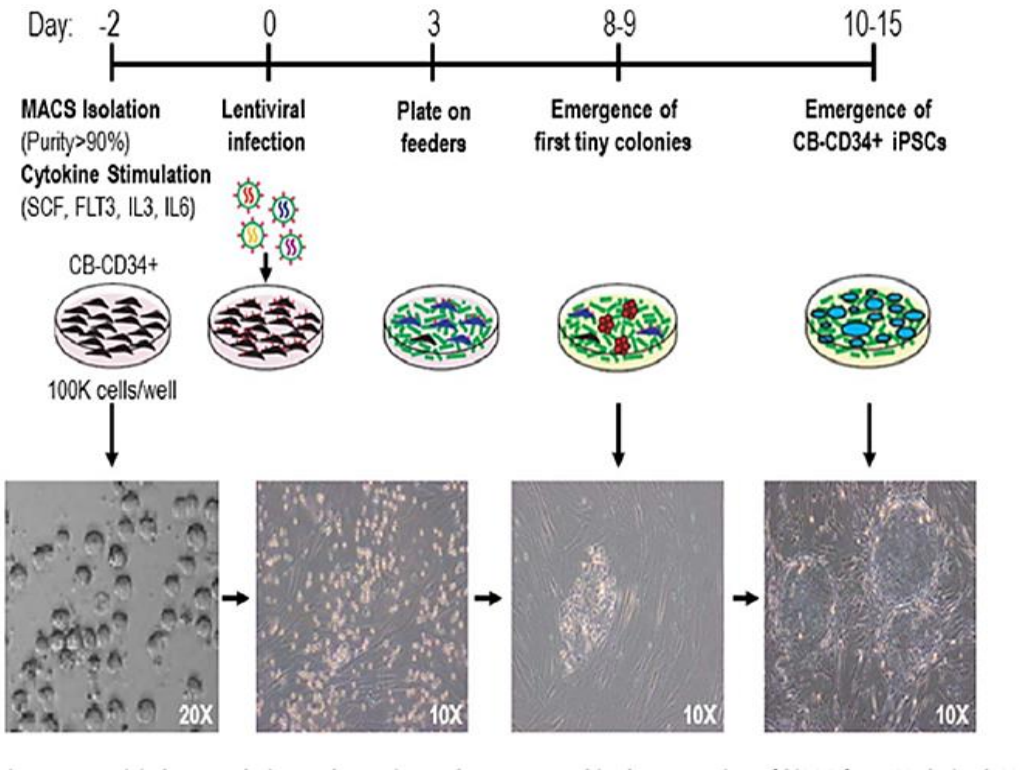


Figure 15. Experimental strategy used in the generation of hiPSCs from $CD34^+$ cells using OSKM lentivector.
Tranceduced $CD34^+$ cells will be plated on feeders three days after tranceduction and by ten days, emergence of first iPS colonies could be detected on the feeder cells **(Source from V Ramos-Mejía et al. 2012).**

3.2.4 hiPSCs maintenance

hiPSCs colonies need to be passaged every 8-10 days. There are different ways for passaging of iPSCs including enzymatic and non-enzymatic methods (Kibschull, Mileikovsky et al. 2011, Beers, Gulbranson et al. 2012).

For passaging of iPSCs, 12-15 colonies were scratched off the dish and transferred into 6 mL hiPSCs maintenance medium. The colonies were then carefully dissociated into smaller aggregates and seeded into the 6 cm dishes with the SNL feeder layer prepared one day before. The medium was changed every day.

3.2.5 Hematopoietic differentiation of iPSCs

iPSCs from healthy individuals, CyN and CN patients were differentiated into hematopoietic cells through embryoid bodies (EBs) based formation, in a feeder-free method (Fig. 16). The cytokine mix was changed in a stepwise manner to recapitulate early embryonic development.

In the first step, which is EB formation, iPSCs colonies after 10-12 days of maintenance were treated with 0.02% of PBS-EDTA for 5-7 minutes. After removing PBS-EDTA, single cells were harvested by gently pipetting in 1-2 ml APEL basal medium. 2×10^4 of cells were split into a 96 well plate containing 100 µl of APEL medium with 20 ng/ml of basic fibroblast growth factor (bFGF) and 10 µM Rock inhibitor (Y-27632). Cells were centrifuged at 1500 rpm for 5 minutes at +5 °C. On day two of the experiment (step 2), 100 µl of APEL medium containing 40 ng/ml of BMP4 and bFGF were added to each well (final concentration of relevant cytokines should rich concentration of 20ng/ml). On day 4 (step 3), EBs were plated on matrigel-coated wells (10 EBs per well) in 1,5 ml APEL medium supplemented with 40 ng/ml VEGF, 50 ng/ml SCF and 50 ng/ml IL-3 final concentrations.

On day 7 of the experiment (step 4), half of the medium was changed using APEL medium supplemented with 50 ng/ml of IL-3 and 50 ng/ml of G-CSF. Every second day a half of the medium was changed and IL-3 and G-CSF were added up to the final concentrations of 50 ng/ml. After 14 days of culture, EBs started to release floating hematopoietic cells. Floating cells were collected for FACS analysis at defined time points.

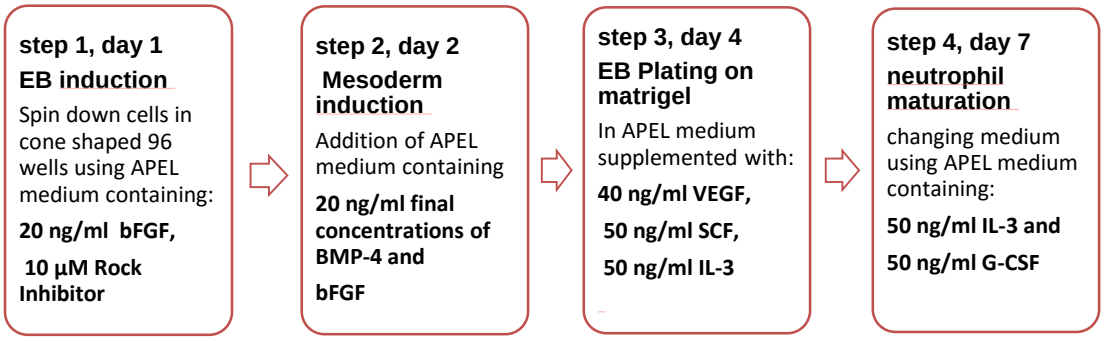


Figure 16. Schema of differentiation protocol of hiPSCs into mature neutrophils.

3.2.6 Flow Cytometry

iPSCs were characterized using evaluation of SSEA4 and Tra1-60 as surface pluripotency markers of iPSCs (Draper, Pigott et al. 2002) by FACS.

Briefly, iPSCs on day 7-8 of culture were harvested after 7 minutes of treatment with acutase and washed with PBS (400g for 5min). For each sample, 1×10^4- 2×10^4 cells were resuspended in 50µl of FACS buffer (PBS with 1% BSA) and were incubated in related antibody for 20 minutes. The anti-human Tra1-85 antibody was used as a detector marker between human and mouse cells. Finally, cells were washed and resuspended in 200 µl of

FACS buffer containing 1 µl/ml of DAPI as a nuclear marker. Measurements were performed on FACS Canto II system.

The early and late antibody panel were used for detection of hematopoietic differentiation at different time points (shown below). The phenotype of cells was studied using monoclonal antibodies against the appropriate antigens. The results were obtained using multicolor FACS using compensation controls with compensation beads. Analysis of the experiments was performed using the FlowJo software package (Version 10.0.8r1, Tree Star, and Ashland, OR, USA).

Early stage of hematopoietic differentiation Antibody panel	**Late stage of hematopoietic differentiation Antibody panel**
CD34 PE-cy7	CD11b PE-cy7
CD235a FITC	CD15 PE
CD41a FITC	CD16 FITC
CD45 BV510	CD45 BV510
7AAD nuclear marker	7AAD nuclear marker

3.2.7 Alkaline phosphatase activity assay

iPSCs were tested for alkaline phosphatase enzyme activity (O'Connor, Kardel et al. 2008)**.** Using NBT/BCIP staining protocol briefly, iPS plates were washed with PBS and fixed using 2 ml of 4% PFA per 6cm plate for 2 min then washed again with PBS. NBT/BCIP (15ml containing: 550 µl NBT, 52,5 µl BCIP and fill up to 15 ml NTM buffer (0,1M Tris pH 9,5 (121,14 g/mol), 0,1M NaCl (58,44 g/mol), 0,05M MgCl (95,21 g/mol) made and stored in 4°*C* staining dye was added to 6 cm plates for 20 min at RT in the dark place. After incubation, each plate was washed with PBS and stored at +4°*C.*

3.2.8 Quantitative real-time RT-PCR (qRT-PCR)

Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) was performed by QIAGEN RNeasy Mini Kit (Qiagen), as described by the manufacturer protocol. The cDNA was synthesized using random hexamer primers and the mRNA expression was measured using SYBR Green qPCR Kit (Qiagen). After normalization to ß-actin or GAPDH or to RSP9, the target genes mRNA expression were expressed as fold change and were represented as arbitrary units (AU) or fold change.

3.2.9 Western blot analysis

Cell lysates were obtained through lysis of the cells in Laemmli buffer and loading buffer. Proteins were separated on 10% SDS-PAGE and the binding was detected by ECL.

3.2.10 DUOLINK using *in situ* proximity ligation (PLA) assay

ES cells cultured with 10 ng/ml of TPO treatment for 1,5 days, used for Duolink In Situ Proximity Ligation (PLA) Assay (Olink Bioscience, Uppsala, Sweden). In this experiment treated cells were detected for Endogenous expression of HIF-1α protein, as described by the manufacturer.

3.2.11 Statistical analysis

Differences in mean values between groups were analyzed using two-sided, unpaired Student's t-tests using the Prism statistical package or the SPSS V. 9.0 statistical package.

4 Results

4.1 Evaluation of the mechanisms operating during the early stages of hematopoiesis using a mouse ES cells model

4.1.1 TPO-induced hematopoietic differentiation evaluation of mouse ES cells

Hematopoietic differentiation of the E14 mouse ES cell line has been performed by the embryoid bodies (EBs) formation experiment. For this reason generated EBs were plated in the medium containing of 10 ng/mL TPO and medium without TPO considered as a control group (Fig. 17a) (Pramono, Zahabi et al. 2016).

mRNA expression of Flk-1 (early hematopoietic and vascular progenitors marker) was induced in EBs after one-day treatment with TPO and reached a maximum before two days. After three days of treatment with TPO, the Flk-1 expression was decreased as shown in the figure 17b (Pramono, Zahabi et al. 2016).

Interestingly, expression of the VEGF-A mRNA (vascular marker) was highly increased after TPO treatment of ES cells for one day (Fig. 17c). Mesoderm and hematopoietic differentiation after TPO treatment were confirmed through elevation of mRNA levels of brachyury gene as a responsible candidate for mesoderm formation and Runx1 for hematopoietic development (Fig. 17d) (Pramono, Zahabi et al. 2016).

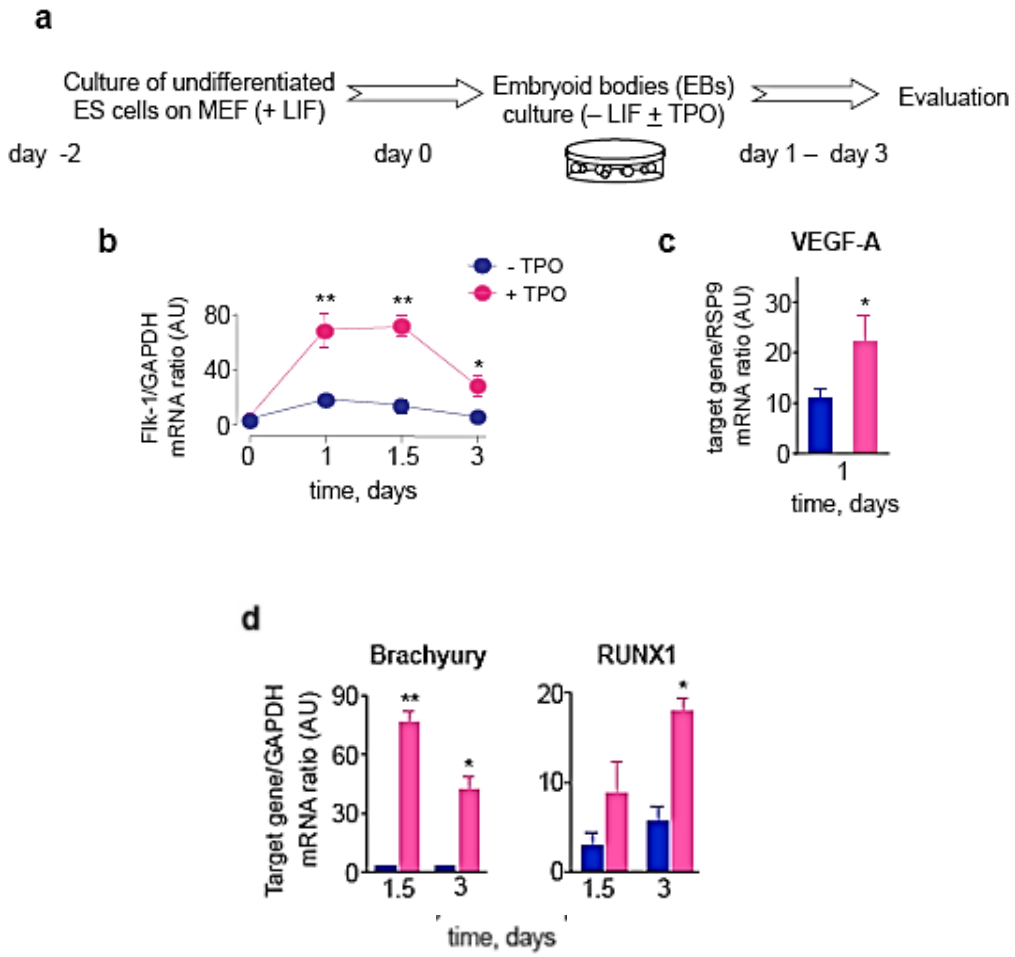


Figure 17. Induction of hematopoietic differentiation of mouse ES cells by TPO

a, schema of hematopoietic differentiation of the E14 mouse ES cell line. **b**, **c** evaluation of **the** mRNA expression for Flk-1 and VEGF-A in the TPO treatment or non-treatment groups is normalized to GAPDH or RSP9. Data is represented as arbitrary units (AU) and means ± s.d. Samples derived from four individual experiments each in triplicates (*, *P*< 0.05; **, *P*< 0.01); **d**, mRNA expression of Brachury and Runx1 genes quantified and normalized to GAPDH and is presented as arbitrary units (AU). data are derived from three individual experiments each in triplicates and is represented by means ± s.d. (*, *P*< 0.05; **, *P*< 0.01) **(Source from Pramono, Zahabi et al. 2016).**

4.1.2 Up-regulation of c-mpl receptor by TPO in mouse EBs

Untreated cells represented a low level of TPO receptor c-mpl mRNA and protein as compared to c-mpl mRNA and protein increased levels in EBs after treatment with TPO (Fig. 18a,b) (Pramono, Zahabi et al. 2016).

