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Characterization of a novel Lin-CD34+CD133+CD41+ HSPC population in  
Myelofibrosis patients and establishment of a long-term co-culture system

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## Table of Contents

Introduction.....	5
Hematopoiesis.....	5
Early stages of hematopoiesis.....	5
HSC heterogeneity.....	8
Characterization of human hematopoiesis .....	9
CD133 as a stem cell marker .....	11
Myeloproliferative neoplasms.....	16
Primary Myelofibrosis.....	17
Essential thrombocythemia and Polycythemia vera.....	18
Genetic mutations associated with MPNs.....	19
Understanding fibrogenesis .....	21
Characterization of the Neoplastic Stem Cell in Myelofibrosis.....	23
Aim.....	23
Materials and Methods .....	25
Materials.....	25
Equipment.....	25
Consumables .....	25
Reagents, media and media components .....	26
Cell lines .....	27
Cytokines.....	27
Fluorochrome-conjugated antibodies .....	28
Lentiviral Gene Ontology Vectors.....	28
Methods .....	28
Transduction with Lentiviral Gene Ontology Vectors .....	28
Functional assays.....	29
Irradiation of stromal cells.....	30
Enzyme-linked immunosorbent assay (ELISA) .....	30
Isolation of mononuclear cells .....	31
Co-cultures with transgenic HS5 cell lines and CD34 <sup>+</sup> .....	32
Cell cycle analysis with propidium iodide and flow cytometry .....	32
Flow cytometry and cell sorting.....	32
Population tracking with Cell Trace Violet™ .....	32
Colony-forming unit (CFU) assays .....	33
Morphological assessment of cells .....	35
Co-culture of transduced stromal cell lines and healthy BM cells.....	36

Long-term colony-initiating cell assay.....	37
Boosting with THPO.....	38
Results.....	38
Establishment of an <i>in vitro</i> co-culture system .....	38
Transduced stroma cells produce high levels of functional cytokines.....	39
Irradiated HS5 do not survive as a stable stromal layer.....	41
Examination of the influence of cytokine expression on normal HSPC cultivated on transgenic stroma cultures.....	44
Characterization of Lin <sup>-</sup> CD34 <sup>+</sup> CD133 <sup>+</sup> CD41 <sup>+</sup> .....	51
Healthy LDP CD41 <sup>+</sup> cells show priming towards megakaryopoiesis <i>in vitro</i> .....	53
Boosting with THPO can induce megakaryocytic differentiation of Lin <sup>-</sup> CD34 <sup>+</sup> CD133 <sup>+</sup> cells .....	56
Lin <sup>-</sup> CD34 <sup>+</sup> CD133 <sup>+</sup> CD41 <sup>+</sup> contain long-term colony-initiating cells.....	62
LDP from MF patients do not show CD38 <sup>+</sup> myeloid progenitor phenotype.....	64
Discussion.....	66
Transgenic stromal cell lines are suboptimal for long-term co-culture systems.....	66
Lin <sup>-</sup> CD34 <sup>+</sup> CD133 <sup>+</sup> CD41 <sup>+</sup> represent a rare population in MF patient PB.....	68
LDP CD41 <sup>+</sup> show multi-lineage potential with priming for megakaryopoiesis.....	70
Heterogenous LDP CD41 <sup>+</sup> population challenges a previous model limiting CD41 expression to megakaryocytic progenitors.....	71
Summary .....	75
Zusammenfassung.....	76
Supplementary material.....	78
List of abbreviations.....	78
Supplementary Table 1: Overview of patients .....	79
References.....	80
Acknowledgments .....	89
Curriculum vitae.....	90

## Introduction

### Hematopoiesis

To understand hematological malignancies, it is essential to investigate and understand hematopoiesis itself. All blood cells in the human body are continuously replenished; roughly 200 billion erythrocytes and 70 billion neutrophils are produced every day. Hematopoiesis, the process of blood cell production, can generally be divided into three phases, two of which occur in the embryo. In the first phase of primitive hematopoiesis, red blood cell precursors, derived from mesodermal cell aggregates, form in the yolk sac as early as the third week of pregnancy. These so-called primitive erythroblasts still contain a nucleus and have a limited developmental capacity. The second phase of hematopoiesis is the definitive phase, taking place in the liver and the spleen between the sixth week and the fifth month of pregnancy. Granulocytes and megakaryocytes are still rare in this phase. Beginning in the fifth month of pregnancy, blood cell formation can be detected in the bone marrow (BM), marking the onset of the medullary phase. This last phase is characterized by the production of all hematopoietic cells in the entire skeleton. Postnatal hematopoiesis is concentrated in the cranium, sternum, vertebrae and pelvis, as well as the ribs and proximal tibia and femur. Reactivation of hematopoiesis in extramedullary organs only occurs in the setting of hematological diseases such as myelofibrosis (Tavian, Biasch et al. 2010, Ulrich Welsch 2014, Singh, Soman-Faulkner et al. 2020).

### Early stages of hematopoiesis

The concept that mature blood cells arise from hematopoietic stem and progenitor cells (HSPC) was established from early experimental assays using *in vitro* culture techniques, which revealed the ability of early hematopoietic cells to form colonies composed of their progeny of mixed or specific lineage types. These *in vitro* colony-forming assays using semi-solid media were complemented by *in vivo* spleen colony-forming assays, which identified an earlier progenitor or stem cell with the ability to form hematopoietic colonies in recipient mice spleen (Becker, Mc et al. 1963, Bradley and Metcalf 1966). Flow cytometric analysis demonstrated that early HSPC populations could be identified by immunophenotypic markers. These surface markers could be targeted by

immunofluorescent antibodies to sort populations via fluorescence-activated cell sorting (FACS) and subsequent analysis with colony assays (Greaves, Robinson et al. 1981, Ansorge and Täger 2014). Analysis of these colony-forming cells led to the identification of several distinct groups of progenitors (Reya, Morrison et al. 2001). Transplantation assays identified a pluripotent long-term HSC (LT-HSC) that can replenish all blood cells, as well as being able to renew itself by asymmetric division (Baum, Weissman et al. 1992, Morrison and Weissman 1994, Takano, Ema et al. 2004). In mice, these very early cells do not carry any of the phenotypic markers of fully differentiated lineage-committed blood cells and are thus termed lineage negative (Lin<sup>-</sup>). Furthermore, HSC were shown to be positive for two other markers: stem cell antigen-1 (Sca-1) and the cell surface protein tyrosine kinase c-kit (Spangrude, Heimfeld et al. 1988, Ikuta and Weissman 1992). The Lin<sup>-</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup> (LSK) HSC compartment was then subdivided based on the expression of CD34 and fms like tyrosine kinase 3 (Flt3) into the LSKCD34<sup>-</sup>Flt3<sup>-</sup> LT-HSC and LSKCD34<sup>+</sup>Flt3<sup>-</sup> ST-HSC and LSKCD34<sup>+</sup>Flt3<sup>+</sup> MPP (Osawa, Hanada et al. 1996, Adolfsson, Borge et al. 2001, Christensen and Weissman 2001, Yang, Bryder et al. 2005).

The hierarchical model, which has functioned as the basis for hematopoietic research for decades, assumes that pluripotent LT-HSC give rise to short-term hematopoietic stem cells (ST-HSC) and multipotent progenitors (MPP), which in turn form more and more committed precursors (Reya, Morrison et al. 2001). All three of these very early cells can reconstitute hematopoiesis in mice, but only LT-HSC are not limited to a maximum period (Morrison and Weissman 1994). ST-HSC are viable for only six to eight weeks and can reconstitute lethally irradiated mice for up to eight weeks. In contrast to MPP, which can also give rise to hematopoietic cells of all lineages for a limited time, ST-HSC can still self-renew (Morrison and Weissman 1994, Osawa, Hanada et al. 1996, Morrison, Wandycz et al. 1997). Overall, every division results in a more lineage-restricted progenitor and further inhibits the self-renewal capacity, eventually producing fully differentiated blood cells (Till and McCulloch 1980, Morrison, Wandycz et al. 1997).

It was proposed that the MPP gives rise to a common myeloid and a common lymphoid progenitor (CMP, CLP), thus separating the myeloid from the lymphoid lineage. Both of these progenitors were shown to have lost the capacity to self-renew upon transplantation. CMP form megakaryocyte erythrocyte progenitors (MEP) and granulocyte monocyte progenitors (GMP) responsible for differentiating into erythrocytes, megakaryocytes,

granulocytes and monocytes, while CLP differentiate into B- and T-lymphocytes (Kondo, Weissman et al. 1997, Akashi, Traver et al. 2000, Manz, Miyamoto et al. 2002).

This classic model of hematopoiesis described thus far dictates a clear separation of the myeloid and lymphoid lineage differentiation as one of the first steps of maturation. Contrary to this assumption, Adolfsson et al. showed that LSKCD34<sup>+</sup>Flt3<sup>hi</sup> cells, which would be categorized as MPP according to previous publications, lack megakaryocyte and erythroid differentiation potential in *in vitro* colony assays. Furthermore, this population also showed a down-regulation of the corresponding genes *GATA-1* and *EpoR* associated with erythroid development, as well as of the megakaryopoiesis genes *SCL/TAL-1*, *NF-E2* and *TpoR*. Instead, the expression of *IL-7R $\alpha$* , a lymphopoiesis gene, is increased. In concordance with this change in gene expression, LSKCD34<sup>+</sup>Flt3<sup>hi</sup> cells were shown to have the capacity to differentiate into both lymphoid and myeloid lineages and were thus termed a lymphoid-primed multipotent progenitor (LMPP). These findings support the proposal of an alternate model of hematopoiesis, wherein the LSKCD34<sup>+</sup>Flt3<sup>-/low</sup> ST-HSC could give rise to both the LMPP as well as to the previously described CMP as shown in Figure 1 (Adolfsson, Mansson et al. 2005).

The complexity and heterogeneity of cells in the LSK compartment was further illuminated when Forsberg et al. showed that LSKCD34<sup>+</sup>Flt3<sup>+</sup> do have *in vivo* megakaryocyte-erythroid repopulation potential. This publication also reported *EpoR* and *TpoR* mRNA expression in this same subset, overall supporting the previous CLP-CMP model instead of the LMPP model. The authors explain their contradictory findings with differences in the number of cells used and the time points analyzed. They integrate the LMPP as a subgroup of the MPP with the 25% brightest Flt3 expression, characterized by lower overall lineage production, but still retaining the potential to differentiate all lineages (Forsberg, Serwold et al. 2006). Mansson et al. further investigated the LSKCD34<sup>+</sup>Flt3<sup>hi</sup> population in fetal liver and adult BM and argued that the very low numbers of cells they observed co-expressing genes of the meg-erythroid and granulocyte-monocyte lineage may represent a transient form of either newly formed LMPP or cells moving toward lymphoid priming. With this data, it could be proposed that a multitude of progenitors are formed after the HSC stage, including LMPP, CMP and CLP, none of which represent a mandatory intermediary step (Mansson, Hultquist et al. 2007).

## HSC heterogeneity

Since the first introduction of models delineating the earliest stages of hematopoiesis, a better understanding of the HSC itself has also been gained, providing evidence of the great heterogeneity of this compartment.

This was brought about by the use of single-cell transplantation assays in the mouse. It was found that phenotypically homogeneous HSC revealed different patterns of lineage reconstitution in transplanted mice (Sieburg, Cho et al. 2006, Morita, Ema et al. 2010, Yamamoto, Morita et al. 2013). Serially transplanting HSC showed clustering of four possible classes of HSC defined as  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ . Each of these subsets produced a different ratio of myeloid to lymphoid progeny in the host after transplantation.  $\alpha$  cells favored the myeloid lineage  $>2:1$ ,  $\beta$  cells exhibited a more balanced ratio in the range of  $1:4$  to  $2:1$ ,  $\gamma$  cells reconstituted the lymphoid lineage  $<1:4$ , and  $\delta$  cells produced  $<1:4$  lymphoid cells with an overall myeloid reconstitution below one percent. Furthermore, only HSC categorized as  $\alpha$  or  $\beta$  showed robust self-renewal despite the phenotypic homogeneity of cells transplanted. Repopulation patterns often remained stable even after secondary transplantation, which suggests that priming towards different lineages already occurs at the earliest stages of hematopoiesis (Dykstra, Kent et al. 2007).

This heterogeneity found in the HSC compartment was confirmed in experiments performed by Morita et al. to assay the SLAM (signaling, lymphocyte activation molecule) family marker CD150 as a stem cell marker. Their group segregated LSKCD34<sup>-</sup> HSC dependent on CD150 expression into high, medium and negative populations. An enrichment of LT-HSC was found among the LSKCD34<sup>-</sup>CD150<sup>hi</sup> cells. This fraction also houses an LT-HSC with only limited myeloid reconstitution potential that can give rise to all lineages upon secondary transplantation, consistent with an  $\alpha$ -HSC. Overall, 75% of LSKCD34<sup>-</sup>CD150<sup>hi</sup> cells investigated in this study were categorized as  $\alpha$ , with the remaining cells falling in the  $\beta$  category. LSKCD34<sup>-</sup>CD150<sup>-</sup> were deemed exclusively to be either  $\gamma$  or  $\delta$ ; CD150<sup>med</sup> were predominantly  $\delta$ , with the remainder spread evenly across the other three classes (Morita, Ema et al. 2010).



## Characterization of human hematopoiesis

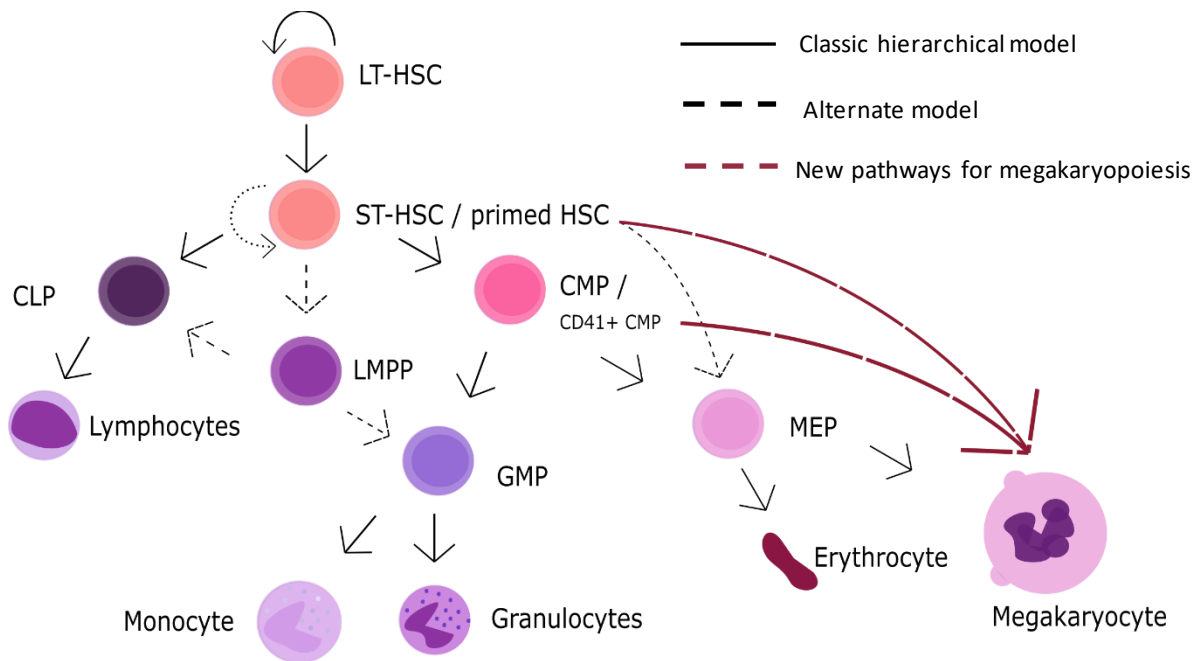
To understand the human system of hematopoiesis, studies on human HSC are necessary to complement the findings reported in mice. The advent of xenotransplantation models, enabling the engraftment of human hematopoietic cells in immunodeficient mice, provided the basis for new studies on human hematopoiesis (Doulatov, Notta et al. 2012). Several *in vitro* assays using only human cells, such as the long-term culture-initiating cell assay supplement, these humanized mouse models (Sutherland, Eaves et al. 1989). The LTC-IC assay is a primitive human HSC assay capable of producing colony-forming cells after a minimum of five weeks of co-culture on a stromal cell layer. Stem cell attributes are attested to these cells, as transplantation of LTC-IC in mice has shown repopulation potential (de Wynter, Buck et al. 1998).