This finding shows that TPO-receptor c-mpl can be up-regulated after TPO treatment of ES cells.

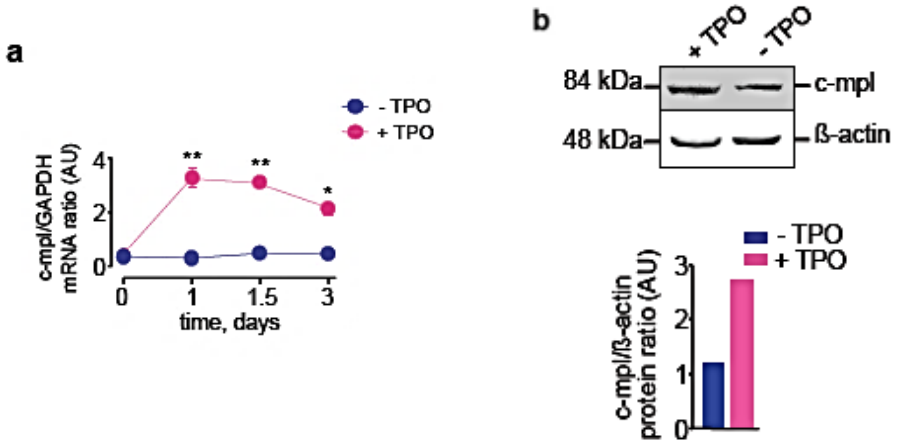


Figure 18. Up-regulation of TPO-receptor, c-mpl after treatment of mouse ES cells by TPO
a, c-mpl mRNA expression levels is normalized to GAPDH and is represented as arbitrary units (AU). data represent means ± s.d. and are derived from four individual experiments each in triplicates (*, $P< 0.05$; **, $P< 0.01$); **b,** graphic bars of the ratio between the optical density of protein bands of c-mpl protein expression to the house-keeping protein, β-actin in western blot analysis 1.5 day after treatment with TPO **(Source from Pramono, Zahabi et al. 2016).**

4.1.3 Activation of BMP4 autoregulation in mouse ES cells by TPO

During the first days of treatment with 10 ng/ml TPO, we found that Flk-1 expression was highly induced in EB culture. Therefore, downstream of

TPO/c-mpl signaling pathway, were evaluated to investigate the role of TPO in the early hematopoietic differentiation of ES cells, in the next step (Pramono, Zahabi et al. 2016).

The mRNA expression profiles of BMP4 and the BMP4 target genes in the Flk-1 expressing or non-expressing mouse ES cells were evaluated. We found a highly up-regulation in the mRNA levels of these genes in Flk-1 expressing but not in Flk-1 non-expressing cells upon TPO treatment (Fig. 19) (Pramono, Zahabi et al. 2016). These data confirms the role of TPO in the development of mouse ES cells derived early hematopoiesis.

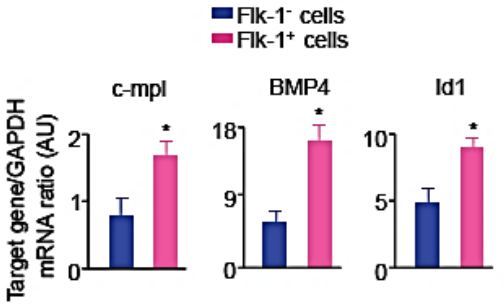


Figure 19. TPO upregulates the BMP4 during hematopoietic differentiation of mouse ES cells
a, mRNA expression levels of BMP4, c-mpl and Id1 in Flk-1 expressing cells as compared to Flk-1 non-expressing cell was normalized to GAPDH. Data is presented as arbitrary units (AU) and are derived from two individual experiments each in triplicates and is represented by means ± s.d. **(Source from Pramono, Zahabi et al. 2016).**

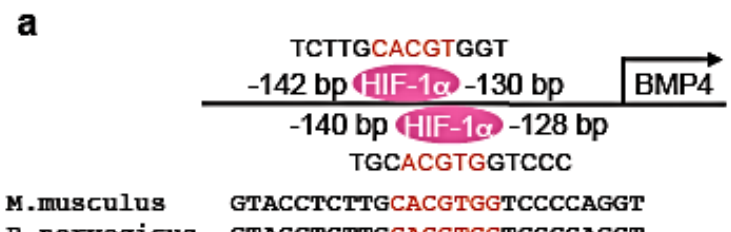
4.1.4 The binding of HIF-1α to the BMP4 gene promoter is induced by TPO in mouse ES cells

In silico, functional analysis of the BMP4 gene promoter was performed in order to understand the molecular mechanisms of TPO treatment on BMP4 synthesis (Fig.20).

Two putative highly conserved binding sites (at the positions –142 bp to –130 bp and –140 bp to –128 bp) for HIF-1α were identified (Fig. 20a) (Pramono, Zahabi et al. 2016). This data confirmed the enhancement of HIF-1α protein stability by TPO in primitive hematopoietic cells.

In addition, endogenous HIF-1α protein expression after 1,5 days treatment of ES cells with 10 ng/ml TPO was detected using DUOLINK based on in situ proximity ligation (PLA), which is a proximity ligation assay. DUOLINK data confirmed the enhancement of HIF-1α protein by TPO treatment of the EC cells as compared to non-treated control (Fig. 20b).

Altogether, this data indicates that TPO regulates BMP4 signaling via HIF-1α transcription factor and induces stabilization and binding of HIF-1α to the BMP4 gene promoter (Pramono, Zahabi et al. 2016). Induction and secretion of BMP4 in an autoregulation pattern induce the upregulation of BMP4 downstream signaling target genes and induction of early hematopoietic differentiation of ES cells (Fig. 20c) (Pramono, Zahabi et al. 2016).



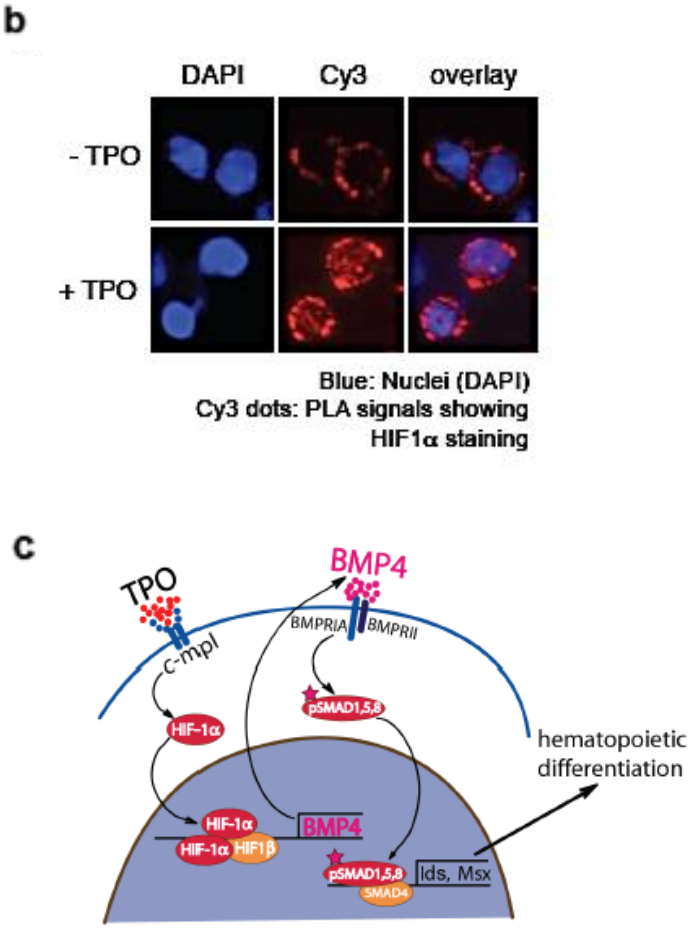


Figure 20. Autocrine synthesis of BMP4 triggered by TPO and BMP4 downstream activation through HIF-1α to the BMP4 gene promoter binding

a, Alignment of the BMP4 upstream promoter with the HIF-1α binding region in the *in silico* experiment marked in red; **b**, I*n situ* PLAs using anti-HIF-1α mouse monoclonal Ab were performed on cytospin slides prepared after 1.5 days of TPO treatment of EBs. Untreated ES cells considered as control group **e**, Schema of TPO-dependent BMP4 regulation: stabilization of HIF-1α protein by TPO and binding of HIF-1α to the BMP4 gene promoter could activate the autocrine synthesis of BMP4 and its target genes that resultes in mouse ES cells derived early hematopoietic differentiation **(Source from Pramono, Zahabi et al. 2016).**

4.2 Evaluation of the mechanisms operating during late stages of hematopoiesis using iPSCs model

4.2.1 Generation of iPSCs using Amaxa 4D nucleofector system

Episomal iPSCs were generated using Amaxa nucleofector 4D (EN-150 program) from either $CD34^+$ or fibroblasts of healthy individuals (Fig. 21a). Generated iPSCs colonies were tested for pluripotency markers such as alkaline phosphatase activity (Fig. 21b) and surface expression of SSEA-4 and Tra1-60 (Fig. 21c,d).

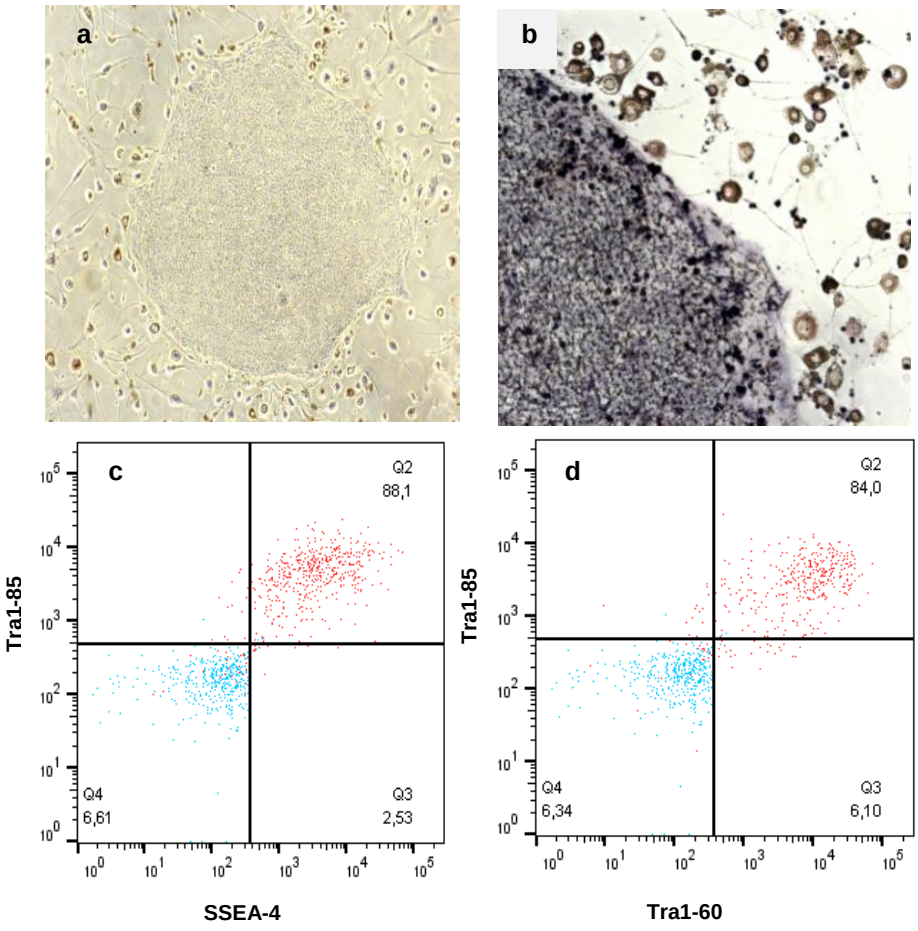


Figure 21. Generation of iPSCs using Amaxa nucleofector 4D
a, representative image of iPSCs derived from healthy human $CD34^+$ cells using AMAXA nucleofection; **b,** representative image of alkhaline phosfatase activity assay of iPSCs derived from healthy human $CD34^+$ cells; **c,** SSEA-4 (PE)-Tra 1-85 (FITC) or **d,** Tra1-60 (PE)-Tra 1-85 (FITC) co-expression on iPSCs derived from healthy human CD34+ cells.

4.2.2 Generation of iPSCs from CN and CyN patients carrying ELANE mutations using OSKM lentivirus

Fibroblasts or CD34$^+$ cells (Fig. 22a,d, respectively) from CyN or CN patients harboring ELANE mutations as well as from healthy individuals were reprogrammed using OSKM lentivirus (Schambach, Bohne et al. 2006) into iPSCs. Three to five days after transduction of cells using OSKM lentivirus, the red signal was appeared in transfected cells (Fig. 22b,e), iPS colonies were generated within 8-10 days after plating on SNL feeder cells (Fig. 22c,f), which was 2-3 times faster than episomal based reprogramming. In both fibroblasts and CD34$^+$ reprogramming conditions, 4-5 of the best iPS colonies were selected for further characterization and differentiation. The efficiency of OSKM lentivirus-based reprogramming was 30-40% higher than that of the episomal reprogramming method.