The first marker found in human populations enriched for HSC and progenitors was CD34, calculated to be positive in >99% of human HSC (Civin, Strauss et al. 1984, Doulatov, Notta et al. 2012). While CD34 is also a marker of early cells in mice, not all markers described in the murine system simply transfer to the human system. For example, as described above, Flt3 has been used to identify different subsets of HSPC in mice, with higher levels of Flt3 expression marking more committed stages of hematopoiesis (Forsberg, Serwold et al. 2006, Mansson, Hultquist et al. 2007). This same marker does not appear to have the same expression patterns in human samples. CD34<sup>+</sup>Flt3<sup>+</sup> cells isolated from cord blood and human BM were shown to be capable of *in vivo* reconstitution of both myelopoiesis and lymphopoiesis. Although CD34<sup>+</sup>Flt3<sup>-</sup> formed colonies *in vitro* and were able to reconstitute hematopoiesis *in vivo*, much higher cell numbers were required to achieve engraftment, suggesting that in humans, Flt3 expression is found in more primitive cells rather than in more committed progenitors (Sitnicka, Buza-Vidas et al. 2003). SLAM markers such as CD150 and CD48 have also successfully been used in mice to identify HSC populations, yet these same markers proved insufficient in enriching for human HSC (Larochelle, Savona et al. 2011). Xenotransplantation in sheep fetuses demonstrated long-term engraftment of CD34<sup>+</sup>c-kit<sup>lo</sup> cells, whereas murine HSC are c-kit<sup>+</sup> (Kawashima, Zanjani et al. 1996).

Although the murine markers could not be directly transferred to human HSC, these cells are now well characterized. Numerous studies identified human HSC as CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup>CD45RA<sup>-</sup> (Lansdorp, Sutherland et al. 1990, Baum, Weissman et al. 1992, Bhatia, Wang et al. 1997, Conneally, Cashman et al. 1997). The integrin  $\alpha 6$  (CD49f) has also been

reported as a marker of human HSC. CD49f expression has been found on both CD90+ and CD90- HSC-candidate populations, with both populations exhibiting HSC activity *in vivo*, whereas CD90-CD49f- cells were only capable of transient short-term engraftment (Notta, Doulatov et al. 2011).

Work published by Manz and colleagues has also provided great insight into early human hematopoiesis. Their experiments confirmed that the cells with the highest self-renewing potential lie within the Lin-CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>+</sup> HSC fraction. However, the fraction of Lin-CD34<sup>+</sup> cells that is positive for CD38 was found to contain human progenitors. *In vitro* colony assays and *in vivo* xenotransplantation of Lin-CD34<sup>+</sup>CD38<sup>+</sup> human BM cells defined three distinct subsets of progenitors that closely match the CMP, MEP and GMP found in mice. Lin-CD34<sup>+</sup>CD38<sup>+</sup>CD123<sup>lo</sup>CD45RA<sup>-</sup> CMP produce progeny of all myeloid lineages and give rise to functional GMP (CD123<sup>lo</sup>CD45RA<sup>+</sup>) and MEP (CD123<sup>-</sup>CD45RA<sup>-</sup>). As would be expected of more committed progenitors, these cells have very limited to no self-renewing capacity (Manz, Miyamoto et al. 2002).



**Figure 1: Models of hematopoiesis.** The classic hierarchical model, as proposed by the Weissmann group, is shown with black arrows. An alternative model, as suggested by the Jacobsen group, shown by black dashed arrows. New pathways for megakaryopoiesis are shown with red arrows, as described by Notta et al. (2016) and Miyawaki et al. (2017).

While differential expression of Flt3 in HSCs in mice and humans has already been shown, using Flt3 could be used to successfully differentiate myeloid progenitors between

candidate CMP, GMP and MEP. Cells that are Lin-CD34<sup>+</sup>CD38<sup>+</sup>Flt3<sup>+</sup>CD45RA<sup>-</sup> produce all myeloid lineages with no lymphoid potential, defining these cells as candidate CMP. Lin-CD34<sup>+</sup>CD38<sup>+</sup>Flt3<sup>+</sup>CD45RA<sup>+</sup> cells do not have any meg-erythroid potential and are thus GMP. MEP was found to be Flt3<sup>-</sup>CD45RA<sup>-</sup>. The same study also identified a multi-lymphoid progenitor (MLP [Lin-CD34<sup>+</sup>CD38<sup>-</sup>CD90<sup>neg-lo</sup>CD45RA<sup>+</sup>]), which surprisingly could not only differentiate down the lymphoid lineages but also give rise to myelomonocytic progeny. This finding thus also questions the assumption that differentiation of myeloid and lymphoid cells is split at the CLP/CMP level in humans (Doulatov, Notta et al. 2010). Notta and colleagues were able to show a further subdivision of the human CMP, GMP and MEP by staining for additional markers within the homogeneous progenitor populations. The erythroid marker CD71 and the megakaryocyte marker CD110 were used to define fractions that were either negative for both, positive only for CD71 or positive for both (Notta, Zandi et al. 2016). Analysis of these subsets showed a great degree of heterogeneity. For example, CMP contained mainly bi-potent and not tri-potent cells, as would be expected based on the classic hierarchical model. Furthermore, single-cell assays revealed an increasingly lower percentage of multi-lineage potential in the different CD34<sup>+</sup> isolated during different stages of development. 40% of CD34<sup>+</sup> cells isolated from fetal liver were multi-lineage, while this percentage decreased to 27% in neonatal cord blood and only 18% in adult BM. Furthermore, a restriction of multi-lineage potential to the CD34<sup>+</sup>CD38<sup>-</sup> stem cell compartment in adult BM was found, whereas multi-lineage potential can also be found in the CD34<sup>+</sup>CD38<sup>+</sup> progenitor department in fetal liver. Overall, their experiments revealed distinct changes between fetal and adult hematopoiesis. In BM, the megakaryocytic lineage appeared to be closely linked to multi-lineage potential, whereas megakaryocytic potential was enriched, but not restricted to the HSC compartment in the fetal liver. These findings provided evidence of alternate pathways of lineage differentiation, especially megakaryopoiesis, outside of the CMP-CLP model (Notta, Zandi et al. 2016).

#### CD133 as a stem cell marker

CD133 (also known as AC133 or prominin-1) was first described as a stem cell marker found exclusively on CD34<sup>hi</sup> HSPC found in human fetal liver, BM and blood by Yin and colleagues

in 1997. It is a five-transmembrane antigen, with a glycosylation-dependent epitope that is recognized by the AC133 antibody used in early work to characterize the molecule (Yin, Miraglia et al. 1997, Mizrak, Brittan et al. 2008). AC133 defined a CD34<sup>+</sup> subset with mixed clonogenic potential *in vitro* and primary and secondary repopulating potential *in vivo*. This would indicate that AC133<sup>+</sup> cells include long-term HSC (Yin, Miraglia et al. 1997). CD133 was also found on leukemic blasts isolated from acute myeloid leukemia (AML) patients (Miraglia, Godfrey et al. 1997). The percentage of AC133<sup>+</sup> in the CD34<sup>+</sup> population varied depending on the sample source. The highest percentage (75.3±0.8%) of AC133<sup>+</sup> cells was found in apheresis samples from PB of patients with various neoplasms in remission, while 36.3±2.2% of CD34<sup>+</sup> cells in healthy BM stained positive for AC133. CD34<sup>+</sup>AC133<sup>+</sup> produced more colony-initiating cells in *in vitro* co-cultures on BM stroma and also produced more non-adherent output cells than CD34<sup>+</sup>AC133<sup>-</sup> cells. In agreement with the *in vitro* data, AC133<sup>+</sup> HSPC showed higher engraftment upon transplantation into NOD/SCID mice (de Wynter, Buck et al. 1998). However, AC133 expression is not restricted to hematopoietic cells and has also been described on skeletal muscle and human neural tissue (Bhatia 2001). Further research on CD133 using different antigens produced the evidence that CD133 could, in fact, be the human equivalent of mouse prominin, which is also found in epithelial cells (Corbeil, Roper et al. 2000). This use of an alternate antibody allowed the identification of CD133 on several different tissues, mimicking the expression patterns found in mice (Florek, Haase et al. 2005, Mizrak, Brittan et al. 2008). Fargeas and colleagues have since proposed nomenclature for CD133, and thus it should be clarified that any further mention of CD133 refers to CD133/AC133 unless explicitly stated otherwise (Fargeas, Corbeil et al. 2003).

Additional evidence of CD133 as a stem cell marker was published by Gallacher and colleagues, who reported an extremely rare CD133<sup>+</sup> subset in the Lin<sup>-</sup>CD34<sup>-</sup>CD38<sup>-</sup> fraction of human cord blood with the capability to produce CD34<sup>+</sup> progeny and engraft in mice. This finding could indicate that CD133 expression occurs before CD34 expression in the stem cell hierarchy (Gallacher, Murdoch et al. 2000). In solid tumors, different antigenic CD133 expression has been found on a variety of cancer stem cells, including pancreatic, colon, prostatic and neural tumors (Mizrak, Brittan et al. 2008). In the context of MPN, previous work in our lab has characterized Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> cells as a neoplastic stem cell population in myelofibrosis. These CD34<sup>+</sup>CD133<sup>+</sup> cells carried the *JAK2V617F* driver

mutation with a variable allele burden and exhibited multi-lineage clonogenic potential *in vitro*. Xenotransplantation into immunodeficient NGS mice validated the repopulating potential of CD34<sup>+</sup>CD133<sup>+</sup> cells and produced a PMF phenotype in mice, including atypical megakaryopoiesis and fibrosis (Trivaii, Stubig et al. 2015).

#### Alternate pathways of megakaryopoiesis

Since the first data highlighting HSC heterogeneity has been published, mounting evidence on possible alternate pathways of differentiation, bypassing the stepwise maturation via mandatory intermediary progenitors, has been collected. Erythrocytes and megakaryocytes are particularly essential for any organism to survive, even in completely sterile conditions. Therefore, it seems reasonable that there may be different ways to generate these vital cells more quickly. Different cytokines are needed to produce these cells. For erythropoiesis, the aptly named erythropoietin (EPO) is essential. It is produced in the kidney and functions as the key regulatory molecule for the differentiation and proliferation of the erythroid lineage (Spivak 1989). While its receptor is mainly expressed on erythroid progenitors and it is considered a regulator of late-stage erythropoiesis, possible effects on more primitive hematopoiesis have not been excluded (Krantz and Goldwasser 1984) (Papayannopoulou and Kaushansky 2016). A lack of EPO, as observed in late-stage kidney disease, results in severe anemia in patients without clinically affecting other lineages (Fried 1971). Thrombopoietin was the last hematopoietic cytokine to be discovered and is the strongest promotor of the production of platelets (Kaushansky, Lok et al. 1994). THPO is mainly produced in the liver and, to a lesser extent, by stromal cells and megakaryocytes in the BM (Sungaran, Markovic et al. 1997, Kuter 2013, Nakamura-Ishizu, Takubo et al. 2014). However, its role in hematopoiesis is not restricted to one lineage. It plays a key role in the proliferation of multiple hematopoietic progenitors, as well as being essential for maintaining stem cell quiescence and thus preserving multi-lineage potential (Kuter 2013, Nakamura-Ishizu, Takubo et al. 2014). This central role is evident in patients lacking either THPO or its receptor, who develop pancytopenia as a result (Ballmaier, Germeshausen et al. 2001).

One alternate pathway of megakaryopoiesis was already described in the early 2000s by Takano et al., who reported a direct differentiation of the myeloid-erythroid lineage

through HSC asymmetric division without a CMP intermediary step (Takano, Ema et al. 2004). During the last decade, subsequent studies have been conducted with both mouse and human HSPC.

The development of a transgenic mouse expressing the Kusabira-Orange (Momen-Heravi, Balaj et al.) fluorescent protein in all hematopoietic cells greatly improved the tracking of progeny in transplanted mice. These mice were used to investigate repopulation patterns from single cells belonging to the LSKCD34<sup>-</sup> fractions that were either CD150<sup>+</sup>CD41<sup>-</sup>, CD150<sup>+</sup>CD41<sup>+</sup> or CD150<sup>-</sup>CD41<sup>-</sup> (Yamamoto, Morita et al. 2013). CD41, also known as the glycoprotein (Gp) IIb/IIIa integrin, plays an important role in coagulation as the platelet receptor for fibrinogen (Debili, Robin et al. 2001). In the study published by Yamamoto et al., LT-HSC, retrospectively defined by secondary transplantation, could only be detected in the LSKCD150<sup>+</sup>CD41<sup>-</sup> population. Interestingly, the phenotypically defined HSC were shown to contain some cells exhibiting myeloid-restricted repopulation patterns, as would be expected of CMP, MEP and MKP, and thus termed common myeloid repopulating progenitor [CMRP], megakaryocyte-erythroid repopulating progenitor [MERP] and megakaryocyte repopulating progenitor [MkRP], respectively. The self-renewing capacity of these myeloid-restricted progenitors suggested the possibility of cells differentiating down the myeloid lineage without the intermediary steps of LMPP and CMP. In fact, *in vitro* and paired daughter cell (PDC) *in vivo* assays, following the individual fate of the first generation daughter cells of a single HSC, showed MkRPs to be directly derived from CD150<sup>+</sup>CD41<sup>-</sup> and CD41<sup>+</sup> HSC (Yamamoto, Morita et al. 2013).

CD41 was also investigated by Miyawaki and colleagues to characterize another pathway of megakaryopoiesis in humans. Using CD41 expression to distinguish two CMP (CD34<sup>+</sup>CD38<sup>+</sup>IL-3 $\alpha$ <sup>dim</sup>CD45RA<sup>-</sup>) populations, they were able to identify CD41<sup>+</sup>CMP as uni-potent megakaryocyte progenitors in adult human BM. CD41 positivity was not detected in GMP and only in 1% of MEP. These CD41<sup>+</sup>CMP were restricted to megakaryocyte colony formation in *in vitro* colony assays and robustly primarily produced platelets after intrafemoral transplantation *in vivo*. Furthermore, CD41<sup>+</sup>CMP expressed megakaryocyte lineage-specific genes and lacked erythroid and myeloid gene expression at the single-cell level, further underlining their uni-potent potential. Stimulation of CD41<sup>-</sup>CMP with thrombopoietin (THPO), a cytokine essential to megakaryopoiesis, produced CD41<sup>+</sup>CMP, which supports the conclusion that CD41<sup>+</sup>CMP lie downstream of their CD41<sup>-</sup> counterparts.

In addition to CD41, another protein essential to platelet aggregation, *von Willebrand factor* (vWF), has been identified as a marker of murine megakaryopoiesis. The vWF gene is among those highly expressed in HSC-enriched populations (Mansson, Hultquist et al. 2007). Thus, prompted to investigate if vWF could be a marker of a megakaryocyte-primed HSC, Sanjuan et al. reported LSKCD34<sup>-</sup>CD150<sup>+</sup>CD48<sup>-</sup>HSC to be 60% positive for vWF. Transplantation of this subset revealed a strong platelet-bias of the vWF<sup>+</sup> fraction that was maintained for 32 weeks and persisted even after serial transplantation, as well as when transplanted as single cells. vWF<sup>+</sup> HSC also showed higher expression of megakaryocyte-associated genes (*Selp*, *Sdpr*, *Gpr64*, *Clu*) and *Mpl*. They also provided data to demonstrate that vWF<sup>+</sup> HSC are higher in the hierarchy, as these cells were able to produce both vWF<sup>+</sup> and vWF<sup>-</sup> HSC, while the latter could only give rise to vWF<sup>-</sup> HSC (Sanjuan-Pla, Macaulay et al. 2013).

All these various findings point toward several additional pathways of megakaryopoiesis. Naturally, this raises the question of how these relate to each other and what factors decide which road is taken. The body's need for platelets can vary greatly, prompting Haas and colleagues to investigate how the increased demand during inflammation would affect the process of megakaryopoiesis. Simulation of inflammation led to an increase of protein expression associated with megakaryopoiesis in HSC (Lin<sup>-</sup>c-kit<sup>+</sup>CD150<sup>+</sup>CD48<sup>-</sup>). These proteins included CD41 and vWF (Haas, Hansson et al. 2015). Testing of the *in vitro* clonogenic output of this HSC population revealed a subset committed to the megakaryocytic lineage (stem cell-like megakaryocyte progenitors [SL-MkP]), corroborating data on MkRP published previously (Yamamoto, Morita et al. 2013). No difference in HSC colony output could be observed between homeostasis and the inflammation setting, supporting the conclusion that changes in the expression of megakaryocytic proteins do not influence lineage fate decisions. However, SL-MkP isolated from mice after inflammation induction exhibited faster maturation within the overall smaller megakaryocytic colonies, as compared to SL-MkP isolated during homeostasis. SL-MkP appear to be mainly quiescent and contribute little to megakaryopoiesis in homeostasis, revealing their megakaryocytic potential only when stimulated, such as in the setting of inflammation. The induction of CD41 expression observed in activated SL-MkP may point towards high CD41 expression as a marker for megakaryocyte commitment. Analysis of the expression of megakaryocyte transcriptome revealed SL-MkP to be a sub-

fraction of the previously described vWF<sup>+</sup>HSC (Sanjuan-Pla, Macaulay et al. 2013, Haas, Hansson et al. 2015).

In summary, it has successfully been shown that differentiation of megakaryocytes does not have to occur gradually through different progenitors, but that these lineages can be directly differentiated from the HSC compartment (Figure 1) (Sanjuan-Pla, Macaulay et al. 2013, Haas, Hansson et al. 2015, Notta, Zandi et al. 2016). Several different pathways detailed above and reviewed by Xavier-Ferruccio and Krause have been described, ranging from direct differentiation from the meg-primed HSC to intermediate steps of either uni-potent megakaryocyte progenitors or bi-potent MEP (Xavier-Ferruccio and Krause 2018). How much each pathway contributes to platelet formation in homeostasis is unknown, as well as what role these fractions could potentially play in the setting of hematological disorders, such as myeloproliferative neoplasms.

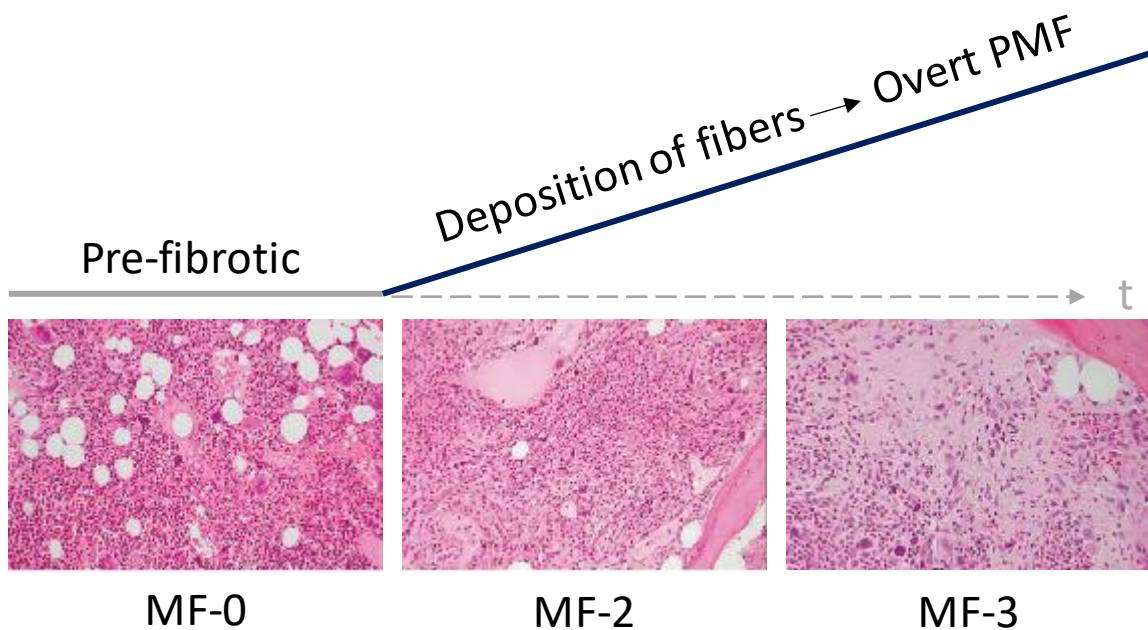
### Myeloproliferative neoplasms

Myeloid neoplasms are a family of blood cancers, which can generally present as either acute or as chronic. The disease is classified as AML when the number of blasts in the BM or circulating in the peripheral blood (PB) is equal to or exceeds 20%. Chronic myeloid neoplasms (with lower blast counts) can be further divided into myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDS), MDS/MPN overlap and myeloid/lymphoid neoplasms with eosinophilia and recurrent rearrangements (Barbui, Thiele et al. 2018). MPN are all defined by clonal HSC proliferation resulting in the expansion of one or more of the myeloid (e.g., erythroid, megakaryocytic, granulocytic or mast cell) lineages of hematopoiesis. Both sexes are affected, and the peak of diagnosis is around the 5<sup>th</sup>–7<sup>th</sup> decade of life. A combination of morphological, clinical and molecular genetic characteristics is employed to differentiate between the classic *BCR-ABL1* negative MPNs (primary myelofibrosis [PMF], essential thrombocythemia [ET] and polycythemia vera [PV], which are the focus of this work, and other MPNs. These include *BCR-ABL1* positive chronic myeloid leukemia (CML), as well as chronic neutrophilic leukemia (CNL), chronic eosinophilic leukemia - not otherwise specified (CEL-NOS), and MPN - unclassifiable (MPN-U) (Swerdlow, Campo et al. 2017).



## Primary Myelofibrosis

PMF affects all three hematopoietic lineages of erythropoiesis, granulopoiesis and megakaryopoiesis. Patients at different stages of the disease may exhibit very heterogeneous symptoms, such as anemia, leukocytosis or leukopenia, thrombocytosis or thrombopenia. While all MPN can ultimately result in BM failure, the pathogenesis leading to it can vary greatly. In PMF, BM failure is caused by myelofibrosis (MF). Erroneous fiber deposition in the BM forces mobilization of HSPC into the bloodstream and necessitates extramedullary hematopoiesis in the spleen and liver, resulting in hepatosplenomegaly.



**Figure 2:** Dynamic of primary myelofibrosis and grading of myelofibrosis. Images of Hematoxylin-Eosin stained BM biopsies with MF grading from Gianelli, Vener et al. 2012

PMF is thought to begin as pre-fibrotic/early PMF, marked by the proliferation of aberrant megakaryocytes and granulocytes in the BM. Over time the disease transforms into overt fibrotic PMF, dominated by myelofibrosis and leukoerythroblastosis in the PB, as shown in Figure 2. Symptoms of overt PMF reflect the consequences of extramedullary hematopoiesis. Splenomegaly, as well as circulating immature cells and teardrop-shaped erythrocytes, are pathognomonic of the fibrotic stage. Transformation to acute myeloid leukemia is also possible (Geyer, Scherber et al. 2014, Swerdlow, Campo et al. 2017).

Meeting all major and one minor diagnostic criterion listed below in Table 1 is required to diagnose the pre-fibrotic and fibrotic phase of PMF respectively, according to the latest 2016 edition of the WHO classification of Tumours of Hematopoietic and Lymphoid Tissues:

Pre-fibrotic/early PMF	Overt PMF
<i>Major criteria</i>	
1. Megakaryocytic proliferation and atypia, without reticulin fibrosis > grade I, accompanied by increased age-adjusted BM cellularity, granulocytic proliferation and often decreased erythropoiesis	1. Megakaryocyte proliferation and atypia accompanied by either reticulin and/or collagen fibrosis (grade II or III)
2. Not meeting WHO criteria for <i>BCR-ABL1+</i> CML, PV, ET, MDS or other myeloid neoplasm	2. Not meeting WHO criteria for <i>BCR-ABL1+</i> CML, PV, ET, MDS or other myeloid neoplasm
3. Presence of <i>JAK2</i> , <i>CALR</i> or <i>MPL</i> mutation or another clonal marker or absence of minor reactive BM reticulin fibrosis	3. Presence of <i>JAK2</i> , <i>CALR</i> or <i>MPL</i> mutation or another clonal marker or absence of evidence for reactive BM fibrosis
<i>Minor criteria</i>	
1. Presence of one or more of the following, confirmed in two consecutive determinations: <ul style="list-style-type: none"> <li>• Anemia not attributed to a comorbid condition</li> <li>• Leukocytosis <math>\geq 11 \times 10^9/L</math></li> <li>• Palpable splenomegaly</li> <li>• LDH level above the upper limit of the institutional reference range</li> </ul>	1. Presence of one or more of the following confirmed in two consecutive determinations: <ul style="list-style-type: none"> <li>• Anemia not attributed to a comorbid condition</li> <li>• Leukocytosis <math>\geq 11 \times 10^9/L</math></li> <li>• Palpable splenomegaly</li> <li>• LDH level above the upper limit of the institutional reference range</li> <li>• Leukoerythroblastosis</li> </ul>

**Table 1:** 2016 WHO diagnostic criteria for PMF

Table adapted from Barburi et al. *Blood Cancer Journal* (2018)8:15 (Barbui, Thiele et al. 2018)

### Essential thrombocythemia and Polycythemia vera

ET primarily involves the megakaryocytic lineage, while PV affects all three myeloid lineages with a clinical dominance of the erythroid lineage. Patients suffering from ET or PV do not always develop myelofibrosis but can transform to post-ET MF and post-PV MF, respectively, or progress to acute leukemic blast phase (Passamonti, Rumi et al. 2005, Swerdlow, Campo et al. 2017). Many patients are asymptomatic at the time of diagnosis. If there are symptoms, they are closely associated with an overabundance of platelets or erythrocytes (e.g., thrombosis and hemorrhage or hypertension) (Swerdlow, Campo et al. 2017). In asymptomatic patients presenting with isolated erythrocytosis or thrombocytosis, it is essential to exclude reactive causes of the change in blood values, such as orthopnea or chronic inflammatory diseases, etc. (Passamonti and Maffioli 2016). Splenomegaly can accompany either disorder, however, it is seen less often than in PMF (20-30%) and is usually not massive (Passamonti, Rumi et al. 2008, Passamonti, Rumi et al. 2010). According to the 2016 WHO diagnostic criteria to diagnose a patient with ET either, all four major criteria or the first three major and a minor criterion must be met. For a PV diagnosis either

all three major criteria or the first two major and the minor criterion has to be fulfilled (Table 2) (Swerdlow, Campo et al. 2017).

Essential thrombocythemia	Polycythemia vera
<b>Major criteria</b>	
1. Platelet count $\geq 450 \times 10^9/L$	1. Hb > 16.5 g/dl ( $\sigma$ ) / > 16.0 g/dl ( $\varphi$ ) or Hct > 49% ( $\sigma$ ) / > 48% ( $\varphi$ ) or increased red cell mass
2. BM morphology: proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant left-shift of neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers	2. BM morphology: hypercellularity for age with trilineage growth (panmyelosis), including prominent erythroid, granulocytic and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)
3. Not meeting WHO criteria for <i>BCR-ABL1</i> + CML, PV, PMF, MDS, or other myeloid neoplasms	3. Presence of JAK2 or JAK2 exon 12 mutation
4. <i>JAK2</i> , <i>CALR</i> or <i>MPL</i> mutation	
<b>Minor criteria</b>	
1. Presence of a clonal marker (e.g., abnormal karyotype) or absence of evidence for reactive thrombocytosis	1. Subnormal serum erythropoietin level

**Table 2:** 2016 WHO diagnostic criteria for essential thrombocythemia and polycythemia vera  
Table adapted from Barburi et al. *Blood Cancer Journal* (2018)8:15 (Barbui, Thiele et al. 2018)

### Genetic mutations associated with MPNs

Distinct mutations have been described as drivers of these diseases. Mutations of the *JAK2*, *MPL* and *CALR* genes are found in all classic MPN. The discovery of these mutations was a breakthrough in understanding the pathophysiology of these disorders (Tefferi 2010). In the early 2000s, the significance of the receptor-associated Janus kinase 2 (*JAK2*) as a common signaling pathway of hematological cytokines such as THPO and EPO was well-established. Binding of a ligand such as THPO to its transmembrane receptor *MPL* activates the cytoplasmic *JAK2* by phosphorylation. This allows the binding of *STAT* (*signal transducer and activator of transcription*) transcription factors and their phosphorylation to induce downstream signaling (Kaushansky 1997, Heinrich, Haan et al. 2014). In addition, several studies indicated that the *JAK2* pathway plays a central role in hematopoietic signal transduction in the setting of PMF and PV (Komura, Chagraoui et al. 2003, Ugo, Marzac et al. 2004).

Screening of MPN patients for genetic mutations in the *JAK2* gene led to the discovery of the single point mutation *JAK2V617F*, which exchanges valine (V) at position 617 for

phenylalanine (F). *JAK2V617F* is detectable in up to 97% of PV patients and 50-60% of patients with ET and PMF. (Baxter, Scott et al. 2005). The *JAK2V617F* mutation, as well as exon 12 mutations found in the remaining PV patients, result in the constitutive activation of the kinase and subsequent activation of the downstream STAT signaling pathway. This activation enables aberrant cytokine-independent cell proliferation (Scott, Tong et al. 2007, Milosevic Feenstra, Nivarthi et al. 2016). In *JAK2* negative patients, the upstream *MPL* receptor was found to harbor mutations in 9% of studied cases. *MPLW515L* and the *MPLW515K* are the two most common *MPL* mutations, which encode for either leucine (L) or lysine (K) instead of tryptophan (W) at codon 515 of the gene (Pardanani, Levine et al. 2006). Similar to the previously described *JAK2* mutation, the somatic *MPLW515L* and *MPLW515K* mutations also constitutively activate the JAK-STAT pathway. Depending on the study, *MPL* mutations are reported in 3-15% of ET and PMF patients overall (Pikman, Lee et al. 2006, Tefferi 2010, Pardanani, Guglielmelli et al. 2011). Following the discovery of the mutated *MPL*, mutations of the calreticulin-encoding gene *CALR* were discovered. Calreticulin has numerous functions as an endoplasmic reticulum chaperone and plays a role in calcium homeostasis. Mutations identified in exon 9 of *CALR* generate proteins with an altered carboxyl-terminus, resulting in the loss of the KDEL endoplasmic reticulum retention signal. The two most common alterations found are categorized as type I, with a 52bp deletion, and type II, with a 5 bp insertion. These frameshift mutations can be detected in PMF and ET, but not PV patients (Klampfl, Gisslinger et al. 2013, Nangalia, Massie et al. 2013). *CALR* mutants can only activate the STAT pathway by binding to the *MPL* receptor (Marty, Pecquet et al. 2016). Thus, although all of the described MPN driver mutations share a constitutive activation of the JAK-STAT pathway, their role within the THPO-MPL induced signaling cascade, which regulates HSC and megakaryopoiesis, is likely the most critical in ET and PMF. Importantly, all three driver mutations have been shown to induce an MPN phenotype with progression to secondary myelofibrosis in mouse models, underlining their crucial role in MPN pathophysiology (Lacout, Pisani et al. 2006, Pikman, Lee et al. 2006, Marty, Pecquet et al. 2016).

If none of the mutations detailed above are found, the patient is classified as triple negative. In recent years, further genetic analysis of ET and PMF patients has led to the discovery of additional mutations in *JAK2* and *MPL* (Milosevic Feenstra, Nivarthi et al. 2016). In addition to driver mutations, mutations in genes that modulate DNA methylation

(e.g., TET2, DNMT3A) are also found in MPN, which likely enhances the observed clonal hematopoiesis (Vainchenker and Kralovics 2017, Gangat and Tefferi 2020). Mutations in genes regulating histone modifications (e.g., EZH2 and ASXL1) or the RNA splicing machinery (e.g., U2AF1 and SRSF2) have also been described in MF patients (Delhommeau, Dupont et al. 2009, Shi, Yamamoto et al. 2016, Triviai, Zeschke et al. 2018, Liu, Illar et al. 2020). As these gene mutations are also found at a high incidence in myelodysplastic syndrome (MDS), it is generally accepted that these mutations contribute to the increased propensity to AML and poor prognosis of this subset of MPN (Wang, Zhang et al. 2019, Liu, Illar et al. 2020).