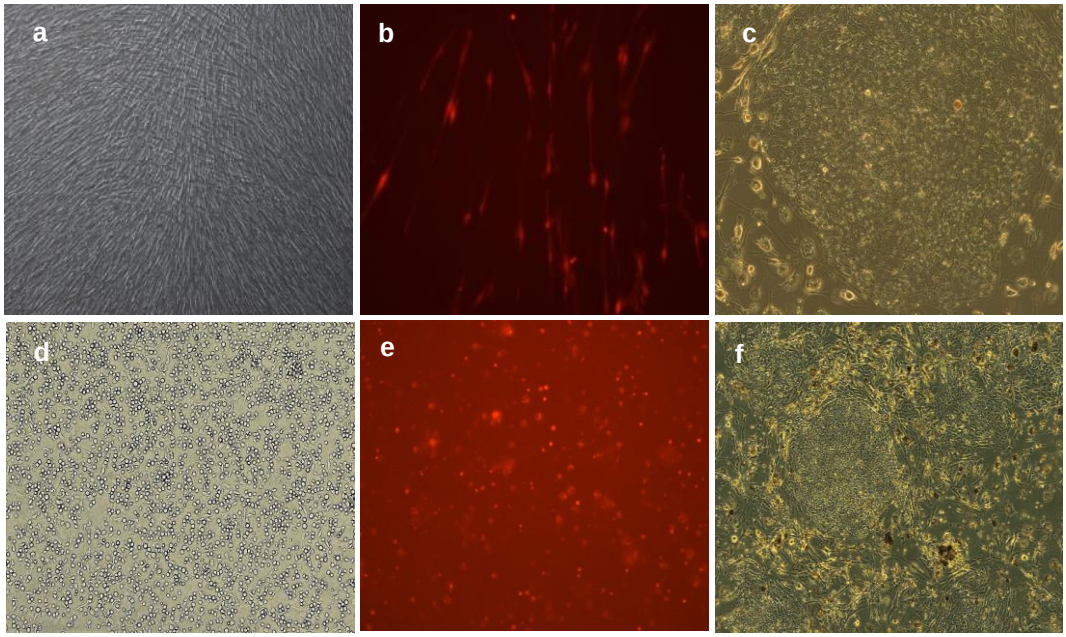


Figure 22. Generation of iPSCs using OSKM lenti virus
a, representative image of human healthy fibroblast used for generation of iPSCs **b,** representative image of human healthy fibroblast transduced by OSKM lenti virus on day 5 of culture **c,** representative image of hiPSCs generated from fibroblasts by OSKM transduction **d,** representative image of human healthy CD34$^+$cells used for generation of iPSCs **e,** representative image of human healthy CD34$^+$cells transduced at day 5 using OSKM lenti virus **f,** representative image of iPSCs generated by OSKM transduction.

4.2.3 mRNA expression levels of pluripotency markers in iPSCs

mRNA expression levels of the iPSCs pluripotency genes such as NANOG and ABCG2 were evaluated on day 7-8 of iPSCs culture using qRT-PCR. As expected, mRNA expression levels of NANOG and ABCG2 were markedly increased in iPSCs derived from cells of CN and CyN patients as well as from a healthy individual, as compared to the expression levels of these markers in the original cells (Fig. 23a-d).

We also evaluated silencing of the OSKM lentivirus after iPSCs generation using primers that amplify the PRE region of the vector. We compared PRE expression in untransduced fibroblasts and CD34+ cells, in these cells on day 5 after transduction and in undifferentiated iPSCs (Fig. 23e,f). Indeed, we found the highest expression levels of PRE in CD34+ cells and in fibroblasts on day 5 after transduction, which were markedly declined in iPSCs.

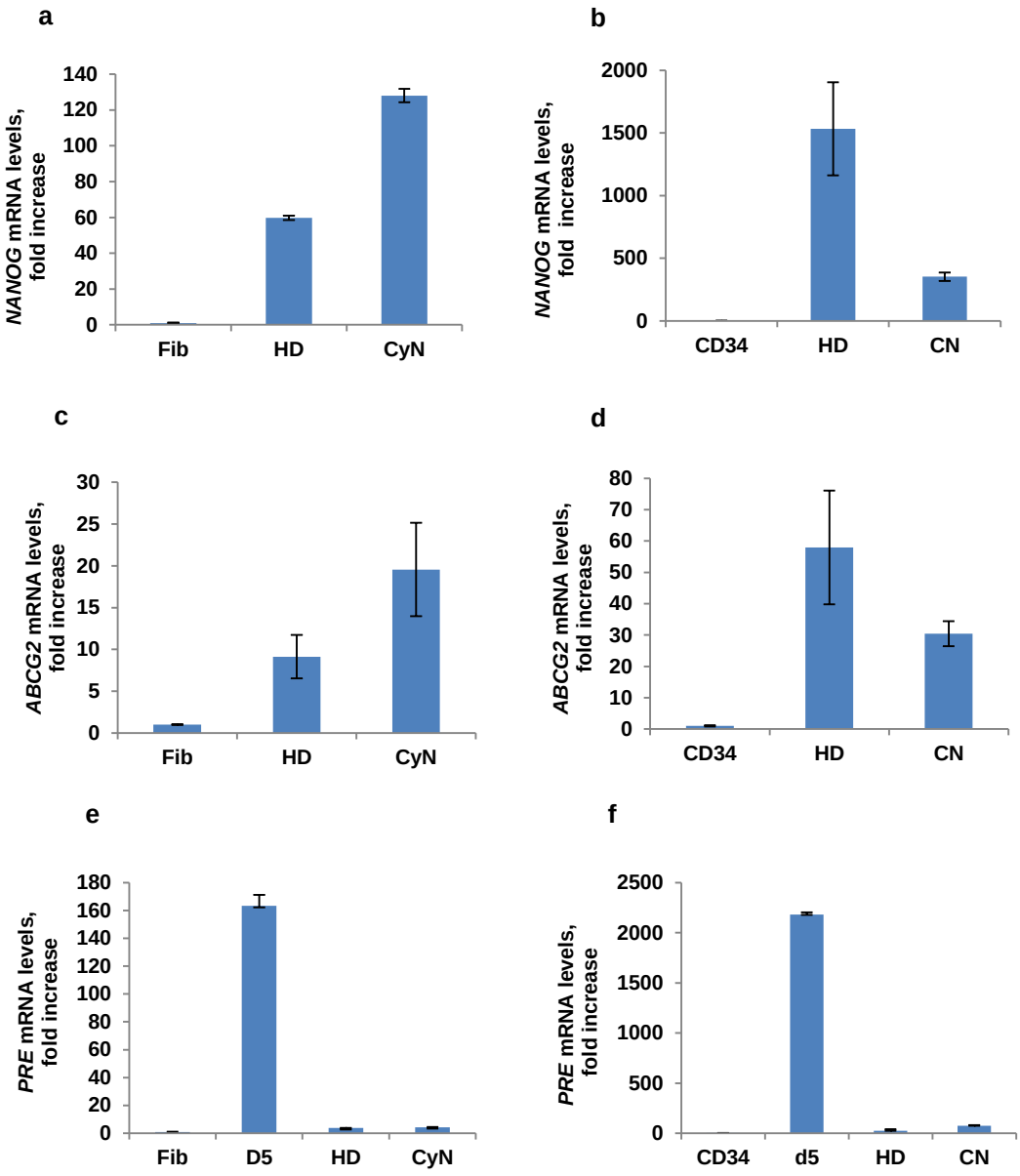


Figure 23. Pluripotency gene expression analysis

mRNA expression levels of the indicated genes in the undifferentiated iPSCs on day 7 of culture was assessed using qRT-PCR. **a-b** Nanog mRNA levels, fold increase of CyN compare to healthy individual and fibroblasts and CN compare to healthy individual and $CD34^+$ cells **c-d** *ABCG2* mRNA levels, fold increase of CyN compare to healthy individual and fibroblasts and CN compare to healthy individual and $CD34^+$ cells **e-f** *PRE* mRNA levels, fold increase of CyN at day 5 of reprogramming compare to healthy individual and fibroblasts or *PRE* mRNA levels, fold increase of CN at day 5 of reprogramming compare to healthy individual and $CD34^+$ cells. Fold change in fibroblast or $CD34^+$ undifferentiated cells was taken as 1. Data are means ± s.d. and are derived from three independent experiments, each in triplicate. By analysis of PRE expression in generated iPSCs, OSKM vector silencing monitored, as compared to day 5 of reprogramming. Fib: fibroblasts; D5: day 5 of reprogramming; H9: Standard human embryonic stem cell line.

4.2.4 Surface expression level of pluripotency markers in iPSCs

iPSCs were analyzed for SSEA4 and Tra1-60 surface marker expression on day 7-10 of culture. We found that more than 85 % of iPSCs generated from the healthy individual (Fig.24a), CyN patient (Fig.24b) or CN patient (Fig.24c) expressed SSEA4 and Tra 1-60 surface markers.

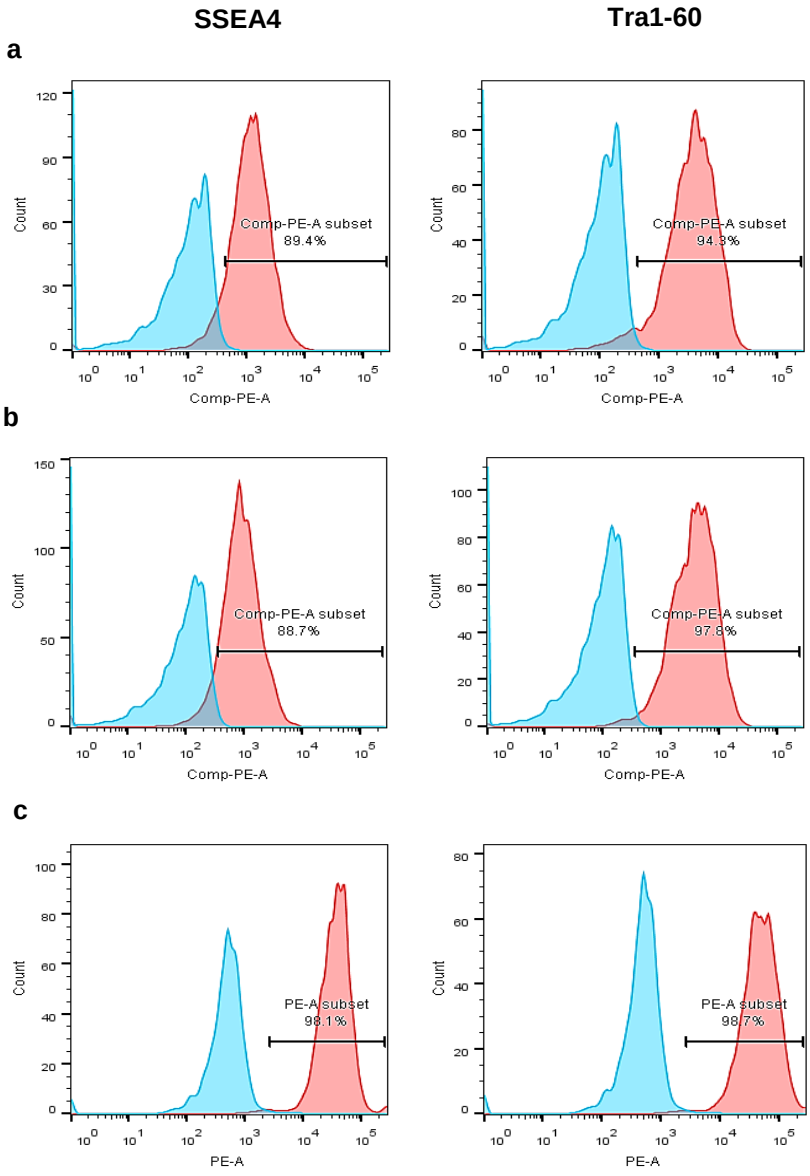


Figure 24. Representative histogram plots of SSEA4 and Tra1-60 surface marker expression on iPSCs measured by FACS

blue: isotype control, pink SSEA4 and Tra1-60 staining **a,** healthy individual SSEA4 and Tra1-60 surface marker expression **b,** SSEA4 and Tra1-60 surface marker expression on CyN derived iPSCs **c,** SSEA4 and Tra1-60 surface marker expression on CN patient derived iPSCs.

4.2.5 Alkaline phosphatase staining

Alkaline phosphatase activity test of iPSCs revealed that all iPSCs colonies generated by us were positive for this stemness indicator marker (Fig. 25a,b,c).

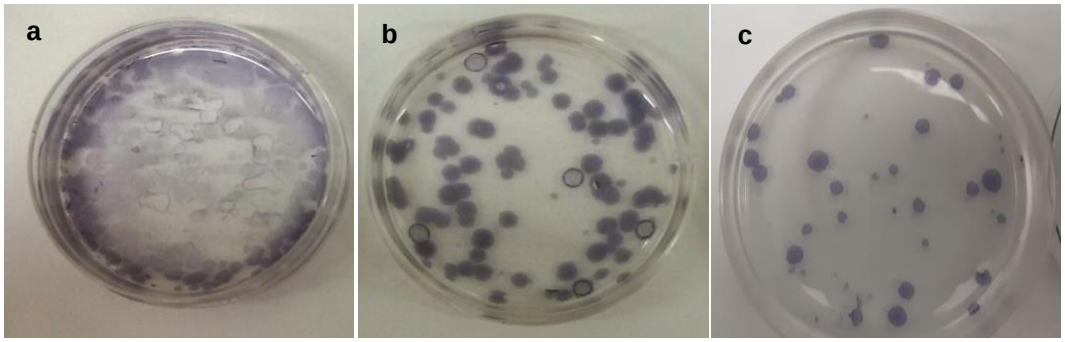


Figure 25. Alkaline phosphatase activity assay
a,b,c representative images of alkaline phosphatase staining of healthy individual derived iPSCs (a) CyN derived iPSCs (b) and CN derived iPSCs (c).

4.2.6 Verification of Germ Layer Markers in Differentiated Pluripotent Stem cells

Immunofluorescence staining of markers characteristic for three different germ layers (Sox17 – marker of early and defined endoderm; Tubulin III – marker of ectoderm; desmin – marker of mesoderm) after three weeks of spontaneous differentiation *via* embryoid bodies of healthy individual, CyN and CN derived iPSCs demonstrated that iPSCs clones are able to differentiate into three germ layers (Fig. 26).

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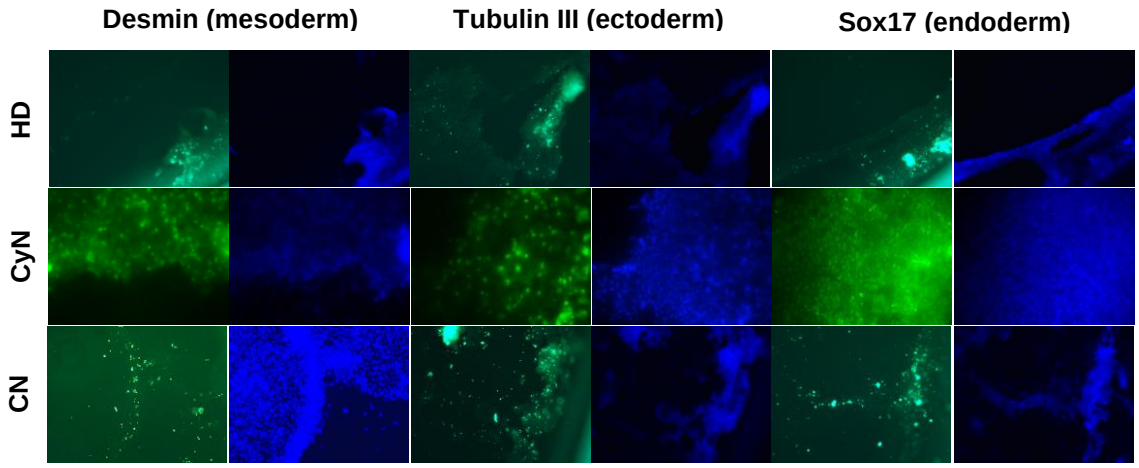


Figure 26. Spontaneous differentiation of iPSCs into three germ layers
Representative images of Immunocytochemistry staining of TUBIII expression as ectodermal marker and Sox17 as endodermal marker and Desmin as mesodermal marker for helthy individual, CyN and CN derived iPSCs. Nuclei (blue) were counterstained with 4,6-diamidino-2-phenylindole (DAPI).

4.2.7 Establishment of the EB-based protocol for myeloid differentiation of human iPSCs

We first established the EB-based system of myeloid differentiation of iPSCs in our lab using iPSCs clones generated from fibroblasts of a healthy donor (Ng, Davis et al. 2008, Lachmann, Happle et al. 2014). EBs were formed by spin-down of single iPSCs in the cone shaped 96 well plates (Fig. 27a,b). EBs were cultured in APEL medium with a step-wise addition of cytokine mixtures, as described in the Material and Methods section. We found that floating hematopoietic cells appeared on day 13 - 15 of EB culture. Floating cells were collected every 3-4 days and the system continuously generated floating cells for up to 35 days (Fig. 27c). Cell morphology of cytospin preparations of floating cells from day 18 or 20 of culture clearly demonstrated the presence of mature granulocytes (Fig. 27d).

FACS analysis of floating cells collected on day 13-15 of culture revealed only one population of cells that consists of mature granulocytes and not yet completely differentiated myeloid cells (Fig. 27e). Interestingly, FACS

analysis of floating cells collected on day 18-20 of culture revealed two cell populations (Fig. 27f). Cell sorting and morphology examination of these two cell populations demonstrated that the upper population (P2) consisted of dying myeloid cells that don't express neutrophil markers such as CD15 or CD16 and the lower population (P1) contained living myeloid cells with high expression of mature neutrophil markers. Therefore, for further analysis of granulocytic differentiation we used the lower (P1) population of myeloid cells.

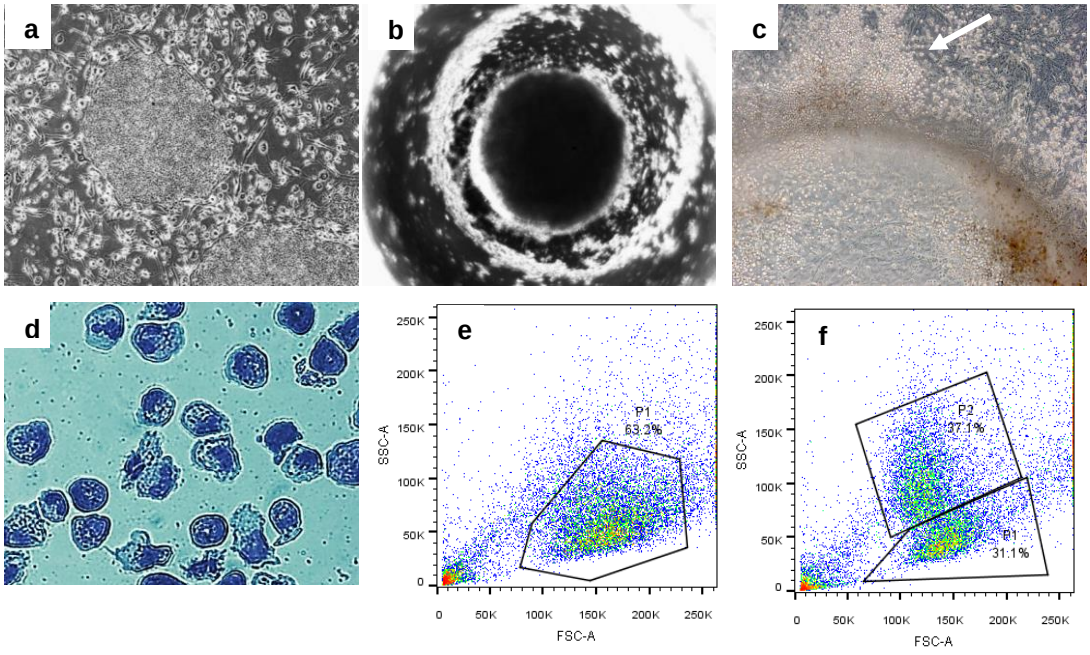


Figure 27. Granulocytic differentiation of iPSCs
a, iPSCs colony on day 10 **b,** spin down EB formation at day 1 **c,** appearance of hematopoietic colonies from day 15 of hematopoietic differentiation culture **d,** Wright-Giemsa–stained images of cytospin preparations from iPSCs derived hematopoietic **e,** FACS analysis of floating cells collected on day 13-15 of culture revealed only 1 population of hematopoietic cells **f,** FACS analysis of floating cells collected on day 18-20 of culture revealed 2 populations of graulocytes.

4.2.8 Evaluation of NAMPT and G-CSF on hematopoietic differentiation of hiPSCs

Previously our group demonstrated that nicotinamide phosphoribosyltransferase (NAMPT) is essential for the G-CSF-induced myeloid differentiation of human hematopoietic $CD34^+$ cells via an NAD^+-sirtuin-1-dependent pathway (Skokowa et al., 2009). Therefore, we evaluated the role of G-CSF and NAMPT on the myeloid differentiation of iPSCs using our EB-based differentiation system. We used different culture conditions, as described in Figure 29. Briefly, on day 4 of differentiation (step 3), in addition to VEGF and SCF, further cytokines were added to the EB culture, as followed: (1) medium alone (control); (2) IL-3 (50 ng/ml) alone (standard conditions); (3) NAMPT (50 ng/ml) alone; (4) G-CSF (50 ng/ml) alone; (5) IL-3+G-CSF or (6) IL-3+NAMPT. Myeloid differentiation was assessed on day 13 and 20 of culture by FACS (Fig. 28).

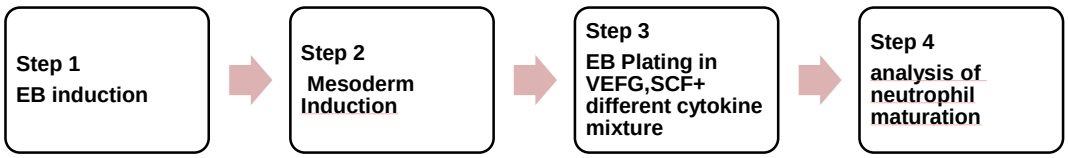
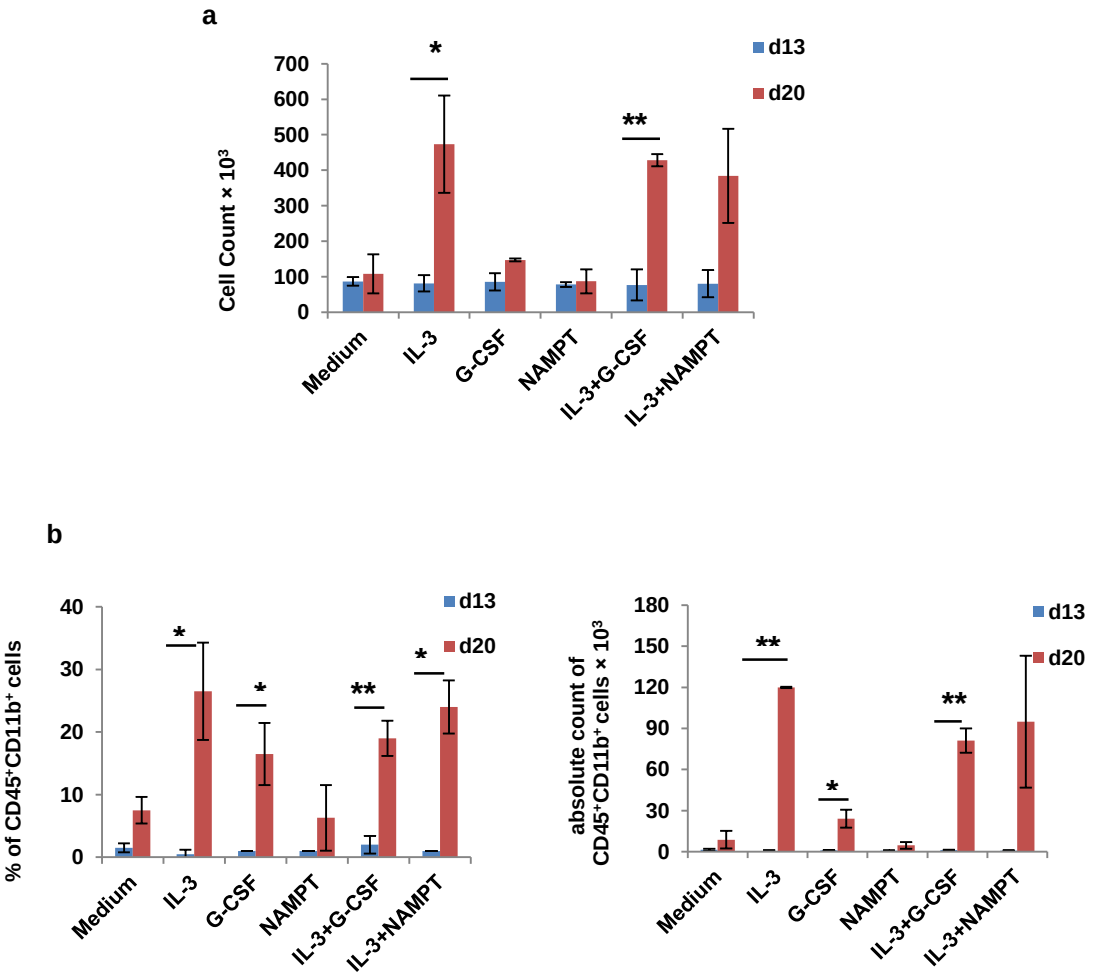


Figure 28. Schema of hematopoietic differentiation of iPSCs to evaluate the effect of NAMPT, G-CSF and IL-3
Different cytokine mixture evaluated in presence or absence of IL-3 as the main hematopoietic differentiation cytokine to test whether NAMPT or G-CSF has any effect on proliferation or maturation of hematopoietic cells.

We found that cell count was dramatically decreased when we omitted IL-3 from the culture (Fig. 29a). We observed an increase in surface marker expression by day 20 in all groups, but absolute cell count results revealed that IL-3 has the most important role on proliferation and myeloid differentiation of iPSCs (Fig. 29b,c,d)

We found that G-CSF alone was able to induce differentiation of $CD45^+CD11b^+$, $CD45^+CD15^+$ and $CD45^+CD16^+$ myelo-granulocytic cells (Fig. 30b-d) on day 13 and day 20 of culture, as compared to the control group (Fig. 29c). NAMPT alone also slightly induced the generation of $CD45^+CD15^+$ and $CD45^+CD16^+$ myelo-granulocytic cells, but to a lesser extent as compared to the IL-3 and G-CSF groups. The addition of NAMPT to IL-3 did not have any synergistic effects on IL-3 and addition of G-CSF to IL-3 slightly enhanced myelo-granulocytic differentiation of iPSCs (Fig. 29).

Altogether, we concluded that IL-3+G-CSF is the best condition to improve our neutrophil maturation protocol for further experiments applicable for CN patients derived iPSCs lines.



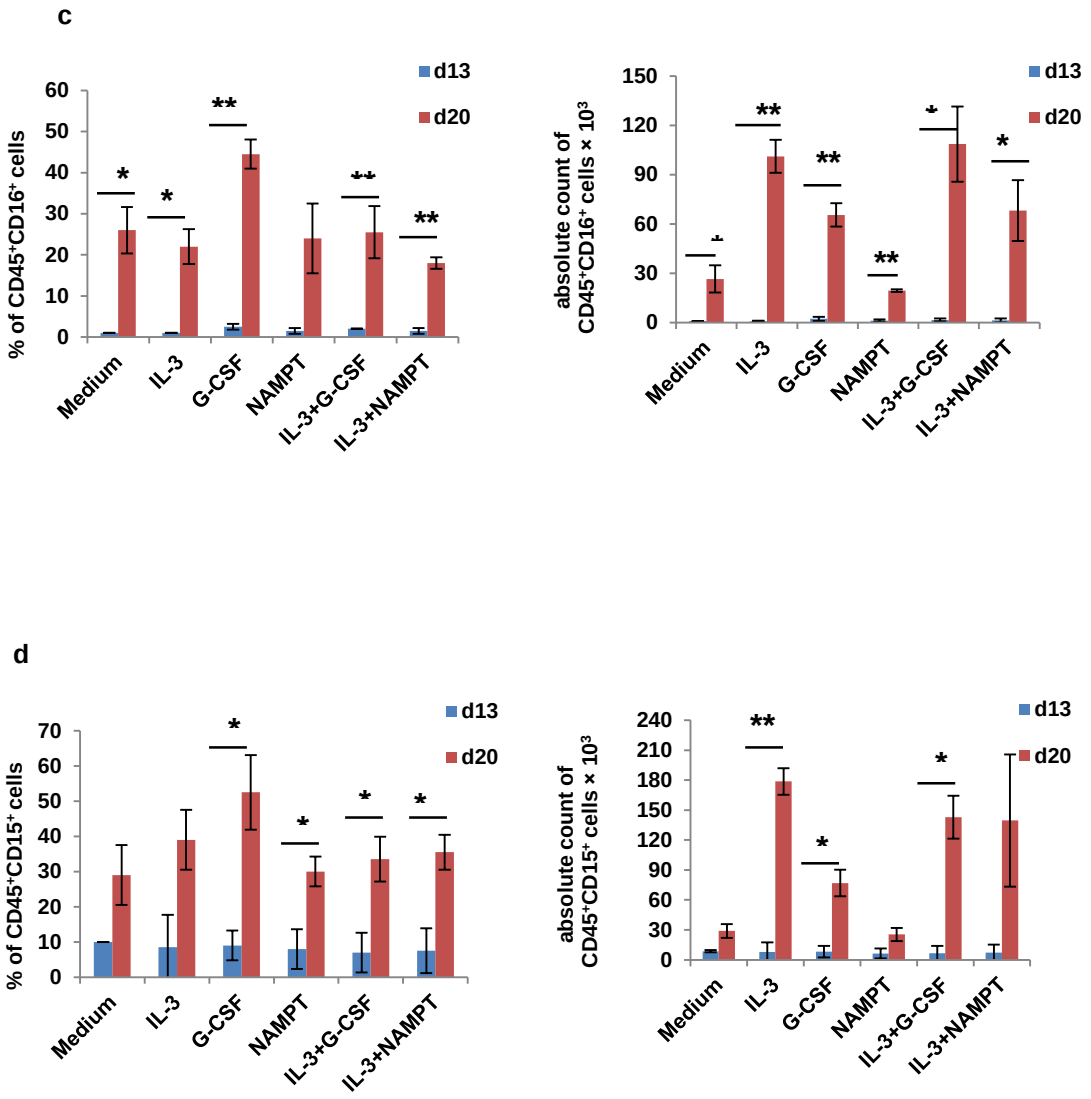


Figure 29. Shema of NAMPT and G-CSF effect evaluation on hematopoietic differentiation of hiPSCs
a, graphic bar represents the cell count of the experiment on different cytokine groups at day 13 and 20 of experiment **b,** graphic bars represent the % of CD45+CD11b+ cells and absolute cell count evaluation of each group **c,** graphic bars represent the % of CD45+CD16+ cells and evaluation of absolute cell count **d,** graphic bars represent the % of CD45+CD15+ cells and evaluation of absolute cell count. data represent means ± s.d. and are derived from two experiments in duplicates (*, P < 0.05; **, P < 0.01, ***, P < 0.001).