The only curative treatment option for myelofibrosis is allogenic stem cell transplantation. Transplanting MF patients results in a near or complete regression of myelofibrosis after one year. The mechanisms governing this remission are also still under investigation (Kroger, Holler et al. 2009).

### Understanding fibrogenesis

Despite many advances in the field of MPN research, the exact mechanisms behind and the primary cell responsible for fibrogenesis in myelofibrosis remain elusive. Myelofibrosis is defined as increased deposition of collagen or reticulin fibers in the BM. The fibrosis of the BM is thought to evolve from myofibroblasts, which have also been shown as a source of fibrosis in pulmonary, cardiac and liver disease (El Agha, Kramann et al. 2017). While fibroblasts produce the extracellular matrix, it is hypothesized that it is the interaction between the hematopoietic cells of the aberrant clone and the HSC niche, which drives fibrogenesis (Schneider, Mullally et al. 2017). The key role of the HSC's microenvironment in the BM in regulating stem cell function is generally accepted. It was first observed in 1978 that changes in cells, which together make up the microenvironment or niche of the HSC, are pivotal to HSC maintenance and regulation. In turn, HSC also influence other cells of their niche (Schofield 1978, Morrison and Spradling 2008). Different HSC niches in the BM have been described, likely reflecting the heterogeneity of the stem cell compartment. It has been shown that HSC can be found both in a perivascular, as well as in an endosteal niche (Morrison and Scadden 2014). The perivascular niche includes mesenchymal stromal cells (MSC) and megakaryocytes, which are often found directly adjacent to HSCs (Bruns,

Lucas et al. 2014, Crane, Jeffery et al. 2017). These cell types are important, as both have been implicated in playing a role in the development of fibrosis. Fibroblasts in the BM are not part of the abnormal clone in MPN (Jacobson, Salo et al. 1978). Instead, it is hypothesized that the healthy cells of the niche reactively produce myelofibrosis as a response to stimuli from the aberrant clone. There is mounting evidence to suggest a relationship between aberrant megakaryocytes found in MPN and myelofibrosis (Burstein, Malpass et al. 1984, Ciurea, Merchant et al. 2007, Gangat and Tefferi 2020). It was shown almost thirty years ago that megakaryocytes can stimulate resident fibroblasts capable of collagen production, thus leading to fibrosis (Castro-Malaspina, Rabellino et al. 1981). Overexpression of THPO in mouse BM also causes the animals to develop myelofibrosis and osteosclerosis, which is salvageable by secondary BM transplantation (Yan, Lacey et al. 1996). Elevated levels of the chemokine C-X-C motif ligand 4 (CXCL-4 or platelet factor 4), a cytokine released from the  $\alpha$ -granules of megakaryocytes, have been detected in MF patients (Burstein, Malpass et al. 1984). Furthermore, megakaryocytes in the HSC BM niche can directly regulate HSC behavior, such as promoting quiescence, through the release of CXCL-4 (Bruns, Lucas et al. 2014). In MDS, aberrant myelopoiesis has been linked to lower levels of CXCL-4 compared to healthy individuals (Aivado, Spentzos et al. 2007).

More recently, Gli1<sup>+</sup> MSC were reported as potential driver cells of myelofibrosis. Gli1<sup>+</sup> MSCs were found in both the endosteal, as well as the perivascular region in human adult BM and stained positive for the MSC markers CD105, CD90 and nestin. (Schneider, Mullally et al. 2017). The THPO-overexpression mouse model was used to investigate the role of Gli1<sup>+</sup> MSC in fibrogenesis. Reconstitution of irradiated mice with THPO-expressing HSC resulted in a significant expansion of Gli1<sup>+</sup> MSC and differentiation towards myofibroblasts. When Gli1<sup>+</sup> MSC were ablated prior to THPO overexpression, the mice did not develop myelofibrosis, indicating that this cell population plays a pivotal role in fibrogenesis. In a different model, transplanting HSC expressing the MPN driver mutation *JAK2V617F* also led to expansion of Gli1<sup>+</sup> cells and led to myelofibrosis. Analysis of cytokine expression in the two myelofibrosis models showed an upregulation of CXCL-4. CXCL-4 also was shown to induce *in vitro* myofibroblasts differentiation in Gli1<sup>+</sup> MSC. Corresponding to the mouse data, significantly higher numbers of Gli1<sup>+</sup> MSC were also found in BM biopsies of MPN patients (Schneider, Mullally et al. 2017)

## Characterization of the Neoplastic Stem Cell in Myelofibrosis

It is generally accepted that the clonal expansion observed in MPNs originates from a neoplastic multipotent stem cell (Mead and Mullally 2017). This hypothesis is based on numerous studies conducted over the last decades. Early evidence for the clonal origin of MPN was provided through clonality studies based on X-chromosome genes (Tefferi 2000). X-chromosome-linked enzymes are an example of naturally occurring cellular mosaicism. In a female individual, heterozygosity for an allele found on the X chromosome will produce a mixed phenotype in the same tissue depending on which allele is active. If a tumor has a clonal origin, only one phenotype is found in the daughter cells. One such study performed by Adamson and colleagues reported a single-phenotype enzyme found in various differentiated hematopoietic lineages of female PV patients, indicating a clonal neoplasm (Adamson, Fialkow et al. 1976). This hypothesis was confirmed by analysis of the driver mutations found in MPNs. *JAK2V617F* correlates with clonal hematopoiesis and has been found at the stem cell level, indicating this compartment as the origin of the disease (James, Ugo et al. 2005, Jamieson, Gotlib et al. 2006).

More recently, reports of the aberrant behavior of CD34<sup>+</sup> HSC from MF patients in both *in vitro* and *in vivo* assays have provided further evidence of the neoplastic HSC in myelofibrosis (Xu, Bruno et al. 2005). Work in our lab has also defined a CD34<sup>+</sup>CD133<sup>+</sup> multipotent cell in the PB of PMF patients that recapitulates many aspects of the disease as detailed above and carries the *JAK2V617F* driver mutation (Trivai, Stubig et al. 2015).

## Aim

A hallmark of ET and PMF is the increased level and aberrant maturation of megakaryocytes, which in turn likely play a vital role in the induction of fibrosis. Thus, cell systems are required to determine the mechanism by which external (e.g., THPO) or internal (e.g., mutated differentiation programs) stimuli influence megakaryocytic differentiation from the CD133<sup>+</sup> neoplastic stem cell. In the early stages of the disease, the neoplastic stem cell resides in the BM, contributing directly or indirectly to the induction of fibrosis. Extensive fibrosis eventually leads to the expulsion of CD34<sup>+</sup> cells from the BM. Although increased CD133<sup>+</sup> neoplastic stem cells are subsequently found in the PB, it is likely that these cells arise from rare stem cells maintained in the BM (despite the extensive

fibrosis) or in the spleen, which has also been shown to harbor neoplastic stem cells (Wang, Prakash et al. 2012). These resident stem cells, therefore, may still interact with a stroma environment.

To better characterize the neoplastic stem cell that drives PMF within a stroma compartment, the first aim of this work was to investigate if the abnormal stem cell microenvironment with increased levels of cytokines would alter the survival as well as the proliferative and differentiation potential of the neoplastic Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> population from MF patients. An *in vitro* long-term co-culture system was established using the human stromal cell line HS5, which was transduced to overproduce THPO or GM-CSF.

In a second approach, the CD133<sup>+</sup> neoplastic stem cells were subject to further immunophenotypic and functional analysis to determine their self-renewal and differentiation potential. During the initial phase of these studies, experimental evidence suggested that commitment to the megakaryocytic lineages occurred at the HSC/MPP stage human adults (Notta, Zandi et al. 2016). Another study reported the existence of a CD41<sup>+</sup> uni-potent megakaryocyte-progenitor at the CMP level in humans, which was also increased in ET patients, making this a possible candidate population sustaining aberrant megakaryopoiesis in MPN (Miyawaki, Iwasaki et al. 2017).

We thus asked the question if this CD41<sup>+</sup> population can also be detected in Myelofibrosis. If so, do these cells represent an early, possibly neoplastic, HSC with megakaryocytic propensity as described in the mouse and hypothesized in humans or is this a uni-potent CMP that expands in patient PB? Co-culture experiments with stroma cultures coupled with colony assays, provide the conditions to assess longevity and differentiation potential to answer these questions.



## Materials and Methods

### Materials

#### Equipment

**Table 3:** List of used instruments

Instrument	Name	Supplier
Centrifuge	Multifuge 3 S-R	Heraeus
Shaking water bath	1083	GFL
Fluorescence microscope	IX81	Olympus
Inverted light microscope	Wilovert S	Hund Wetzlar
Pipettes (10 µl, 200 µl, 1000 µl)	Research plus	Eppendorf
Pipetting aid	Easy Pet 3	Eppendorf
Vortexer	Reax 2000	Heidolph
Safety cabinet	Hera Safe	Heraeus
Incubator (colony assays)	Hera Cell	Heraeus
Incubator (cell culture)	Hera Cell 240	Heraeus
Cytocentrifuge	Cytospin 2	Shandon
Cell Counter	KX-21N	Sysmex
Apotome Microscope	Axiovert 200 MAT	Zeiss
Flow cytometry cell sorters	FACS Aria Fusion, FACS Aria III, FACS Canto II	BD
Microcentrifuge	MC-13	Heraeus
ELISA washer	Columbus Pro	Tecan
ELISA reader	Infinite F200 Pro	Tecan

#### Consumables

**Table 4:** List of used consumables

Item	Size/Details	Supplier	Art. Nr.
Filter tips	100 µl 200 µl 1250 µl	Sarstedt (Biosphere)	70.1186.210 70.760.212 70.760.211
Filter tips	10 µl	Greiner-bio-one	771 261
Serological pipettes	5 ml 10 ml	Sarstedt	86.1253.001 86.1254.001
Serological pipettes	2ml 25ml	Falcon	356507 356525
Centrifuge tubes	15 ml 50 ml	Cellstar by Greiner Bio One	188 271 227 261
FACS tubes	12x75 mm round-bottom tube	Falcon	352054

<b>Microtubes</b>	1.5 ml Safe Seal	Sarstedt	72.706.400
<b>Low-protein-binding tubes</b>	Protein LoBind Tube 1.5ml	Eppendorf	022431081
<b>Cryovials</b>	1.8 ml	Thermo Scientific	363401
<b>Cell culture plates</b>	6 wells 24 wells 96 wells	Cellstar by Greiner Bio One	657 160 662 160 655180
<b>Cell culture flasks</b>	T25 T75	Cellstar by Greiner Bio One	690175 658175
<b>Cell culture dish</b>	35x10 mm (Nunclon Delta)	Thermo Fischer Scientific	153066
<b>Cell culture dish</b>	10cm	Sarstedt	83.3902
<b>Chamber slides</b>	Double chambers	Stemcell Technologies	04813
<b>Filter cards and spacer membranes</b>	For double chamber slides	Stemcell Technologies	04911
<b>Cell strainers</b>	CellTrics 30 µm	Sysmex	04-004-2326
<b>Syringe filters</b>	Filtropur S 0.2 20µm	Sarstedt	83.1826.001
<b>Filter cards</b>	Shandon Filter cards (thick)	Thermo Scientific	5991022
<b>Cytoslide</b>	Microscope slides for Shandon CytoSpin	Thermo Scientific	5991056
<b>Syringes</b>	Single-use syringe 2ml	B.Braun	4606710V
<b>Cannula</b>	Dialysis cannula 1.6mm	Togo Medikit	SP502-16(33)

## Reagents, media and media components

**Table 5:** List of used reagents, media and media components

Reagents/Media/Media Component	Supplier	Art. Nr.
<b>Dulbecco's phosphate buffered saline (DPBS 1x)</b>	Gibco	14190-094
<b>Distilled water</b>	B.Braun	387875
<b>Heat inactivated fetal bovine serum (FBS)</b>	Gibco	10500
<b>Penicillin-Streptomycin (10000 U/ml) (PenStrep)</b>	Gibco	15140122
<b>0.25% Trypsin-EDTA</b>	Gibco	25200-056
<b>Minimum essential medium (Nishikii, Kanazawa et al.) Alpha</b>	Gibco	12561-056
<b>Iscove's modified Dulbecco's medium (IMDM 1x)</b>	Gibco	12440-053
<b>MethoCult H4435, enriched</b>	Stemcell Technologies	04435
<b>MegaCult-C</b>	Stemcell Technologies	04951
<b>MyeloCult H5100</b>	Stemcell Technologies	05100
<b>Hydrocortisone</b>	Sigma	H0888
<b>Collagen solution</b>	Stemcell Technologies	04902
<b>RPMI 1640</b>	Gibco	11875085
<b>Dulbecco's Modified Eagle Medium (DMEM)</b>	Gibco	11995065
<b>Red cell lysis buffer (10x)</b>	BioLegend	420301
<b>RosetteSep Human Bone Marrow Progenitor Cell Pre-Enrichment Cocktail</b>	Stemcell Technologies	15027
<b>Ficoll-Paque PLUS</b>	GE Healthcare	17-1440-03
<b>TRIzol reagent</b>	Ambion	15596-026
<b>Trypan blue solution (0.4%)</b>	Sigma	T8154
<b>Cell Trace Violet™</b>	Thermo Scientific	C34557
<b>Giemsa solution</b>	Merck	1.09204.0500

May-Grünwald's solution	Merck	1.01424.0500
Tablets for buffer acc. to WEISE	Merck	111374
Methanol	Roth	4627.1
Acetone	ChemSolute	2614.1000
Sodium chloride	Roth	9265.2
StemSpan SFEM II	Stemcell Technologies	09605
StemSpan Megakaryocyte Expansion Supplement	Stemcell Technologies	02696
Potassium chloride	Sigma	P9333
Trizma base	Sigma	T1503-1KG
Magnesium chloride	Roth	2189.2
Tween 20	Roth	9127.1
Levamisole solution (200mM)	Vector Laboratories	SP-5000
Skimmed milk powder	frema Reform	0201
Vector Red	Vector	SK-5100
Nitro-blue tetrazolium chloride/5-Brom-4-chlor-3-indoxylphosphat (NBT/BCIP)	Sigma	B1911
Avidin/Biotin Blocking kit	Vector	SP-2001
Alkaline phosphatase	Vector	AK-5000
QIAamp DNA Micro Kit	Qiagen	56304
Human Thrombopoietin DuoSet ELISA kit	R&D Systems	DY288
Ethanol absolut	ChemSolute	2273.1000
Triton X-100	Sigma	9002-93-1
Propidium iodide	Life technologies	P3566
RNase A	Thermo Fischer	12091021

## Cell lines

**Table 6:** List of used cell lines

Cell line	Cell Type	Tissue	Supplier
HS5 (ATCC CRL-11882)	HPV-16 transformed E6/E7	Bone marrow /stroma	ATCC
TF-1 (ATCC CRL-2003)	Erythroblast	Bone marrow	ATCC
UT7/TPO	Megkaryoblastic leukemia	Bone marrow	Kindly provided by the Division of Hematology of the Jichi Medical School

## Cytokines

**Table 7:** List of used cytokines and growth factors

Cytokine	Supplier	Art. Nr.
SCF	PeproTech	300-07
EPO	PeproTech	100-64
IL3	Gibco BRL	13269-030
IL11	PeproTech	200-11
THPO	PeproTech	300-18
FLT3L	PeproTech	300-19
b-FGF	R&D Systems	234-FSE

## Fluorochrome-conjugated antibodies

**Table 8:** List of used fluorochrome-conjugated antibodies

	Clone	Supplier	Art. Nr.
<b>CD2 FITC</b>	RPA-2.10	BD Pharmingen	555326
<b>CD3 FITC</b>	HIT3a	BD Pharmingen	555339
<b>CD4 FITC</b>	RPA-T4	BD Pharmingen	555346
<b>CD8 FITC</b>	DK25	Dako	F0765
<b>CD11b FITC</b>	ICRF44	BD Pharmingen	562793
<b>CD14 FITC</b>	RMO52	Beckman Coulter	IM0645
<b>CD19 FITC</b>	HIB19	BD Pharmingen	555412
<b>CD20 FITC</b>	2H7	BD Pharmingen	555622
<b>CD34 APC</b>	581	BD Pharmingen	555824
<b>CD34 PerCP Cy5.5</b>	581	BioLegend	343522
<b>CD38 APC-H7</b>	HB7	BD Pharmingen	656646
<b>CD41 BV510</b>	HIP8	BioLegend	303736
<b>CD45 APC</b>	HI30	BD Pharmingen	555485
<b>CD45 BV605</b>	HI30	BioLegend	304041
<b>CD45 eFluor 450</b>	2D1	eBiosciences	48-9459-41
<b>CD45RA</b>	HI100	BD Pharmingen	550855
<b>CD123</b>	6H6	BioLegend	306032
<b>CD56 FITC</b>	B159	BD Pharmingen	562794
<b>CD90 PE-Cy7</b>	5E10	BD Pharmingen	561558
<b>CD133 PE</b>	293C3	Miltenyi Biotec	130-090-853
<b>CD235a FITC</b>	JC159	Dako	F0870
<b>CD235a PE</b>	HIR2	eBioscience	12-9987-82
<b>CD73 APC</b>	AD2	BD Pharmingen	580847

## Lentiviral Gene Ontology Vectors

**Table 9:** List of used LeGo Vectors

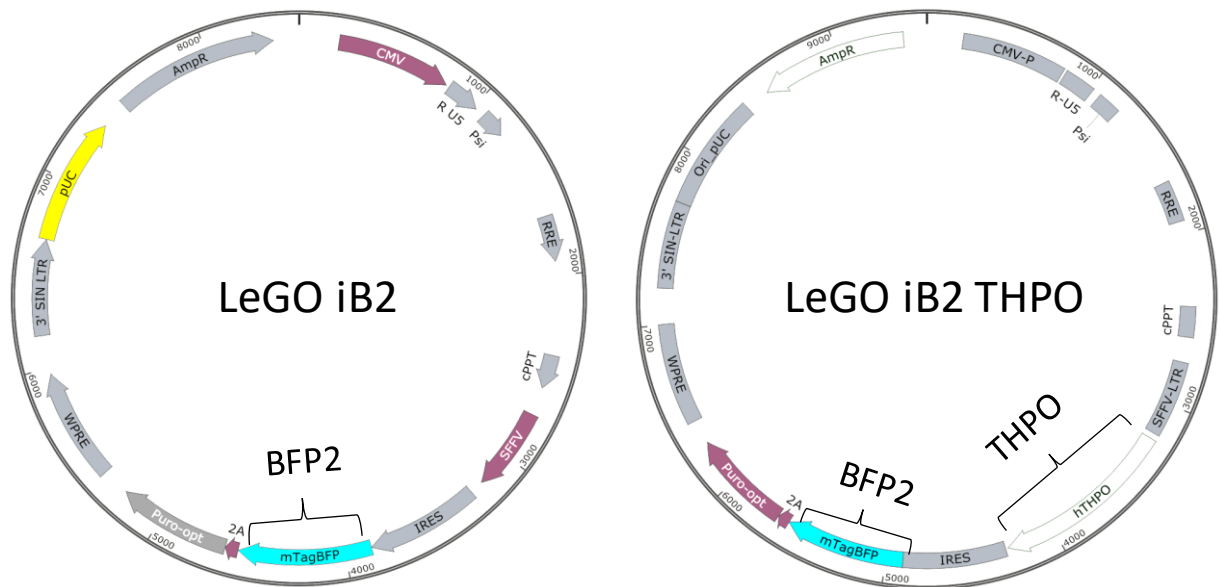
Name	Fluorescent Marker	cDNA
<b>LeGO iB2</b>	BFP2	none
<b>LeGO iB2 THPO</b>	BFP2	ThPO
<b>LeGO iB2 GM-CSF</b>	BFP2	GM-CSF

## Methods

### Transduction with Lentiviral Gene Ontology Vectors

HS5 stroma cells were seeded at a density of  $2.6E4/cm^2$  and allowed to attach for a minimum of 4.5 hours in DMEM + 10% FBS + 1% penicillin/streptomycin. Lentiviral Gene Ontology Vectors (LeGO) were kindly provided by the lab of Boris Fehse and produced in a previous project by Timofey Lebedev. LeGO vectors can express a fluorescent marker protein, in this case BFP2, in addition to cDNA specific for a gene of interest (Weber, Bartsch et al. 2008). LeGO iB2 Puro, LeGO iB2 Puro THPO, as shown in Figure 3 and LeGO iB2 Puro

GM-CSF vectors were added, the culture plate sealed with paraffin and centrifuged for 1h at 1000xg. After an incubation period of two to four days, cells were analyzed by flow cytometry. BFP2 positive cells were considered successfully transduced and sorted for further experiments.



**Figure 3:** LeGo iB2 Puro and LeGo iB2 THPO Puro viral plasmids used for lentiviral transduction of HS5 cell lines

### Functional assays

To validate the successful transduction of our stroma cell lines, functional assays using cytokine-dependent cell lines were performed. To confirm the synthesis and functionality of the stroma-cell-produced GM-CSF and THPO, the cytokine-dependent cell lines TF-1 cells and UT7/TPO were used, as detailed below.

### Isolation of condition media

Transduced stroma cells (6E5 per 10cm petri dish) were seeded with 10ml of DMEM + 10% FBS + 1% penicillin/streptomycin. The cells were then incubated for three days at 37°C, 5% CO<sub>2</sub>. On day three, the medium was removed and replaced with 10ml of fresh medium without FBS. Following an incubation period of one day, the condition medium was aspirated and frozen at -20°C for further use.

### Growth curves using condition media

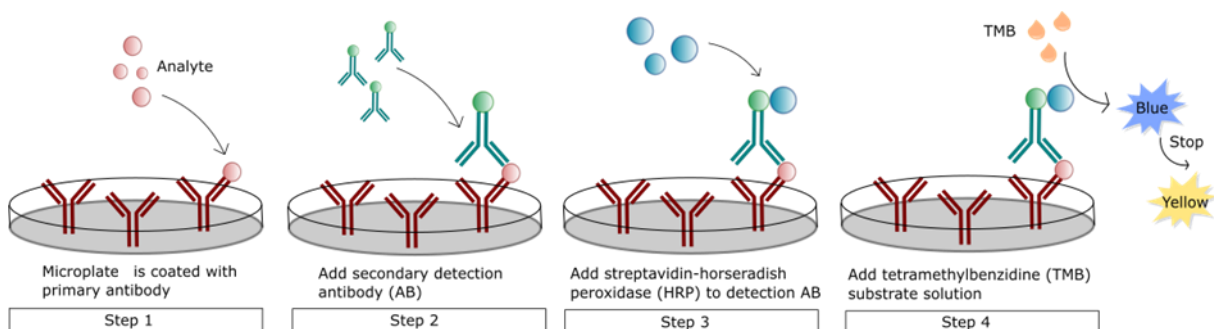
Cytokine-dependent cells were seeded at a concentration of  $1 \times 10^6$ /ml and cultured for 4 days in their regular medium supplemented with condition medium isolated from HS5 BFP2/ BFP2 GMCSF / BFP2 THPO or recombinant human cytokine as a control. Cell numbers were counted daily using a Neubauer counting chamber.

### Irradiation of stromal cells

Stromal cells (HS5, HS5 BFP2 and HS5 BFP2 THPO) were seeded at  $2 \times 10^5$  cells/cm<sup>2</sup> and allowed to adhere overnight. Cultures were then irradiated with 40Gy on day 0. The wells were photographed every two days to assess their morphology. Condition medium was isolated every two days and saved for later analysis. In addition, the BFP2 fluorochrome expression after irradiation was analyzed by flow cytometry.

### Enzyme-linked immunosorbent assay (ELISA)

To quantify the amount of cytokine produced by transduced stroma cells, enzyme-linked immunosorbent assays using cell-culture condition media were performed (Figure 4). Microplates were coated with a capture antibody specific for the tested cytokine and the analyte added (step 1). Afterward, the detection antibody is bound to the immobilized analyte on the plate. Streptavidin-horseradish peroxidase (HRP) was then added to the second detection antibody (step 3) to convert its substrate, tetramethylbenzidine (TMB) (step 4) creating a blue color. The enzymatic reaction was stopped, changing the color of the well from blue to yellow. The amount of cytokine in the analyte was then determined by measuring the absorbance of the color at 450nm.



**Figure 4:** Direct sandwich enzyme-linked immunosorbent assay.

The ELISA kit used to measure human thrombopoietin levels in cell culture supernatant had a detection range of 93.8-6E3 pg/ml. Cell culture supernatants were previously acquired by centrifugation and aspirating and measured at 1:100, 1:1000 and 1:10000 dilutions. The ELISA was then conducted according to the manufacturer's instructions.

#### Isolation of mononuclear cells

PB and BM samples were obtained after informed consent and used to isolate mononuclear cells. To enrich for stem and progenitor cells, 20 $\mu$ l of RosetteSep™ Human Bone Marrow Progenitor Cell Pre-Enrichment Cocktail was added per 1ml of sample and incubated for 20min at room temperature. RosetteSep™ is a negative selection method that uses tetrameric antibodies against the lineage markers CD3, CD11b, CD14, CD16, CD19, CD56, CD66b and CD235a to crosslink erythrocytes and lineage positive cells to form so-called rosettes. These rosettes pellet in the erythrocyte phase instead of the MNC phase during the subsequent density gradient centrifugation, hereby enriching for lineage negative progenitors in the MNC phase.

Following the incubation with RosetteSep™, the sample is diluted to 35ml with MACS buffer and carefully layered onto 15ml of Ficoll-Paque in a 50ml falcon tube and centrifuged without brake and at low acceleration for 15min at 2000rpm. Ficoll PM400 is a density gradient centrifugation medium consisting of a neutral, highly branched, high-mass, hydrophilic polysaccharide with a density of 1.077 g/ml. In Ficoll-Paque it is combined in an aqueous solution with sodium diatrizoate and calcium disodium ethylenediamine-tetra acetic acid. Due to the different properties of hematopoietic cells, density centrifugation media such as Ficoll-Paque separate these cells into different layers based on their differential migration during centrifugation. Erythrocytes and rosetted cells are aggregated by the Ficoll and sediment at the bottom of the falcon. Granulocytes have a high enough density to pass through the media and are therefore found above the erythrocytes. The target MNC layer, which also contains platelets, is located between the medium and serum, as its density is too low to migrate through the medium. The MNC layer is aspirated from the falcon using a 5ml glass pipette and then centrifuged to remove any remaining serum and medium.

### Co-cultures with transgenic HS5 cell lines and CD34<sup>+</sup>

Stroma cells were seeded at sub-confluency on day -1, irradiated at 40Gy and allowed to adhere overnight in a 24-well-plate. CD34<sup>+</sup> (1E5 /well) cells from healthy donor BM were added on day 0 of the assay. Demi-media changes were performed every two days for three weeks. On days 7, 14 and 21, two wells per set-up were used for FACS analysis and colony assays.

### Cell cycle analysis with propidium iodide and flow cytometry

Cells were sorted into PBS and then resuspended in 50µl cold PBS before fixation in 500µl ice-cold 70% ethanol solution. Fixed cells were incubated at -20°C for at least 24h. The ethanol was then removed via centrifugation, and the cells washed with PBS. After washing, the cells were permeabilized with 470µl of PBS+0.25% Triton X and stained with 5µl of propidium iodide (PI) and 25µl of RNase A. Cells were vortexed gently and incubated at 4°C for at least 4h or overnight. Samples were then measured in the PE channel at the Canto II.

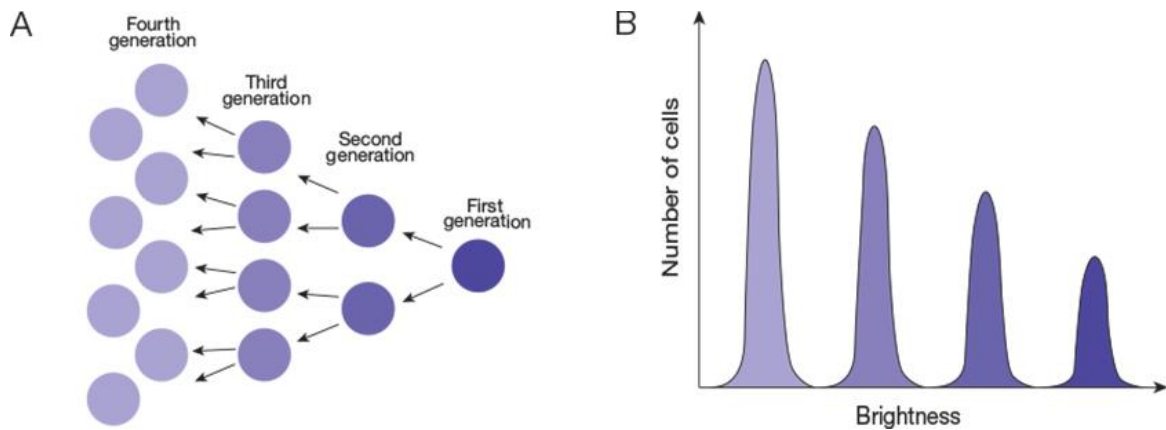
### Flow cytometry and cell sorting

Cells were stained in MACS Buffer at either room temperature (fresh samples) or on ice (thawed samples) with antibodies (Table 8) and incubated for 30min in the dark at 4°C. Fractions of interest were sorted into pre-coated low-protein binding microtubes containing MACS buffer, PBS or IMDM. Flow cytometric data was analyzed using Flowjo V10.

### Population tracking with Cell Trace Violet™

The cytoplasmic dye Cell Trace Violet™ was used to track the proliferation of populations in culture. A baseline intensity of the dye is measured in flow cytometry at the start of the experiment and used to map the continuous dilution of the dye along downstream generations, as shown in Figure 5.





**Figure 5: Principle of Cell Trace Violet™ proliferation tracking.** A. Dilution of the cytoplasmic dye following each cell division. B. Resulting peaks in flow cytometry. Undivided cells exhibit a high fluorescence, and low cell number and subsequent divisions reduce the brightness of the fluorochrome but result in higher cell numbers (Thermo Fischer Scientific - Cell Trace Reagents for Cell Proliferation)

Reconstituted dye (1 $\mu$ l/ml) was added to sorted cells in PBS. Cells were briefly vortexed and incubated for twenty minutes at 37°C, 5% CO<sub>2</sub>. Five times the original volume of medium with 10% FBS was added to stop the staining reaction and incubated for an additional five minutes before centrifuging the sample and re-suspending it in the desired new media. Cell Trace Violet™ was measured by flow cytometry and assessed using Flowjo V10 software.

#### Colony-forming unit (CFU) assays

Methylcellulose- and collagen-based colony-forming unit assays were performed to assess clonogenic and lineage potential by plating sorted cells in semi-solid medium.

For methylcellulose-based assays, cells were suspended in 150  $\mu$ l of IMDM with 2% FBS, 1% penicillin/streptomycin and 1% ciprofloxacin. The cell suspension was then transferred into a 1.6ml aliquot of Methocult™ H4435 and vortexed for 15 seconds to distribute cells in the semi-solid medium evenly. Air bubbles in the tube were allowed to rise for 15 minutes before aspirating 1.5ml using a dialysis catheter syringe. Medium (1.1ml) was then expelled into a 35mm petri dish. Any unwanted air bubbles were removed with a pipette, and the dish twisted to distribute the medium evenly. No more than two 35mm petri dishes were placed in a 10cm dish, with an additional open 35mm dish filled with distilled water to increase humidity and incubated for 14 days at 37°C, 5% CO<sub>2</sub>.

After two weeks of incubation, colonies were counted and categorized according to their morphology using an inverted light microscope at 4x magnification. The number of colonies was used to calculate the clonogenic potential of the cells in the assay.