4.2.9 Evaluation of Nicotinamide (NA) as NAMPT substrate on hematopoietic differentiation of hiPSCs

To evaluate the role of Nicotinamide (NA), as a substrate of NAMPT enzyme, in EB-based differentiation of hiPSCs, EBs were plated in APEL medium containing VEGF (40 ng/ml), SCF (50 ng/ml) and NA (50 nMol) or IL-3 (50 ng/ml) on step 3 of culture (Fig. 30). Medium without any cytokines was considered as a control. Floating cells were collected on day 22 of culture for further evaluation of myeloid differentiation by FACS.

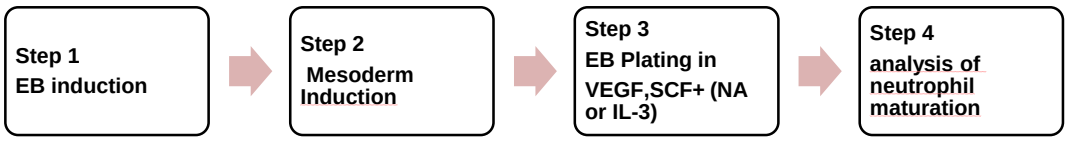
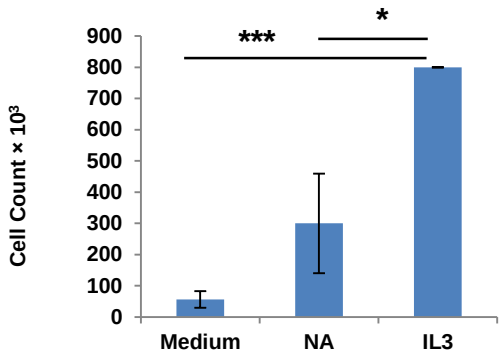


Figure 30. Schema of hematopoietic differentiation of iPSCs to evaluate the effect of NA in comparison to IL-3
NA as substrate of NAMPT enzyme was evaluated compare to IL-3 as the main cytokine group to test whether NA has any effect on proliferation or maturation of hematopoietic cells.

We found that addition of NA alone resulted in a slightly increased total number of floating cells, as compared to the control group (Fig.31a), but using IL-3 the cell numbers dramatically increased. Addition of NA to the culture medium resulted in generation of low amounts of $CD45^+CD11b^+$ and $CD45^+CD15^+$ myelo-granulocytic cells (Fig.31b,d), while the effect of IL-3 was considerable (Fig.31b,c,d). Based on these observations, we concluded that NA alone at a concentration of (50 nMol) is not a proper factor for myeloid differentiation of iPSCs.

a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700
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100
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Cell Count × 10³
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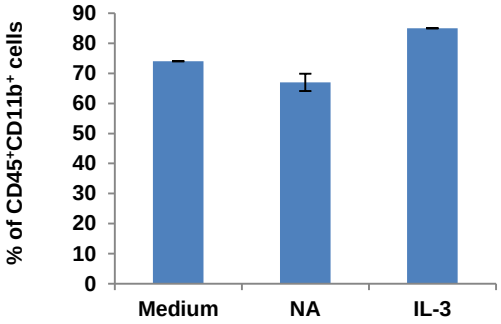
IL3

b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% of CD45⁺CD11b⁺ cells
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NA
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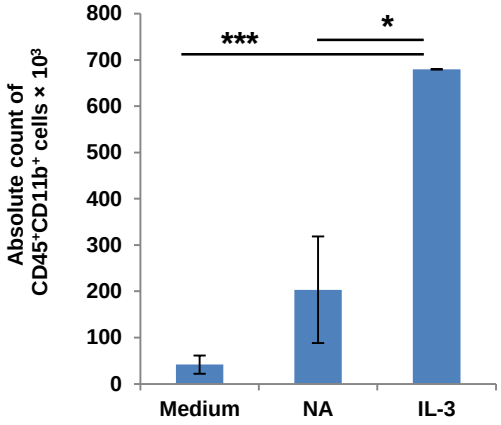
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Absolute count of CD45⁺CD11b⁺ cells × 10³
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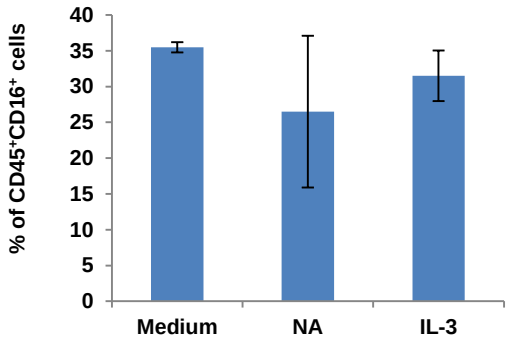
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% of CD45⁺CD16⁺ cells
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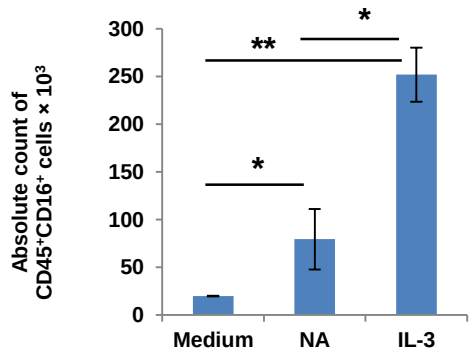
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300
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Absolute count of CD45⁺CD16⁺ cells × 10³
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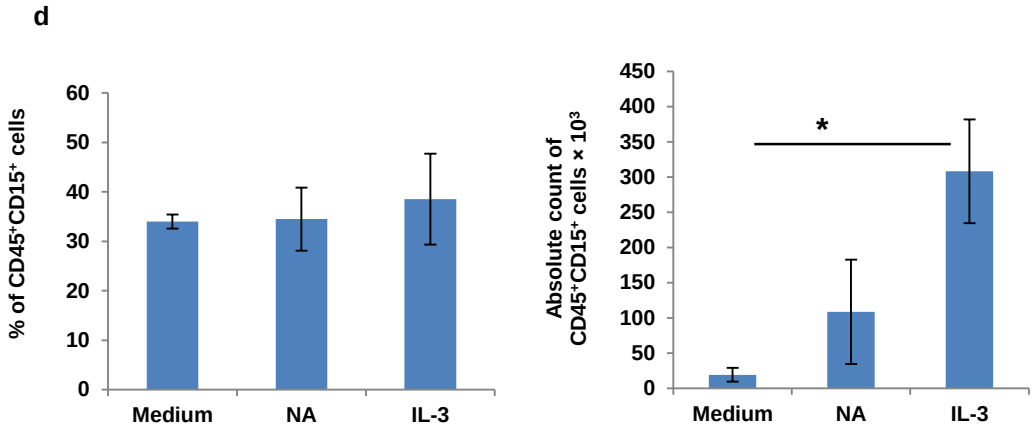


Figure 31. Evaluation of NA effect on hematopoietic differentiation of hiPSCs
a, graphic bar represents the cell count of the experiment in different groups at day 22 of experiment **b,** graphic bars represent the % of CD45+CD11b+ cells and absolute cell count evaluation of each group **c,** graphic bars represent the % of CD45+CD16+ cells and evaluation of absolute cell count **d,** graphic bars represent the % of CD45+CD15+ cells and evaluation of absolute cell count. data represent means ± s.d. and are derived from two experiments in duplicates (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

4.2.10 Evaluation of GM-CSF effect on hematopoietic differentiation of hiPSCs

Recent studies demonstrated that GM-CSF and IL-3 possess in some aspects redundant, but in other, completely different functions. They share same β-chain receptor subunit, but different receptor α-chains (Kitamura, Sato et al. 1991, Sakamaki, Miyajima et al. 1992, Shearer, Rosenwasser et al. 2003) Therefore, we compared the effect of GM-CSF and IL-3 on the myeloid differentiation of hiPSCs using the EB-based differentiation system. For this, EBs were plated in APEL medium containing (1) VEGF (40 ng/ml), SCF (50 ng/ml) and GM-CSF (50 ng/ml) or (2) VEGF, SCF and IL-3 (50 ng/ml) in step 3 of our protocol (Fig. 32). Floating cells were collected on day 22 of culture for further analysis by FACS.

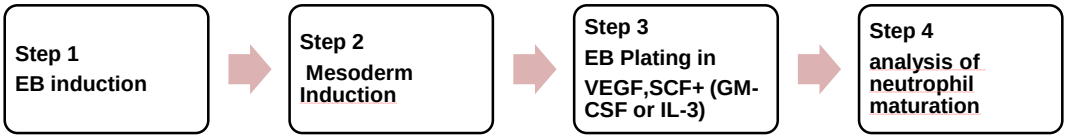


Figure 32. Schema of hematopoietic differentiation of iPSCs to evaluate the effect of GM-CSF in comparison to IL-3

GM-CSF which has the same β chain receptor compare to IL-3 and shared some similar function was evaluated compare to IL-3 as the main cytokine group to test whether GM-CSF has any effect on proliferation or maturation of hematopoietic cells.

Interestingly, we found that GM-CSF-triggered myelo-granulocytic differentiation was similar to the IL-3-triggered differentiation (Fig. 33a-c). Therefore, we concluded that GM-CSF also has effects on both proliferation and myelo-granulocytic maturation of iPSCs in the EB-based system.

In order to improve the EB-based myeloid differentiation of iPSCs derived from CN or CyN patients, we aimed to identify the best combination of cytokines for more efficient generation of granulocytes. We found that GM-CSF could be also a good candidate to improve the differentiation system into granulocytes, but still IL-3+GCSF was the best cytokine combination that we could use for our CN or CyN patient-derived iPSCs.