The detection of megakaryocytic potential was performed using collagen-based media (MegaCult-C™). Cells were suspended in IMDM without FBS and 1% penicillin/streptomycin and 1% ciprofloxacin. The cell suspension was then transferred to 500µl of MegaCult-C containing 50ng/ml SCF and 20 U/ml of EPO. 308µl of collagen solution was added and mixed well, before plating 750µl in each chamber of a two-chamber slide. The assay was then incubated at 37°C, 5% CO<sub>2</sub> for ten days.

To fix the samples after colonies have formed, the chamber structure on top of the slide was removed, and excess liquid absorbed with a filter card and spacer. The filter card was removed as soon as it was soaked, and the slide placed in a fixation solution of 1:3 acetone and methanol for twenty minutes at room temperature. Slides were left to air-dry before dual immunohistochemical staining.

The following staining protocol was used to stain collagen-based CFU assay slides after fixation. On the day of the staining, the reagents listed below were freshly prepared.

- Blocking Buffer: (45ml PBS, 50µl Tween 20 (0.1%), 2,5g skimmed milk powder (5%), 2.5 ml FBS (5%))
- Alkaline phosphatase buffer (NTMT): (100µl 5M NaCl, 500µl 1M Tris pH 9.5, 250µl 1M MgCl<sub>2</sub>, 5µl Tween20, 4.15ml ddH<sub>2</sub>O, 50µl Levamisole (200mM)) with a final pH of 9.4-9.5
- 10x TBST: 14ml 5M NaCl, 1.5ml 1M KCl, 12.5ml 1M Tris pH 7.5, 500 µl Tween20, 20.16ml ddH<sub>2</sub>O

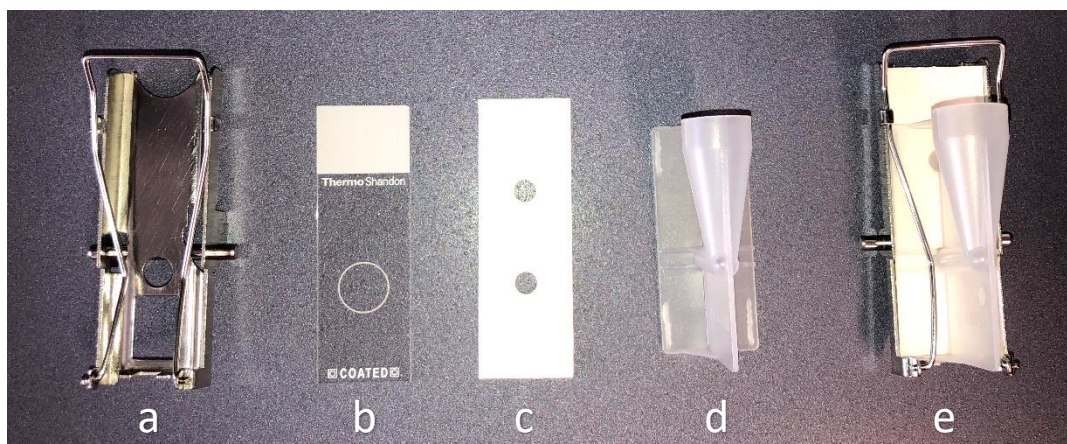
Step	Min
1. Let slides warm to room temperature	20
2. Soak in PBS	10
3. Block in 500 µl Blocking Buffer + 2 drops Avidin per slide	30
4. Rinse with PBS	
5. Add 500µl of 1 <sup>st</sup> AB (Rat anti-human CD235a) in 1:1500 dilution in BB + 2 drops Biotin per slide	30
6. Rinse 3x with PBS	
7. Add 500µl of 2 <sup>nd</sup> AB (Goat anti-Rat Biotin in BB at 1:1500 dilution)	30
8. Rinse 2x with PBS	
9. Add 500µl of ABC Complex	30
10. Rinse 2x with PBS	
11. Rinse 2x with TBST	
12. Rinse 3x with NTMT	
13. Add 500µl of BCIP/NBT	8
14. Rinse 1x and then soak in TBST	2
15. Optional fixation overnight: a.) Rinse twice with dH <sub>2</sub> O and then 2x with 50% MeOH	

b.) Fix for 5min in MeOH c.) Air dry and keep at 4°C in the dark	
16. Rinse 2x with PBS (5min each if rehydrating)	
17. Block in 500µl Blocking Buffer + 2 drops Avidin per slide	30
18. Rinse with PBS	
19. Add 500µl of 3 <sup>rd</sup> AB (Mouse anti-human CD41a in BB at a 1:1500 dilution) + 2 drops of Biotin per slide	30
20. Rinse 3x with PBS	
21. Add 500µl of 4 <sup>th</sup> AB (Goat anti-mouse Biotin XX in 1:1000 in BB), centrifuge at max speed for 7min, use supernatant for staining	30
22. Rinse 2x with PBS	
23. Add ABC Complex	30
24. Rinse 2x with PBS	
25. Rinse 1x and soak in 0.1M Tris pH 8.5	5
26. Add 500µl of Vector Red	30
27. Rinse 2x with PBS	
28. Rinse 1x and soak in dH2O	1
29. Air dry in the dark	

Slides were photographed in bright field light microscopy using the 5x EC Plan-Neofluar objective of the Zeiss ApoTome (Zeiss Axiovert 200M) with the Axiovision 4.8.2 imaging software at the UKE Microscopy Imaging Facility. Colonies were counted using ImageJ software.

#### Morphological assessment of cells

Cytospins were performed to allow morphological assessment of cells with bright field microscopy. Cells were suspended in 500µl of PBS or MACS buffer and transferred into the assembled cytofunnel (see Figure 6).



**Figure 6:** Components and assembly of the cytofunnel. (a) Cytofunnel assembly clip (b) microscopy slide (c) filter paper (d) plastic cytofunnel (e) assembled cytofunnel

Slides were centrifuged in the cytocentrifuge for 10min at 400rpm and left to dry horizontally before Pappenheim staining.

Staining after Pappenheim allows for good discrimination of hematopoietic cells by combining the Giemsa and May-Grünwald staining. Table 10 gives an overview of the staining behavior of blood cells after Pappenheim staining (Mulisch 2010).

**Table 10:** Overview of cell component color after Pappenheim staining

Cell component	Color after Pappenheim staining
<b>Nuclei</b>	Red-purple to blue
<b>Erythrocyte cytoplasm</b>	pink
<b>Lymphocyte cytoplasm</b>	Light blue
<b>Monocyte cytoplasm</b>	Grey-blue
<b>Eosinophilic granules</b>	Deep red
<b>Basophilic granules</b>	Dark purple
<b>Neutrophilic granules</b>	Light purple

Staining solutions were prepared as per the manufacturer’s instructions. Slides were stained vertically in a staining rack and submerged in May-Grünwald solution for 4 minutes. After this first step, the slides were incubated for 3 minutes in May-Grünwald solution diluted 1:1 with buffer solution before rinsing in distilled water. For the second staining, the rack was placed in Giemsa solution for 7 minutes and then rinsed again with distilled water before air-drying.

#### Co-culture of transduced stromal cell lines and healthy BM cells

Stromal cells were irradiated at 40Gy and seeded at  $1.5E5/cm^2$  on a 24-well plate. Cells were allowed to adhere overnight. On day 0 of the culture, CD34+ cells from frozen healthy donor BM ( $1E5$  per well) were added. Half of the culture media was changed every two days, and the health of the culture assessed by light microscopy. After 7, 14 and 21 days of culture, cells were trypsinized and stained for flow cytometric analysis using the antibodies listed in Table 11.

**Table 11:** Antibodies used for FACS for co-cultures with stromal cell lines

Fluorochrome	Antibody
BUV 395	CD 34
BV786	CD10
BV510	CD123
PE	CD45
FITC	Lineage (CD2, 3, 4, 8, 11b, 14, 19, 20, 56, 235a)
PerCP eF710	CD13
PE-Cy7	CD90
Bv650	CD33
APC H7	CD38
APC	CD45RA

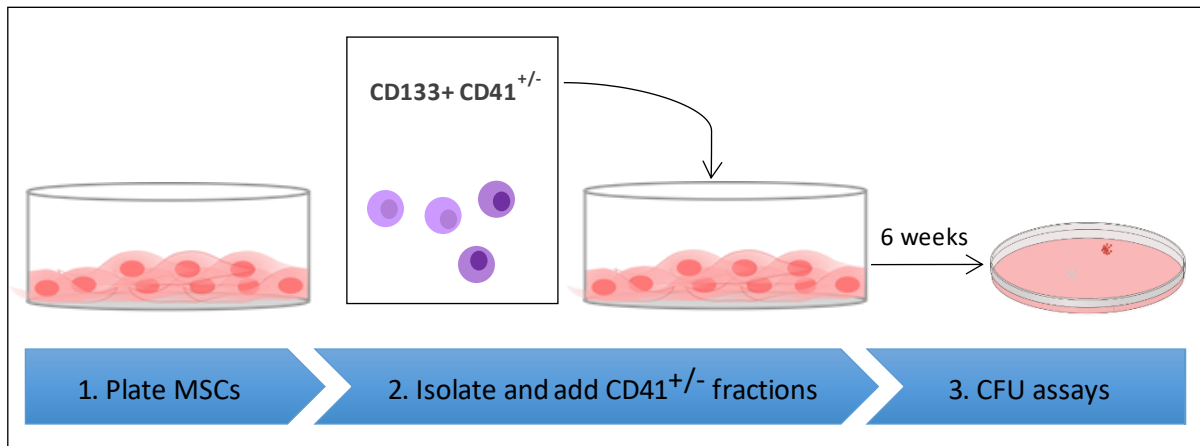
#### Long-term colony-initiating cell assay

Co-cultures over a period of six weeks were performed to enrich for stem and progenitor cells. Primary mesenchymal stromal cells isolated from healthy human donors (1.8E5/well) in  $\alpha$ -MEM with 10% FBS and 1ng/ml bFGF were plated per well in a six-well plate. After sorting the cell fractions of interest, hematopoietic cells were plated at various concentrations in 2.5ml Myelocult H5100 with 20ng/ml of THPO, SCF and IL-3, 1% penicillin/streptomycin and  $10^{-6}$ M hydrocortisone on top of the MSCs. Two control wells with MSC alone in media ( $\alpha$ -MEM + 10%FBS + 1% penicillin/streptomycin + 1ng/ml bFGF) or Myelocult (with cytokines, hydrocortisone and penicillin/streptomycin) were included. Media changes were performed every week by aspirating 500 $\mu$ l of medium at the side of the well and replacing it with 1ml of fresh medium to balance out evaporation. After six weeks in culture, the cells were harvested using 0.25% Trypsin + EDTA. The cells were washed and stained, as listed in Table 12. A schematic overview of the LTC-IC assay is shown in Figure 7.

**Table 12:** Antibodies used for FACS for LTC-IC assay

Fluorochrome	Antibody
FITC	Lineage markers (CD2, 3, 4, 8, 11b, 14, 19, 20, 56, 235a)
PerCP Cy5.5	CD34
PE	CD133
APC H7	CD38
APC	CD73
BV510	CD41
PE Cy7	CD90

Various cell fractions were sorted after the LTC-IC assay and assessed for their clonogenic potential.



**Figure 7:** Long-term colony-initiating cell assay.

### Boosting with THPO

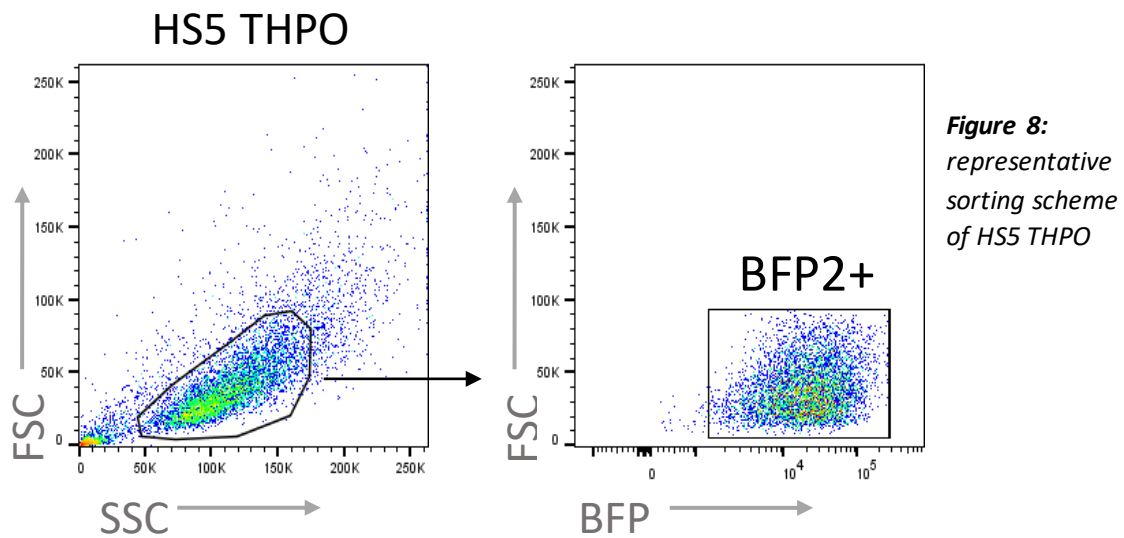
To boost the proliferation of normally quiescent HSPC, sorted cell fractions were seeded directly in StemSpan SFEM II with 1% Megakaryocyte Expansion Supplement or stained with Cell Trace Violet™ before being incubated in liquid culture for seven days. After the boosting period, cells were used for colony assays and analyzed for their proliferation behavior using flow cytometry.

## Results

### Establishment of an *in vitro* co-culture system

To study Lin-CD34+CD133+ cells, we first established an *in vitro* co-culture system using human stromal cells. The purpose of the co-culture was to simulate a possible hematopoietic niche in the setting of chronic myelofibrosis, by locally overexpressing THPO, which has been shown to induce myelofibrosis. A co-culture using GM-CSF would be used to observe the effects of a strong proliferative cytokine. To study long-term effects, the length of the culture was aimed at a minimum of six weeks. The human stromal cell line HS5 was transduced with lentiviral plasmids to produce either one of two human cytokines, THPO and GM-CSF, and the BFP2 fluorochrome. Transduced cells with blue fluorescence intensity above  $10^3$  in flow cytometry were deemed BFP2+ and sorted as pure populations

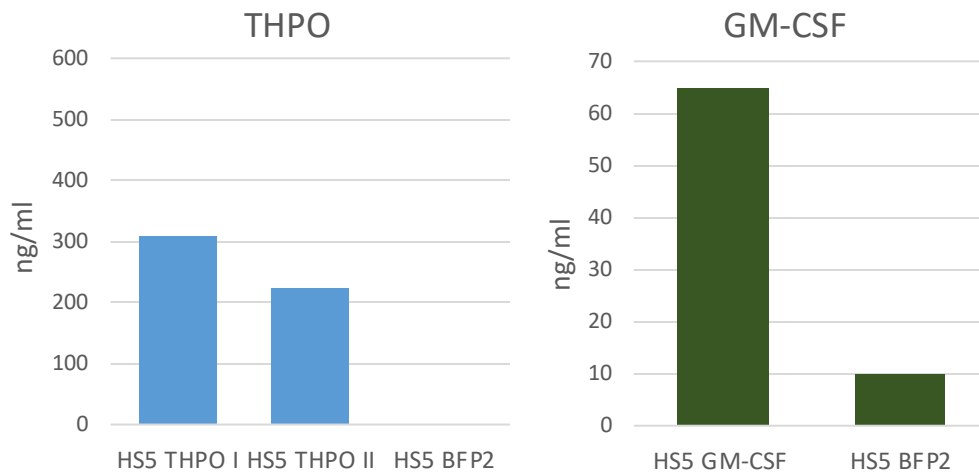
of HS5 THPO, HS5 GM-CSF, or control HS5 BFP2. A representative sort by flow cytometry is shown in Figure 8.