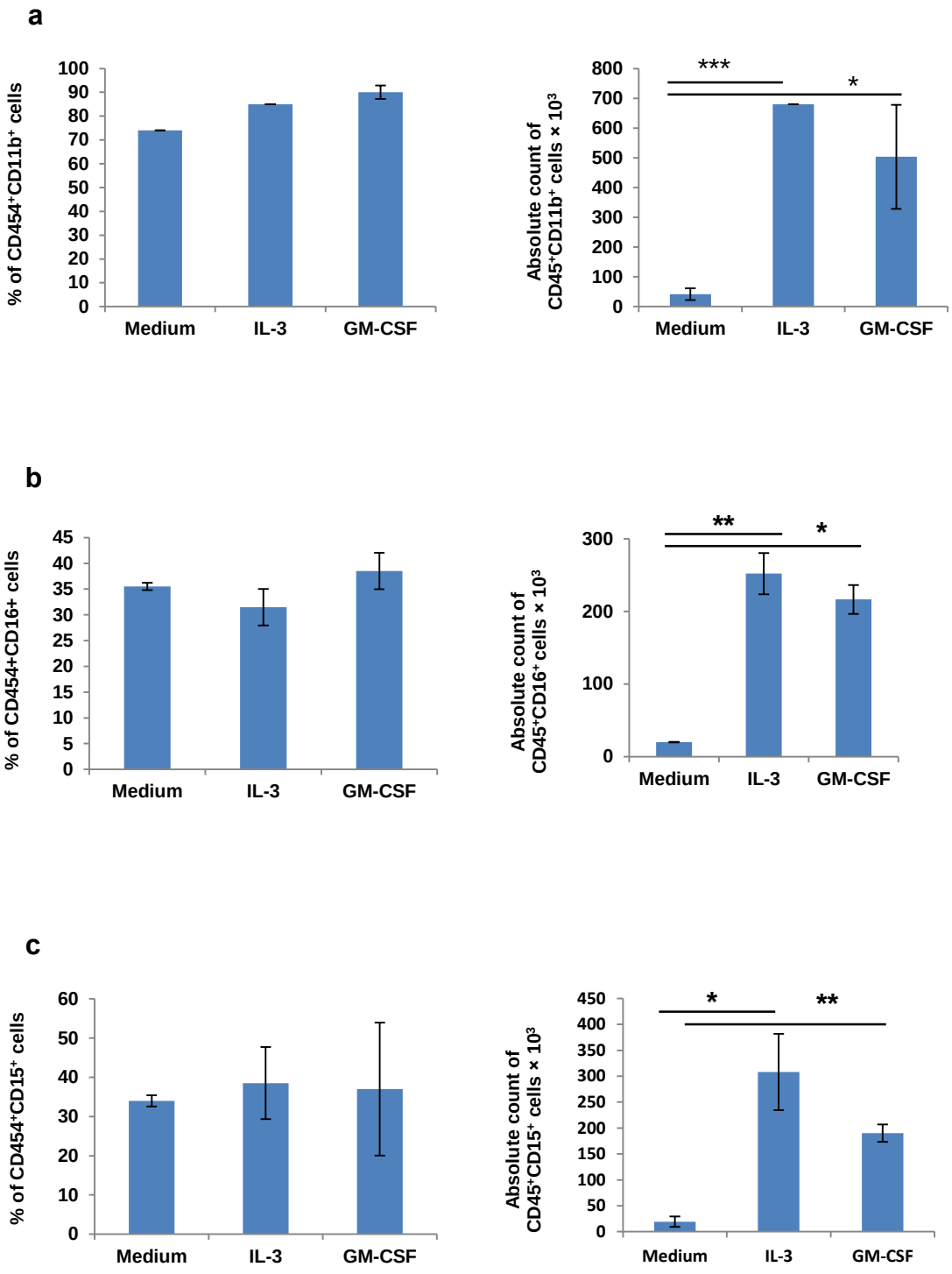


Figure 33. Evaluation of GM-CSF on hematopoietic differentiation of hiPSCs

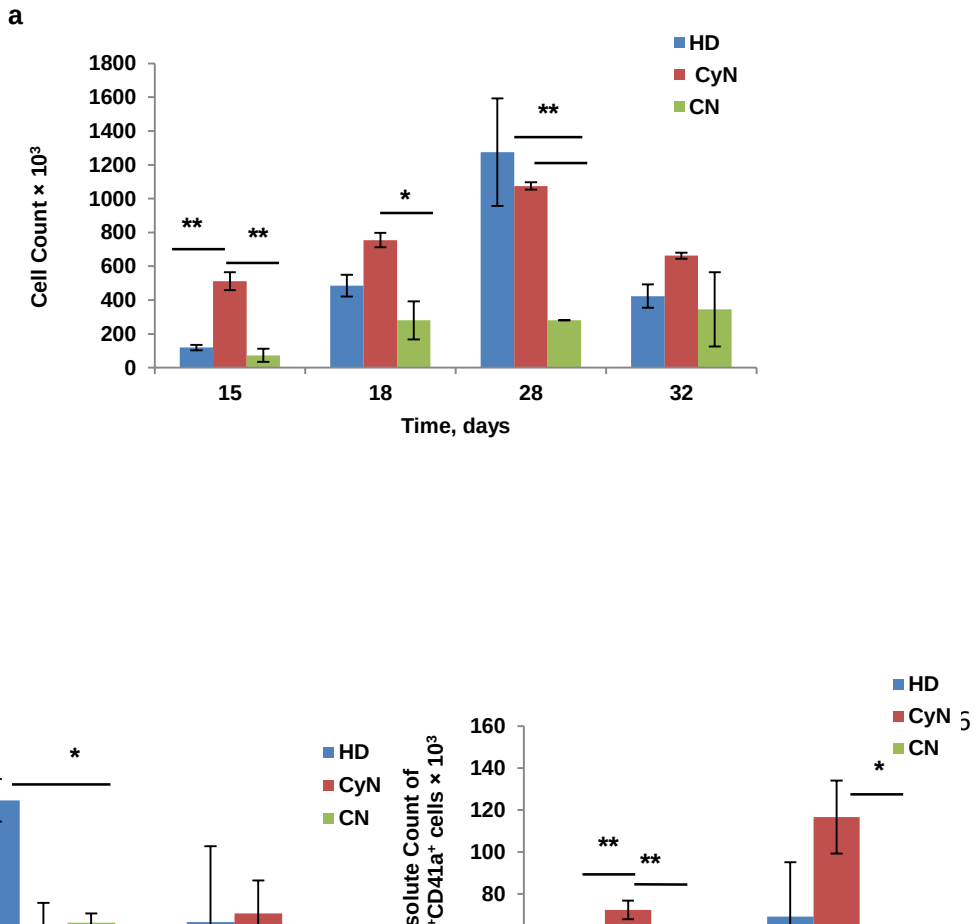
a, graphic bars represent the % of CD45+CD11b+ surface markers and absolute cell count evaluation of each group **c,** graphic bars represent the % of CD45+CD16+ cells and evaluation of absolute cell count **d,** graphic bars represent the % of CD45+CD15+ cells and evaluation of absolute cell count. Data represent means ± s.d. and are derived from two experiments in duplicates. (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

4.2.11 Comparison of hematopoietic differentiation of CyN, CN and healthy individual derived hiPSCs

We further compared the EB-based myeloid differentiation of iPSCs derived from a healthy individual, with that derived from a CyN or CN patients. For this, we monitored the early and late stages of hematopoietic and myeloid differentiation using two multicolor FACS panels (Fig. 34).

We found that EBs derived from iPSCs of a healthy individual and from a CyN patient continuously generated increasing numbers of floating hematopoietic and myeloid cells, while CN patient-derived iPSCs represent constant low cell numbers during *in vitro* differentiation (Fig. 34a). Interestingly, CyN patient-derived iPSCs did not show any cycling behavior during *in vitro* culture.

Interestingly, the absolute counts of floating $CD45^+CD41a^+$ and $CD45^+CD34^+$ hematopoietic stem cells generated on day 15 and 18 of culture were comparable between iPSCs of the healthy donor and the CyN patient but were markedly decreased in iPSCs of the CN patient (Fig. 34b,c).



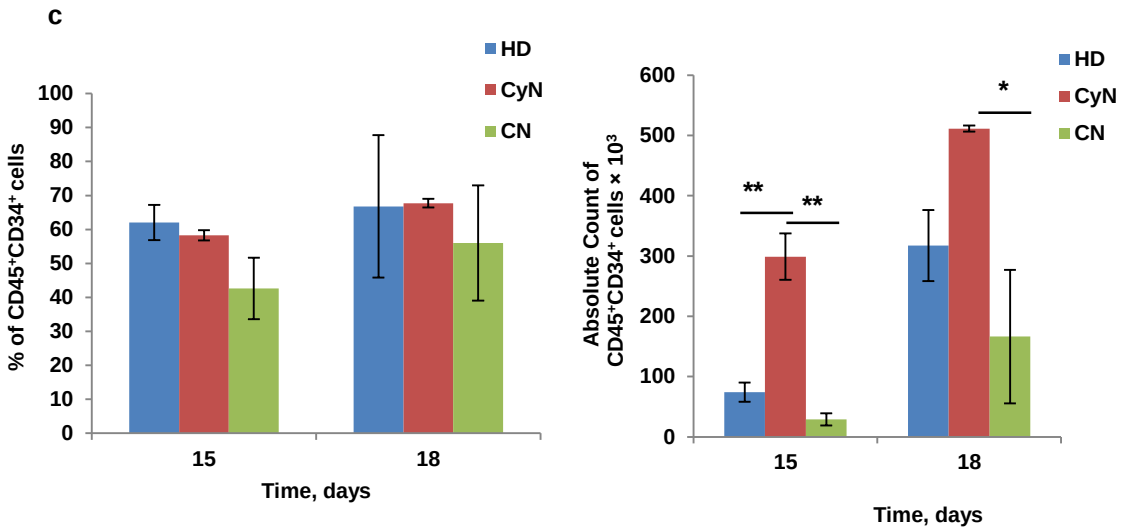
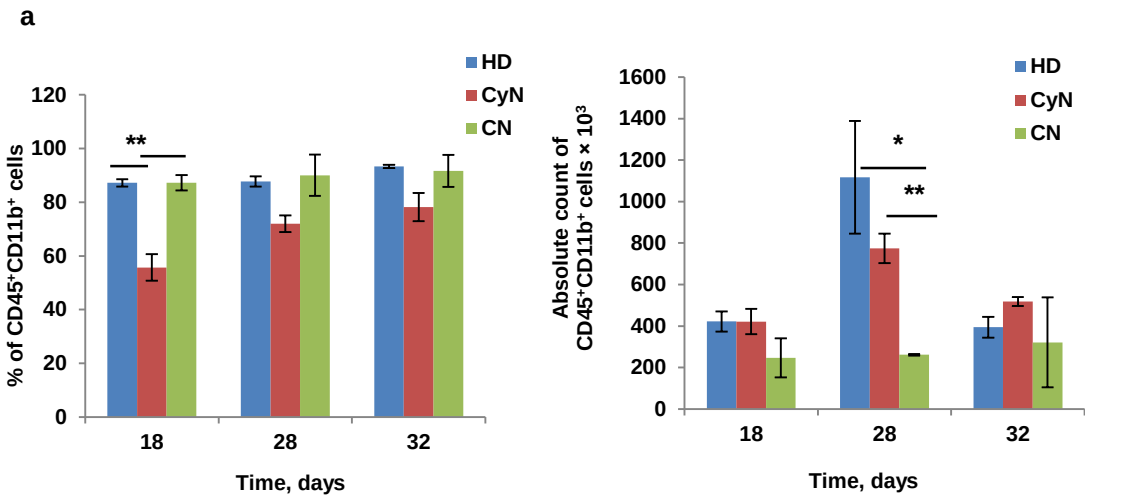


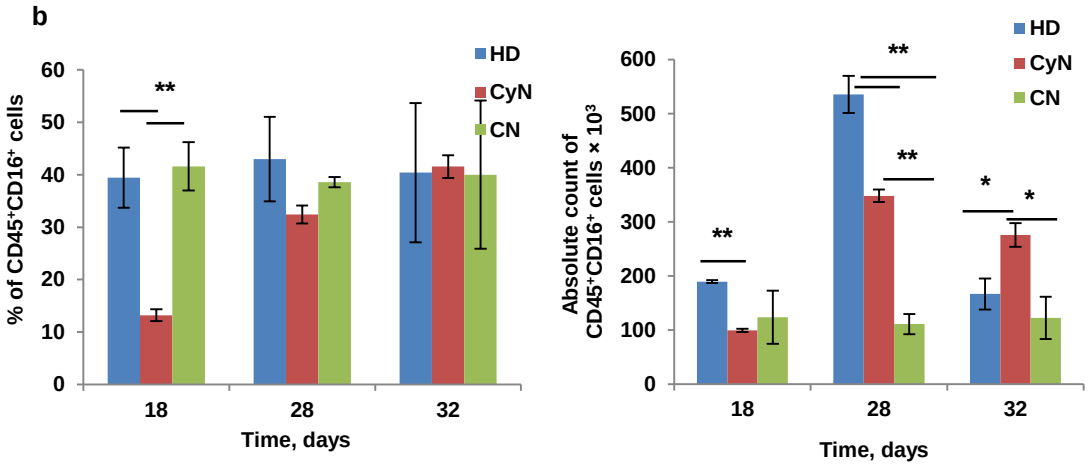
Figure 34. Evaluation of early hematopoietic differentiation differences between healthy individual and CyN or CN patients derived iPSCs

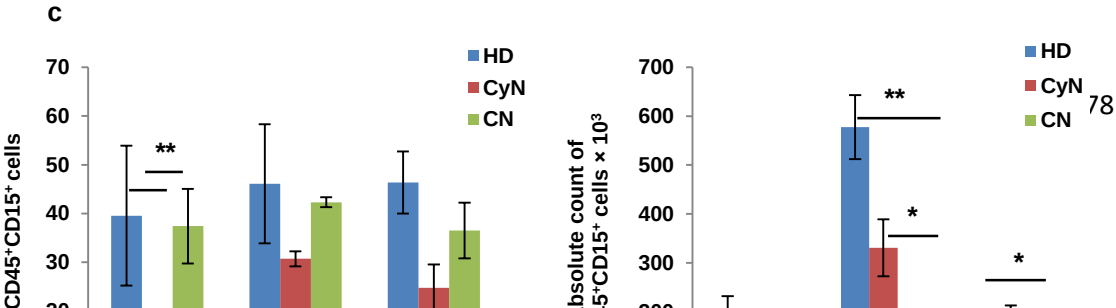
a, graphic bars represent the cell count of 3 different iPS cells at different time points. **b,** graphic bars represent the % of CD45+CD41a+ surface markers of each group and related absolute count of each group **c,** graphic bars represent the % of $CD45^{+}CD34^{+}$ cells and related absolute count of each group. Data represent means ± s.d. and are derived from two experiments in duplicates. (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

To evaluate myeloid differentiation of iPSCs of a healthy individual, CN patient and CyN patient, cells were collected on day 18, 28 and 32 of culture and were assessed by FACS. In this experiment, iPSCs derived from healthy individuals produced maximal numbers of myeloid and granulocytic cells on

day 28, but not on day 32 of culture. Most probably, EBs slowly stopped to differentiate on day 32. We detected diminished absolute numbers of $CD45^+CD11b^+$ myeloid cells and of granulocytic cells ($CD45^+CD16^+$) that were derived from iPSCs of the CyN patient, as compared to that of the healthy individual on day 28 of culture. We also found a remarkable reduction of absolute numbers of the myeloid and granulocytic cells that were generated from iPSCs of the CN patient, as compared to these cells of the healthy individual on day 28 of culture (Fig. 35a-d).







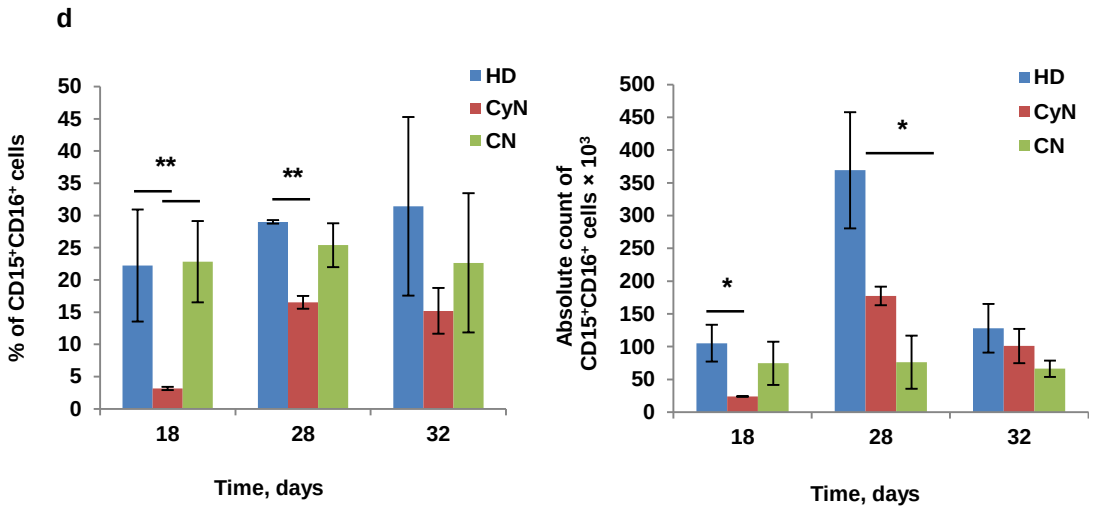


Figure 35. Evaluation of late hematopoietic differentiation differences between healthy individual and CyN or CN patients derived iPSCs

a, graphic bars represent the % of CD45+CD11b+ surface markers of each group and related absolute count of each group **b,** graphic bars represent the % of CD45+CD16+ cells and related absolute cell count of each group **c,** graphic bars represent the % of CD45+CD15+ cells **d,** graphic bars represent the % of CD15+CD16+ surface markers of each group and related absolute count of each group. Data represent means ± s.d. and are derived from the experiments in duplicates. (*, P < 0.05; **, P < 0.01; ***, P < 0.001).

4.2.12 Cell morphology

We further evaluated the cell morphology of Wright-Giemsa–stained cytospin preparations of myeloid cells generated on day 28 of culture from the

previous experiment (Fig. 36). We were able to detect mature neutrophils in healthy individual (Fig. 37a), CyN (Fig. 37b) and CN (Fig. 37c) derived iPSCs in our differentiation culture system, but the absolute cell numbers of mature neutrophils were markedly lower in the CN group, as compared to the healthy individual and CyN patient-derived iPSCs.

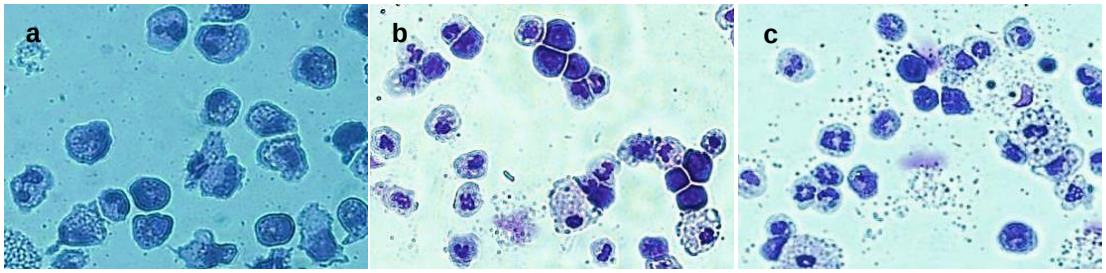


Figure 36. Wright-Giemsa–stained images of cytospin preparations from iPSCs-derived hematopoietic cells
a, healthy individual in day 28 of culture **b,** CyN iPSCs in day 28 of culture **c,** CN iPSCs in day 28 of culture.

Taken together, we found that using this protocol we can differentiate iPSCs into neutrophils from the healthy individual, CyN and CN patient-derived iPSCs, but not in the same quantity, as we described in Figure 34a.

5 Discussion

Use of ESCs and iPSCs will help us to improve our knowledge of the transcriptional networks on the lineage commitment and hematopoietic differentiation. Understanding of the new strategies for treatment of hematopoietic disorders and improvement of hematopoietic stem cell (HSC) transplantation needs more knowledge about stem cells and iPSCs has opened a promising field of therapeutic applications based on their efficient differentiation into hematopoietic cells.

By imitating signaling pathways of the hematopoietic system, we have developed a stepwise *in vitro* protocol for mouse ESCs and human iPSCs differentiation for the efficient generation of hematopoietic precursors and mature myeloid cells.

Using this approach we have shown that hematopoietic differentiation of mouse-derived ES cells induced through a connection between the BMP4 and TPO/c-mpl signaling pathway (Pramono, Zahabi et al. 2016). Treatment with TPO and enhancement of BMP4 target genes in ESCs and iPSCs may provide a new protocol for the generation of HSCs in clinical and experimental aspects.

It has been shown that BMP4 will be regulated by sonic hedgehog (Shh) and effective concentrations of BMP4 for blood cell formation has been confirmed (Bhardwaj, Murdoch et al. 2001). VEGF provides autocrine support for the survival of HSCs in the early stages of hematopoiesis (Gerber, Malik et al. 2002). It is also known that TPO can activate the VEGF production of ES cells during the first days of hematopoietic development (Kirito, Fox et al. 2005).

The synergic contribution of VEGF and BMP4 to stimulate the formation of hematopoietic cells from differentiating ESC-derived EBs has been identified (Nakayama, Lee et al. 2000). Our data suggest that TPO/c-mpl signaling could initiate the production of BMP4 and enhancement of the hematopoietic differentiation of EC cells respectively (Pramono, Zahabi et al. 2016).

We have proposed a direct binding of HIF-1α as a transcription factor which is TPO-dependent to the BMP4 gene promoter for production of sufficient concentrations of BMP4 by undifferentiated ES cells for induction of hematopoietic cells (Fig.37) (Pramono, Zahabi et al. 2016).

It is known that mesoderm formation and embryonic hematopoiesis induced under hypoxia condition; therefore HIF-1α is a crucial factor which is required for cell adaptation to low oxygen level conditions and it has an important role in primitive hematopoiesis (Huang and Bunn 2003).

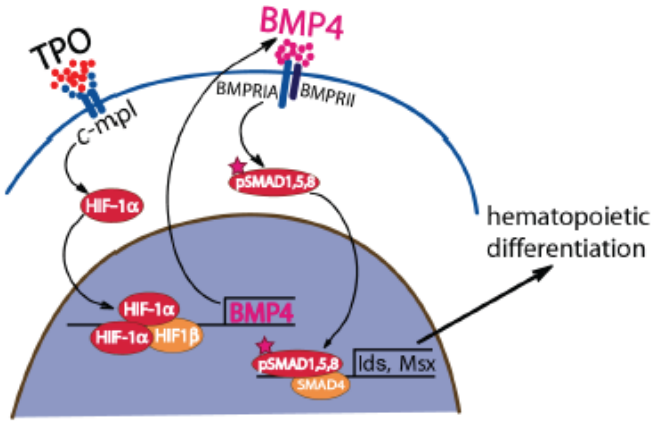


Figure 37. Schema of TPO-dependent BMP4 regulation
stabilization of HIF-1α protein by TPO and binding of HIF-1α to the BMP4 gene promoter could activate the autocrine synthesis of BMP4 and its target genes that results in in mouse ES cells derived early hematopoietic differentiation **(Source from Pramono, Zahabi et al. 2016).**

We believe that using safe iPSCs generation methods and combination of synergic effects of hematopoietic cytokines with developmental regulators,

such as the morphogen BMP4, Wnt or Shh, we could provide useful methods for production of HSCs as a proper source of patient-specific cells.

In the present study, we described the generation of iPSC lines of patients with CN and CyN. iPSCs have several advantages in comparison to postnatal hematopoietic stem cells (Ye, Chou et al. 2012). iPSCs use allows the clonal expansion of cells with defined genetic signatures and their possible genetic modifications, which is difficult to perform in HSCs. Patient-specific iPSCs are ideal sources for gene and cell therapy. We could use iPSCs of CN and CyN patients generated in this study for further drug screenings, in order to identify small molecules or other components capable of overcoming maturation arrest of granulopoiesis seen in CN patients. We could also use iPSCs of CN and CyN patients as an experimental model to study the mechanisms of leukemogenic transformation.

The current challenges of the iPSCs technologies are the optimization of reprogramming methods for generation of safe iPSCs, and development of the efficient differentiation protocols for the generation of functional cell types (Ye, Chou et al. 2012).

iPSCs are capable of differentiating into various lineages (Ebert, Yu et al. 2009, Zhang, Wilson et al. 2009, Zahabi, Shahbazi et al. 2011) including endothelial and hematopoietic cell lineages (Choi, Yu et al. 2009, Lengerke, Grauer et al. 2009, Ye, Zhan et al. 2009, Feng, Lu et al. 2010, Slukvin, Choi et al. 2014). However, it is still challenging to differentiate the progenitor cells of the desired lineage with high efficiency and purity from iPSCs.

There are two major approaches for hematopoietic differentiation of ESCs and iPSCs. One method involves co-culturing iPSCs with stromal cells such as murine OP9, C3H10T1/2, S17 or MS-5 as feeders (Wang, Au et al. 2007), (Kaufman, Hanson et al. 2001, Vodyanik, Bork et al. 2005, Miharada, Hiroyama et al. 2006). For the clinical applications and in order to prevent contamination with feeder cell products or antigen transmission, the second feeder-free culture system approach of iPSCs using embryoid body (EB)

formation would be more desirable (Pick, Azzola et al. 2007, Salvagiotto, Burton et al. 2011, Woods, Parker et al. 2011, Park, Zimmerlin et al. 2013). Efficient production of $CD45^+$ hematopoietic cells from iPSCs has been reported by both culture methods using high-dose of hematopoietic growth factors (Ng, Davis et al. 2008, Nostro, Cheng et al. 2008, Pearson, Sroczynska et al. 2008). However, we still need to know how culture conditions and differentiation protocols affect lineage commitment.

To establish the optimal conditions for hematopoietic differentiation of iPSCs, profound knowledge of the mechanisms operating during different distinct stages of differentiation are essential. The differentiation process *in vitro* into blood cells is divided into different stages with particular signals for mesodermal differentiation, hemato-endothelium, and hematopoietic stem cell- and progenitor cell specification, maturation, and expansion (Woods, Parker et al. 2011).

ES cells can be enforced to differentiate into mesoderm and then into hematopoietic progenitors by specific cytokines. The most important cytokines at this stage are BMP4, a ventral mesoderm inducer (Chadwick, Wang et al. 2003), and VEGF (Nakayama, Lee et al. 2000, Park, Afrikanova et al. 2004). The critical role of BMP4 in mesoderm formation and further hematopoietic differentiation in the posterior mesoderm has been identified in many species (Cerdan, McIntyre et al. 2012). We know that endothelial and hematopoietic cells can be formed from the mesoderm lineage in the extra-embryonic yolk sac following gastrulation (Cerdan, McIntyre et al. 2012).

It is also known that VEGF increases hemogenic endothelial cell proliferation and formation of hematopoietic cells. CD43 is the first marker, which is expressed in hematopoietic progenitors and which discriminate hematopoietic cells from endothelial cells during *in vitro* ES cells differentiation (Wang, Li et al. 2004). VEGF is identified as an inducer of $CD31^+$ enriched hematoendothelial (HE) cells from early mesodermal cells

and commitment inducer of HE cells into hematopoietic progenitors (Wang, Tang et al. 2012).

In the present work, we established an efficient and directed differentiation method to generate neutrophils from human iPSCs using an EB based, feeder-free, serum-free, completely defined system. This protocol may be particularly suitable for studying human hematopoietic development and generating *in vitro* mature cells for cell and gene therapy.

We designed an optimized 3-week protocol using serum free APEL medium, by step-wise addition of mesodermal induction cytokines (BMP4 and bFGF) to induce initial loss of pluripotency and appearance of the specification of myeloid $CD11b^+$ cells. By further addition of hematopoietic growth factors (SCF, VEGF and IL-3), we induced differentiation of hematopoietic cells with production of approximately 40% of $CD34^+$ hematopoietic cells and ca. 90 % of $CD45^+$ cells on day 20 of culture.

We also examined the myeloid differentiation potential of different hematopoietic cytokines, IL-3, GM-CSF and G-CSF in the conditions of our EB-based protocol of iPSCs differentiation. Interestingly, we achieved the best results using IL-3.

In the context of adult hematopoiesis, IL-3 has been identified to support myelopoiesis, especially when used in combination with other growth factors such as G-CSF or GM-CSF (Krumwieh, Weinmann et al. 1990). More recently, IL-3 was also proven to elevate the development, proliferation and survival of early HSCs (Guzeloglu-Kayisli, Kayisli et al. 2009).

It has been reported that IL-3 is a supportive cytokine for the proliferation of a wide range of hematopoietic cells (Wiles and Keller 1991), while G-CSF and M-CSF are cytokines for specific myeloid differentiation. Appointed the emerging role of IL-3 in early primitive as well as definitive hematopoietic differentiation (Yang, Ciarletta et al. 1986), the fundamental importance of G-CSF and M-CSF is rather in terminal granulocyte and monocyte/macrophage

differentiation (Clark and Kamen 1987, Welte, Bonilla et al. 1987, Sengupta, Liu et al. 1988). These observations may explain less potential effects of G-CSF on the myeloid differentiation of iPSCs, as compared to IL-3. Interestingly, we observed that GM-CSF activity toward myeloid cells proliferation and maturation from iPSCs was similar to IL-3. It is known that IL-3 and GM-CSF exhibit similar effects in hematopoietic cells by usage of the same receptor ß-subunit. However, it is known that GM-CSF failed to induce granulopoiesis in CN patients. Even upon treatment with a high dose of GM-CSF we did not observe an elevation in neutrophil numbers in the peripheral blood of CN patients. G-CSF remains the only option for the treatment of CN patients.

We have previously shown that G-CSF-triggered NAMPT expression and its product NAD^+ in myeloid cells (Skokowa, Lan et al. 2009). NAMPT is a rate-limiting enzyme which also known as a pre–B cell colony-enhancing factor (PBEF) (Zhang, Sang et al. 2010). Conversion of nicotinamide into NAD^+ by NAMPT has been shown to be crucial for SIRT1 activation and granulocytic differentiation of $CD34^+$ cells (Skokowa, Lan et al. 2009). Notably, vitamin B3 as a substrate of NAMPT, led to neutrophilia after treatment of healthy individuals which shows a crucial role of NAMPT and NAD^+ in myeloid differentiation (Skokowa, Lan et al. 2009).

Therefore, we sought to investigate the combined use of IL-3 with NAMPT or its substrate nicotinamide (NA) in our EB-based hematopoietic differentiation protocol of iPSCs. Our results showed that NAMPT or its substrate nicotinamide (NA) alone could not support properly *in vitro* myeloid cell proliferation and differentiation, while in the presence of IL-3, the number of floating hematopoietic and myeloid cells were dramatically increased. It would be interesting to study possible dose- and developmental stage-dependent effects of NAMPT and NA on the hematopoietic differentiation of iPSCs. Also, comparison of iPSCs from healthy individuals and from CN or CyN patients may be intriguing.