**Figure 8:**  
*representative  
sorting scheme  
of HS5 THPO*

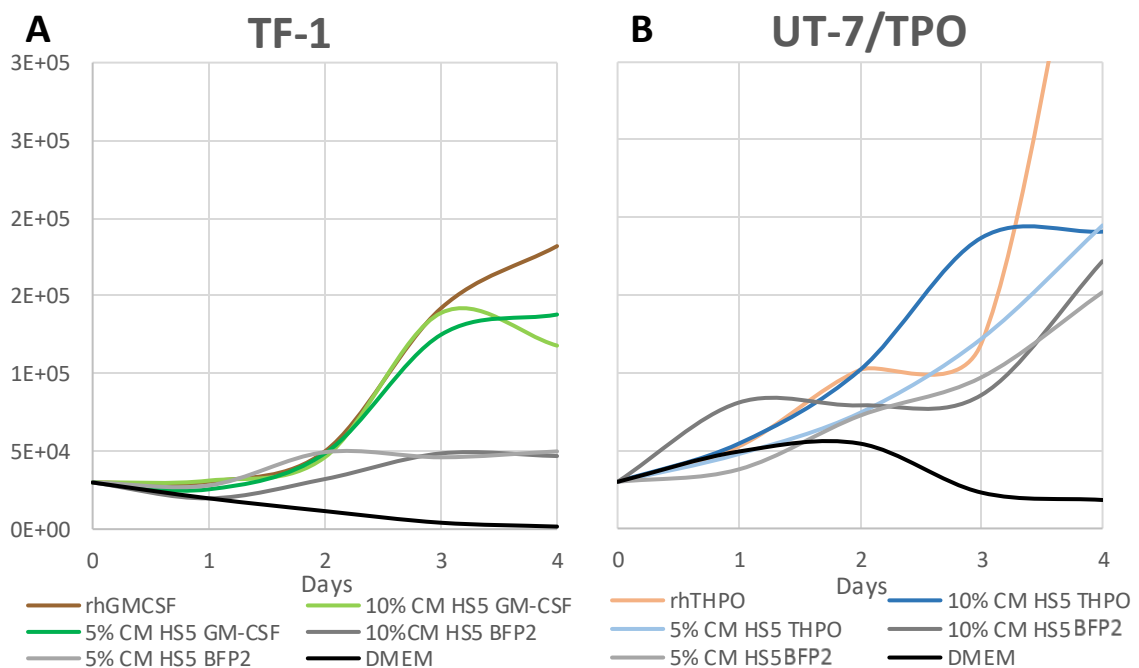
Transduced stroma cells produce high levels of functional cytokines

To quantify the amount of THPO and GM-CSF produced in the HS5 transduced cell lines, the level of THPO and GM-CSF in the condition media of HS5 BFP2 THPO and HS5 BFP2 GM-CSF, respectively, were measured using an ELISA. Two separately transduced HS5 BFP2 THPO cell lines both produced very high levels of THPO (308 and 225 ng/ml). The control HS5 BFP2 cell line did not produce any detectable THPO, as would be expected (Figure 9). The HS5 GM-CSF cell line produced 64.8ng/ml of GM-CSF, while the HS5 BFP2 also produced 9.8ng/ml of the cytokine.



**Figure 9:** Measurement of cytokine concentrations in cell culture supernatant of transduced HS5. THPO levels produced by two separately transduced HS5 THPO cell lines and a control HS5 iB2 cell line is shown on the left. Concentration of GM-CSF produced by transduced HS5 GM-CSF and HS5 is pictured on the right.

To assess if the cytokines produced by the transgenic cell lines are also biologically functional, the cytokine-dependent cell lines TF-1 and UT-7/TPO were used. These cell lines die in the absence of GM-CSF and THPO, respectively, and should, therefore, be a good indicator of the functionality of the produced cytokines. For the assay, the cell culture medium was supplemented with condition media from HS5 GM-CSF or HS5 THPO, or the recombinant human cytokine as a control and cell numbers were counted over a period of four consecutive days.



**Figure 10:** Growth curves of cytokine-dependent cell lines TF-1 (A) and UT-7/TPO (B). Value for day 5 of UT7/TPO total cells cultured with rhTHPO not shown was  $4.79E5$  cells.



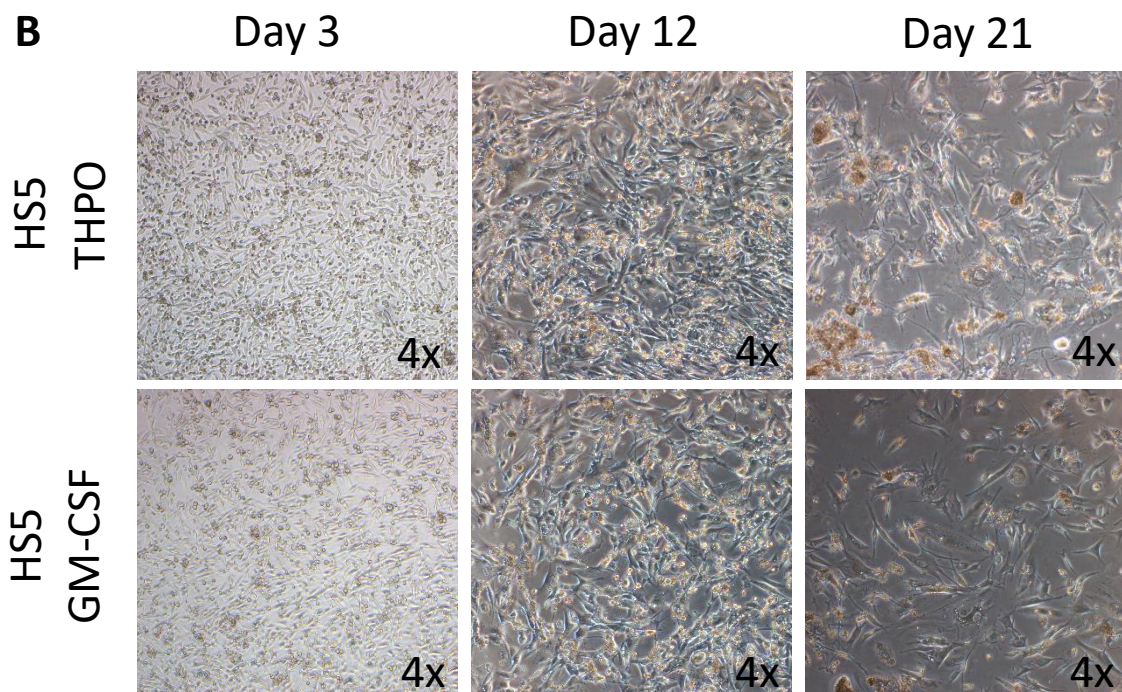
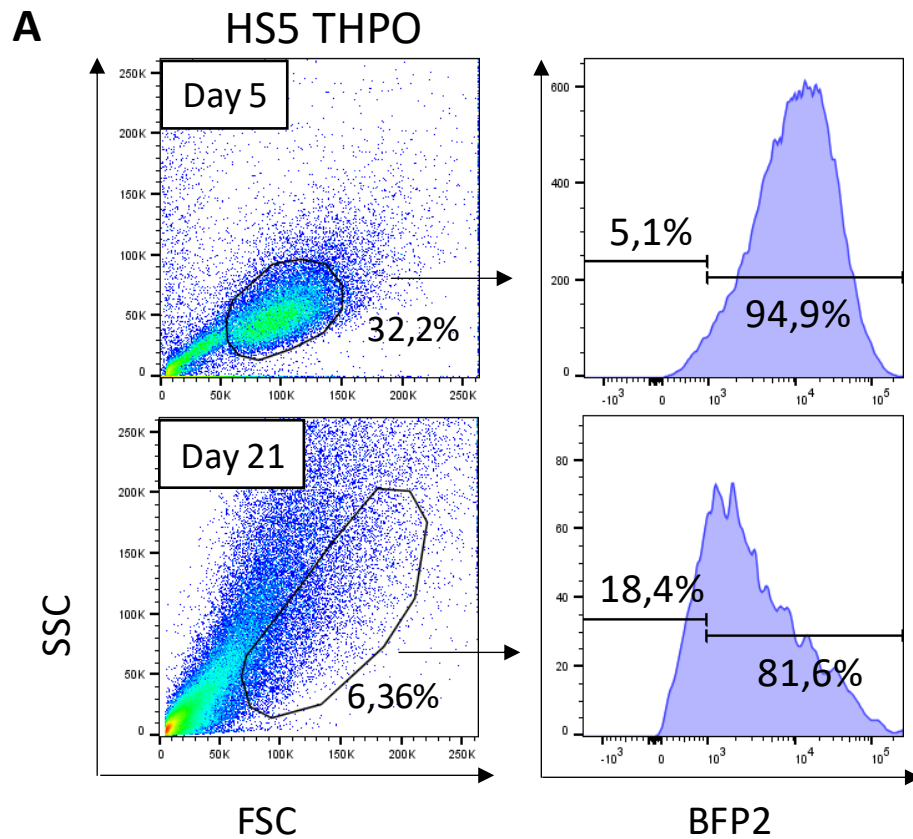
As seen in Figure 10A, condition media from HS5 BFP2 GM-CSF were able to stimulate the proliferation of GM-CSF dependent TF-1 at both a 5% and a 10% concentration. Supplementation with condition media from HS5 BFP2 only resulted in marginal proliferation. TF-1 incubated in DMEM without any cytokine supplementation died, proving their cytokine-dependency. The GM-CSF produced by the transgenic HS5 BFP2 GM-CSF can thus be considered biologically functional.

The THPO-dependent cell line UT-7/TPO showed proliferation with all supplemented HS5 BFP2 condition media, comparable with control recombinant THPO (Figure 10B). Proliferation was marginally greater when condition media from THPO producing cell lines was added compared to HS5 BFP2. UT-7/TPO did not proliferate without the supplementation of condition media or recombinant human THPO.

Taken together, two human stroma cell lines were established that expressed either THPO or GM-CSF at concentrations of circa 200 ng/ml and 60ng/ml of cell culture supernatant, respectively. A biological assay confirmed the functional activity of GM-CSF.

#### Irradiated HS5 do not survive as a stable stromal layer

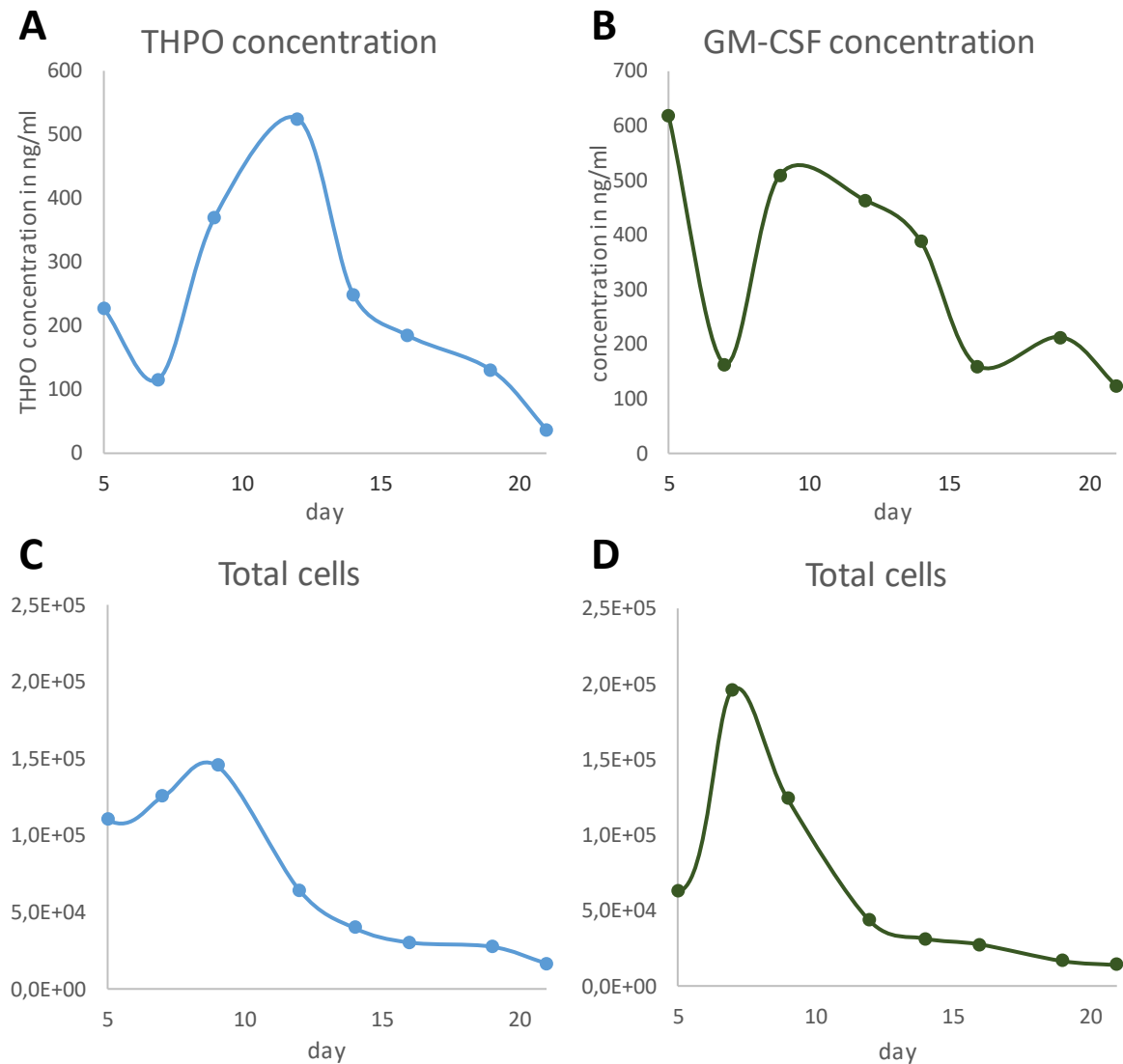
To assess the impact of ectopic expression of cytokines on the stem cell compartment, it is necessary to maintain the CD34<sup>+</sup> cells for a minimum of three weeks on stroma cells. To prevent the stroma cells from overtaking the culture, stromal cells were irradiated at 40Gy to prohibit proliferation and provide steady-state conditions. Different irradiation doses were tested, and a dose of 40Gy was selected by morphological analysis of cell culture health via microscopy.



**Figure 11:** Effects of 40 Gy irradiation on HS5 THPO and HS5 GM-CSF stromal cells. A: representative flow cytometric analysis of the BFP2 expression of HS5 THPO cells on day 5 and day 21. B: representative light microscopy of cell culture wells on day 3, 12 and 21 taken at 4x magnification.

Flow cytometric analysis showed a clear population with high intensity of the transduced fluorochrome after one week of the experiment (Figure 11A) and healthy cell culture

morphology (Figure 11B). During the first week, cell numbers also briefly increased (Figure 12C/D). In the second week, cell numbers were reduced, and the HS5 THPO/GM-CSF showed signs of stress and apoptosis. After three weeks, no clear population could be detected by flow cytometry, although BFP2 expression was still high. Microscopic imaging of the culture confirmed a steady decline in cell numbers and increasing cell death in the last week of culture.



**Figure 12:** Growth and cytokine dynamics of HS5 cultures from day 5 to day 21 after irradiation with 40Gy. THPO (A) and GM-CSF (B) concentrations in the culture supernatant, as measured by ELISA. Growth curves of HS5 THPO (C) and HS5 GM-CSF (D).