Using the EB-based protocol, we compared the potential of hiPSC lines generated from healthy individuals and from patients with CyN or CN to differentiate into hematopoietic progenitors and into mature myeloid cells. One of the strengths of generating patient-derived iPSCs is that the genetic signature of the patients is maintained. Thus, the described system will make it possible to differentiate CN and CyN iPSCs in parallel and compare the granulopoiesis as described above.

Interestingly, we observed a logarithmic increase in generation of floating cells using iPSCs of the healthy individual and CyN patient. We observed the appearance of floating hematopoietic cells on day 14 of culture. Cell counts were continuously increased and reached a peak on day 30 of culture with subsequent decline until day 40- 45 where EBs stopped producing floating hematopoietic and myeloid cells. We found that myeloid differentiation of iPSCs derived from the CyN patient was comparable to that of the healthy individual derived iPSCs, as revealed by the analysis of the percentage and absolute numbers of $CD45^+CD11b^+$, $CD45^+CD16^+$ and $CD45^+CD15^+$ cells as well as by the examinations of the cell morphology on cytospin preparations. We could not detect any cycles of granulopoiesis using the EB-based protocol of differentiation of iPSCs derived CyN patients. Typically, peripheral blood neutrophil counts of CyN patients have approximately 21-day period cycles. This could be explained by the fact that in our system we measured mixed cell populations produced simultaneously by different EBs.

As expected, we found that iPSCs generated from the CN patient produced markedly reduced numbers of myeloid cells at early and late stages of differentiation, as compared to iPSCs generated from a healthy donor and from the CyN patient. Interestingly, although the absolute cell counts of iPSCs derived from the CN patient were much lower than in the other groups, morphological studies showed the presence of mature neutrophils in the CN group, which was comparable to CyN and healthy donor groups. Based on these observations, we were not able to recapitulate "maturation arrest" of granulopoiesis, seen in the bone marrow of CN patients before initiation of

G-CSF therapy. These observations may be explained by the experimental conditions used in our study. We used albumin-reach APEL medium and IL-3/G-CSF stimulation. G-CSF is used for the life-long treatment of CN patients (Skokowa, Lan et al. 2009), however, daily injections of G-CSF doses between (100–1,000 times more than physiological levels) are needed for the enhancement of neutrophil counts in patients with CN disorders (Skokowa, Lan et al. 2009). Therefore, high-dose G-CSF is capable of differentiating HSCs of CN patients into granulocytes, but with very low efficiency. Our observations from *in vitro* EB-based differentiation of CN patient-derived iPSCs also confirmed the low efficiency of cytokine-triggered granulocytic differentiation.

Taken together, we observed reduced hematopoiesis in iPSCs derived from CN patients in this study as compared to healthy individual and CyN derived iPSCs. We found that although CN derived iPSCs could produce mature neutrophils, the potential for proliferation is very low compared to CyN and healthy individual derived iPSCs.

Unaffected myeloid differentiation of iPSCs derived from the CyN patient could be explained by the fact that in CyN patients *in vivo* additional humoral or bone marrow niche components might influence myeloid cell cycling and additional modifier gene mutations or epigenetic defects in combination with mutated ELANE might be responsible for the pathogenesis of CyN.

6 Outlook

Reprogramming of somatic cells into pluripotent stem cells is a brilliant method that could be broadly used in the future for patient-specific stem cell-based therapies. However, many issues remain to be solved before hiPSC-

based therapy will enter the clinic. Virus-free reprogramming of somatic cells would be the most important issue in this direction.

Safety of iPSCs is the main concern for iPSC-based therapy in clinical applications. Establishment of safe reprogramming methods to generate iPSCs and differentiation potential of iPSCs require further evaluation. Recent updates in iPSCs generation are: (1) reprogramming using the Sendai virus, (2) DNA-based episomal reprogramming, (3) induction of pluripotency via mRNA or protein expression.

Methods for large-scale production of mature cells of specific lineages from iPSCs in defined feeder-free conditions must be developed for translational studies. Understanding the disease pathogenesis and defining targets for the development of novel drugs for treatment or correcting defective signaling molecules is another challenging step.

Gene modification and transplantation of HSCs demand the highest quality in iPSCs research in the near future. Recent developments in gene targeting technologies such as CRISPR/Cas9 technologies have provided a valuable tool for research and for therapeutic applications of iPSCs.

In addition, usage of iPSCs for transplantation applications requires the quick cell preparations for universal purposes. Patient-specific iPSCs will prove to be useful for disease modeling In the near future. Moreover, unclear pathogenic mechanism based diseases or genetically induced disorders are obvious targets for patient-derived iPSCs therapeutic applications.

In conclusion, the application of iPSCs to patients has the potential to provide a new light to clinical research. Indeed, safety limitations of using iPSCs must thoroughly be considered. Patient-specific iPSCs provide crucial new tools to improve our knowledge about disease pathogenesis and treatment strategies. Cell therapy with autologous patient-specific iPSCs is useful due to immune tolerance of autologous unlimited sources of the cells. These advantageous attributes should persuade further discoveries in the field of

iPSCs for translational research, disease modeling, and regenerative medicine, in both laboratory and clinical applications.

Zusammenfassung

Das Ziel dieses Forschungsvorhabens ist es, die Mechanismen der hämatopoetischen Differenzierung, angefangen von sehr frühen Stadien bis zur myeloischen Differenzierung, zu untersuchen. Dafür haben wir die Differenzierung von murinen embryonalen Stammzellen (ES Zellen) Zellen sowie von humanen induzierten pluripotenten Stammzellen (iPS Zellen) verwendet. iPS Zellen sind re-programmierte somatische Zellen, die alle Merkmale embryonaler

Stammzellen tragen. Sie werden durch die Einführung der definierten Transkriptionsfaktoren (*Oct 3/4*, *Sox2*, *Klf4*, mit oder ohne *c-MYC*) in differenzierte somatische Zellen (e.g. Fibroblasten, $CD34^+$ hämatopoetische Knochenmarkzellen, Keratinozyten) generiert. Die Untersuchungen an iPS Zellen können für das Verstehen der Prozesse der Lineage-Spezifizierung und Differenzierung von verschiedenen hämatopoetischen Reihen entscheidend sein.

Im ersten Teil der Arbeit stellte sich heraus, dass ES-Zellen einen Rezeptor für Thrombopoetin (TPO), c-mpl exprimieren. Interessant ist, dass die Stimulation von ES Zellen mit TPO zusammen mit VEGF (Vascular Endothelial Growth Factor) die hämatopoetische Differenzierung dieser Zellen induziert. Es erfolgte durch Aktivierung von HIF1α (hypoxia-inducible factor-1 alpha) ein Transkriptionsfaktor, der an den Promoter des BMP4-Gens bindet und eine autokrine Sekretion von BMP4 bei diesen Zellen aktiviert.

Im zweiten Teil der Arbeit haben wir ein Protokoll für die effiziente hämatopoetische Differenzierung von humanen iPS-Zellen etabliert. Wir haben auch die Effekte von IL-3, G-CSF, GM-CSF, NAMPT und Nikotinamide (NA) auf diese Differenzierung verglichen und haben festgestellt, dass die Kombination von IL-3 und G-CSF für unsere Zwecke am besten geeignet ist. Wir haben weiter die iPS Zellen von gesunden Spendern sowie von Patienten mit schwerer kongenitaler (CN) oder zyklischer (CyN) Neutropenie hergestellt und die myeloische Differenzierung dieser iPS Zellen verglichen. Wir haben entdeckt, dass die iPS-Zellen von CN-Patienten eine stark erniedrigte myeloische Differenzierung im Vergleich zu den iPS Zellen von CN-Patienten aufweisen. Im Gegensatz zeigten die iPS Zellen von CyN-Patienten nur eine leicht reduzierte myeloische Differenzierung.

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Curriculum Vitae

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Education

MSc, Cell and Molecular Biology (2007-2010)

Azarbayjan University of Tarbiat Moallem and Royan Inst. Of Tehran

BSc, Microbiology (1997-2001)

Azad university of Tehran

RESEARCH INTERESTS

Stem cells research and therapeutics

Genetic and Epigenetic

Molecular and cellular basis of degenerative diseases

Gene manipulation

RESEARCH EXPERIENCE

Royan Inst. for Stem Cell Biology & Technology- Azarbayjan University of Tarbiat Moallem:

Project 1: Differentiation of human Normal iPS and ES cells into Retinal Pigmented Epithelial cells and transplantation into subretinal space of sodium iodate injected Rat model of retinal degeneration (MSc Project from 2008-2011, supervisor: Hossein Baharvand, Hamid Ahmadieh)

Royan Inst. for Stem Cell Biology & Technology:

Project 2: Behavior of Human Embryonic Stem Cell-derived Neural Progenitors toward Electrospun Nonfibrillar Surface *in vitro*. (As Research assistance in Royan Institute, 2008-2010, supervisor: Hossein Baharvand)

Project 3: Differentiation of retinal patient iPSCs into RPE cells (2011, supervisor: Hossein Baharvand- Hamid Ahmadieh)

Neuroscience Research Center (NRC) Shahid Beheshti University of Medical Science

Evaluation of Testosterone, Estradiol and Gemfibrozil on H2O2 induced *in vitro* PC12 cell apoptosis via anti-inflammatory and mitochondrial biogenesis mechanisms (2012, supervisor: Fariba Khodagholi)

Pediatric Haematology and Oncology Medical School Hannover:

Hematopoietic differentiation of iPSCs generated from CN and CyN patients carrying the same ELANE mutation (2013, Supervisors: Prof.Dr Karl Welte, Prof.Dr. Skokowa Julia)

Department of Hematology, Oncology, Immunology, Rheumatology and Pulmonology,University Hospital Tübingen, Germany

Modeling of hematopoiesis using ES and patients-derived iPS cells (2014-2017)

PRESENTATIONS AND HONORS

First member of retinal group in ROYAN institute and applying iPS-derived neural stem cells (NSCs) and retinal pigmented epithelial (RPE) cells for treatment of the eye diseases such as age-related macular degeneration (AMD)

Received national grants for a retinal research program in Royan Institute.

Winner as a best oral presentation in Ophthalmology Research Center of Labbafi-Nejad Institute, 2010 national congress, shahid Beheshti university, Tehran, Iran.

Oral presentation for international Asia ARVO 2011 Singapore ophthalmology congress, Sentosa Island

Poster presentation for ISSCR 9th annual congress, 2011 Canada and winner of grant as visa gift card and free congress registration fee for poster presentation

Poster presentation for Royan international congress, 2011 Tehran.

Oral presentation in Ophthalmology Research Center Farabi Hospital-Tehran University of Medical Sciences,2012, Tehran, Iran

Poster presentation at the 57th ASH Annual Meeting and Exposition (December 5-8, 2015) in Orlando, FL.

Abstract submission for poster with presentation in 21st Congress - European Hematology Association

Abstract submission for oral presentation in DGHO Leipzig Congress

Employment and Work Experiences

Working in Bernard medical laboratory in microbiology section (2001-2006)

Royan institute stem cell workshops, summer school, international congress and pre-congress activities (2008-2011)

Research assistant in Neuroscience Research Center (NRC) Shahid Beheshti University of Medical Sciences, Tehran, Iran (2011-Oct.2012)

Research assistant in Pediatric Haematology and Oncology Medical School Hannover

Research assistant in University Clinic Tubingen (UKT)

Language Skills

PERSIAN (FARSI): Mother language, Native

ENGLISH: Fluent in both speaking and writing

German: A2 Basic course 2 level

Publications

A New Efficient Protocol for Directed Differentiation of Retinal Pigmented Epithelial Cells from Normal and Retinal Disease induced Pluripotent Stem Cells (Stem Cells Dev. PMID: 22145677. manuscripts ID: SCD-2011-0599.R1)

Interactions of Human Embryonic Stem Cell-derived Neural Progenitors with an Electrospun Nanofibrillar Surface *in vitro*. Int J Artif Organs. 2011 Jul; 34(7):559-70. doi: 10.5301/IJAO.2011.8511. PMID: 21786255

Regenerative Therapy for Retinal Disorders (review article) (J Ophthalmic Vis Res2010; 5 (4): 250-264)

Thrombopoietin Induces Hematopoiesis from mouse ES Cells Via HIF-1α-dependent activation of a BMP4 autoregulatory Loop (2016 New York Academy of Sciences. PMID: 27447537 DOI:10.1111/nyas.13138)

ATTENDED CONFERENCES AND WORKSHOPS

Training workshop at Pasteur Research Institute of Iran on basic methods in biotechnology (summer 2006), Tehran, Iran.

Participate in the 4th, 5th and 6th Royan International Congress on Stem Cell Biology and Technology Aug 27-29, 2008, Tehran, Iran.

Participate in ophthalmology congress of Shahid Beheshti University Tehran Ophthalmology Research Center of Labbafi-Nejad Institute), Iran, 2010.

Participate in international Asia ARVO 2011 ophthalmology congress Sentosa Singapore.

Participate in international ISSCR 9th annual meeting Toronto Canada.

Participate ASH 2015 annual meeting USA Orlando Florida USA.

Participate EHA 2016 annual meeting Denmark Copenhagen.

Participate DGHO 2016 annual meeting Leipzig Germany.

COMPUTER SKILLS

Microsoft office (Word, Excel, PowerPoint), Photoshop, EndNote (reference managing)

WinMDI (analytical flow cytometry), flow Jo.

Primer designing software: Perl Primer, Gene Runner

Image analysis software: ImageJ 1.41 v

Statistical software: SPSS 13.0 v

MEMBERSHIP

EHA (European Hematology Association)

ResearchGate network

Linked In network

Declaration of Contributions to the Dissertation

The dissertation work was carried out at the Department of Molecular Hematopoiesis, Hannover Medical School (first year) and at the Division of Translational Oncology, Department of Oncology, Hematology, Immunology, Rheumatology and Pulmonology, University Hospital Tuebingen, under the supervision of Prof. Dr. med. Julia Skokowa, Ph.D.

The study was designed by Prof. Dr. med. Julia Skokowa in collaboration with Prof. Dr. med. Karl Welte.

After training by Dr. Tatsuya Morishima, I carried out experiments on the generation and hematopoietic differentiation of iPS cells with the assistance of Benjamin Dannenmann and Regine Bernhard. With the assistance of Andri Pramono, I studied the role of thrombopoietin-induced hematopoietic differentiation of mouse ES cells.

Statistical analysis was carried out under the supervision of Dr. Tatsuya Morishima by myself.

I confirm that I wrote the Dissertation myself under the supervision of Prof. Dr. med. Julia Skokowa and that any additional sources of information have been duly cited.

Signed_____

on _____ in Tübingen