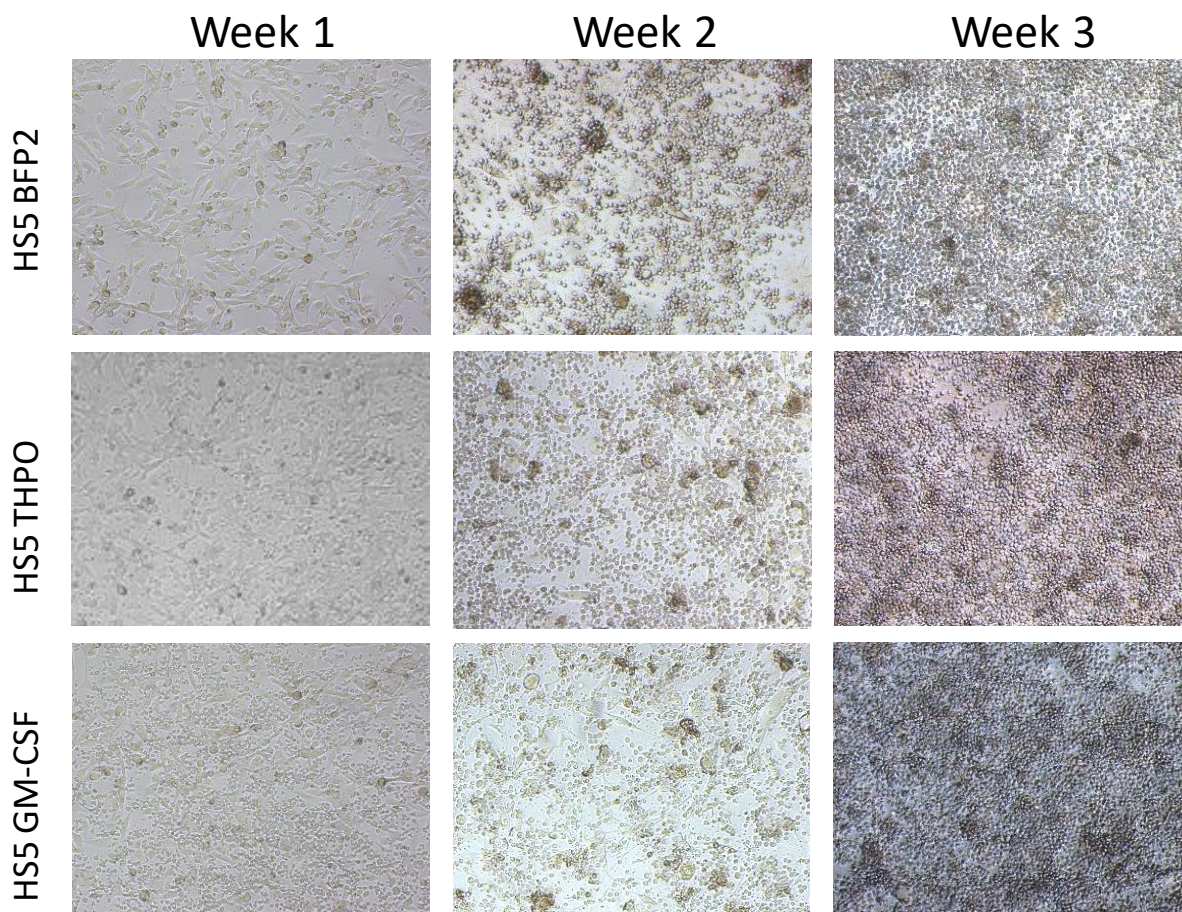
Cell culture supernatants of irradiated HS5 THPO were also measured to assess any changes following irradiation (Figure 12). Following the proliferation of cells during the first week, the concentration of both THPO and GM-CSF also briefly increased. The concentration of THPO peaked on day twelve at 515 ng/ml before decreasing rapidly to 37 ng/ml on day 21.

The concentration of GM-CSF peaked on days 5 and 12 at 620 ng/ml and 464 ng/ml, respectively, before steadily falling to a minimum of 124 ng/ml on day 21.

In summary, the transgenic stroma cell lines HS5 THPO and HS5 GM-CSF lose proliferation capacity over three weeks of culture after irradiation with 40Gy. Nevertheless, cytokine levels are sufficiently elevated to determine their influence on the survival, proliferation, and differentiation status of co-cultured HSPC.

#### Examination of the influence of cytokine expression on normal HSPC cultivated on transgenic stroma cultures

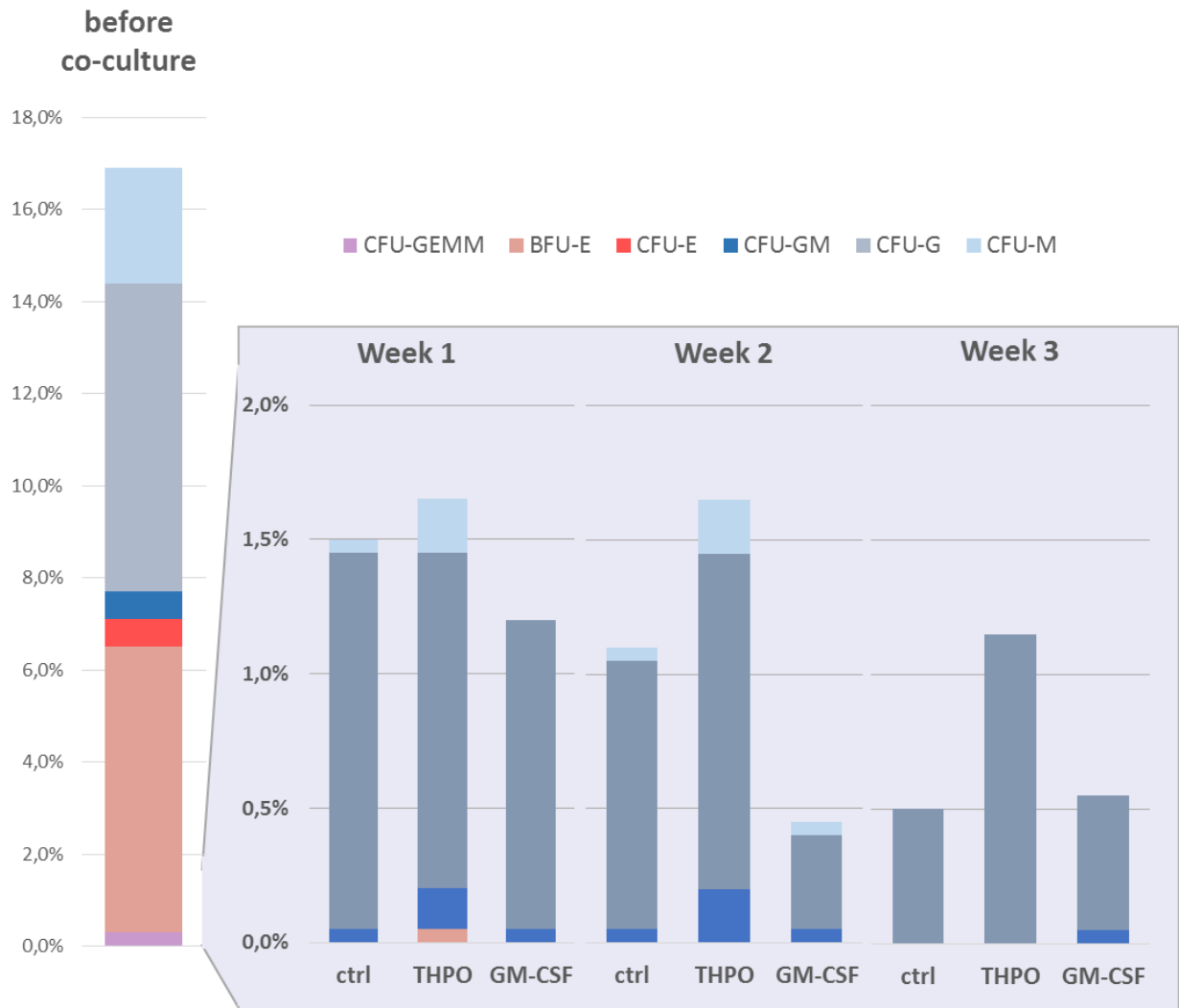
Co-cultures with normal HSCP were established to test the impact of aberrant cytokine expression on self-renewal resulting in increased numbers of stem cells and decreased proportion of differentiated cells. For this, we co-cultured CD34<sup>+</sup> cells from healthy donor BM with HS5 BFP2, HS5 THPO or HS5 GM-CSF for three weeks. The morphology of the culture was monitored via light microscopy (Figure 13). The health of the stromal cells markedly decreased over the course of three weeks. The co-cultured hematopoietic cells proliferated greatly and covered the entire surface of the well by the end of the assay period. The formation of cobblestones could not be observed, possibly due to the cell death of the stromal layer. No morphological differences were found between the different co-cultures.



**Figure 13:** light microscopy of co-cultures of transduced stromal cell lines and CD34<sup>+</sup> cells at 4x magnification during weeks one, two and three (from left to right).

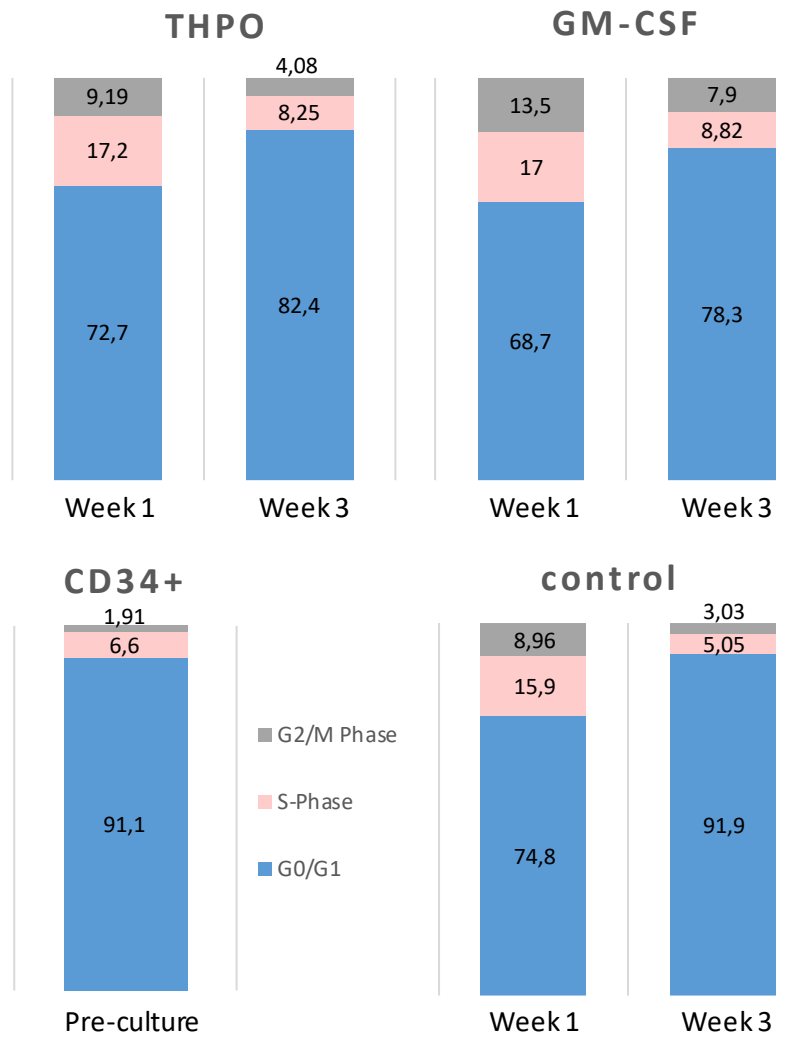
To determine the number of early progenitors in the different culture conditions, the clonogenic potential of the hematopoietic (CD45<sup>+</sup>) cells was tested in Methocult™ colony assays after one, two and three weeks of co-culture (Figure 14). As a reference, the baseline clonogenic potential of CD34<sup>+</sup> cells before co-culture was determined to be 16.9% and produced colonies of all lineages. CFU-G (6.7%) and BFU-E (6.2%) were the most frequent, followed by CFU-M (2.5%) and CFU-GM (0.6%) and CFU-E (0.6%). CFU-GEMM were the rarest type of colony at only 0.3%. Overall, the clonogenic potential and distribution were as could be expected of healthy CD34<sup>+</sup> HSPC.

Co-cultured CD34<sup>+</sup> cells had a distinctly lower clonogenic potential ranging from 1.7% to 0.5%. CD34<sup>+</sup> cells cultured in the presence of THPO had higher clonogenic potential than those cultured with GM-CSF or without cytokines and preserved their multi-lineage potential after one week. CD34<sup>+</sup> cells cultured with GM-CSF had the lowest clonogenic potential and only produced G/M lineage colonies. Across all co-cultures, the cloning potential decreased over the course of the assay.



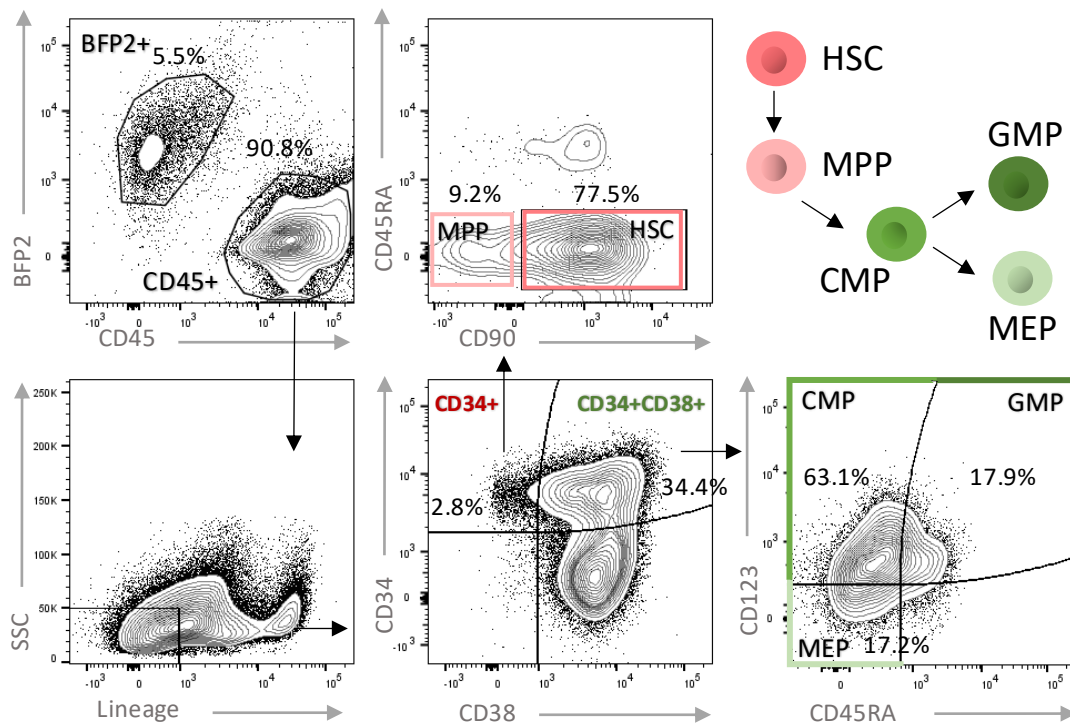
**Figure 14:** Clonogenic potential of CD34+ cells before co-culture (left) and sorted CD45+ cells after one, two and three weeks (column triplets left to right) of co-culture with control HS5 BFP2 (right), HS5 THPO (middle) and HS5 GM-CSF (right).

Cell cycle analysis of sorted CD45+ hematopoietic cells after co-culture (Figure 15) corroborated the proliferation of cells, as seen via light microscopy. While the overwhelming majority of CD34+ cells were not in cycle before the co-culture (91.1% in G0/G1-Phase), the percentage of cells in cycle was higher after one week in culture. 26.4% of cells cultured with THPO and 30.5% of cells cultured with GM-CSF were in either S- or G2/M-Phase at this time. A greater percentage of CD34+ exposed to cytokines were in cell cycle as compared to the control culture (24.9%). After the end of the assay period of three weeks, the percentage of cells in cycle had decreased again to 12.3% for THPO and 16.7% for GM-CSF. The control had the lowest percentage of cells in cycle at 8.1%. Overall, all co-cultured cells were more in cycle during the co-culture than prior to it, with GM-CSF stimulating cell proliferation the most.



**Figure 15:** Cell cycle analysis of CD34+ before culture (bottom left) and sorted CD45+ co-cultured on HS5 BFP2 (bottom right), HS5 THPO (top left) or HS5 GM-CSF (top right). Percentages of cells in G0/G1-/S- or G2/M-Phase are shown after propidium iodide staining and flow cytometric analysis.

We performed flow cytometric analysis of the co-cultured cells after one, two and three weeks to determine the differentiation status of the hematopoietic cultures (Figures 16 and 17). The BFP2 fluorochrome, as well as antibodies against CD45, lineage markers and markers for early progenitors (CD34, CD38, CD123, CD45RA and CD90), were used to first separate the CD45+ hematopoietic cells from the BFP2+ stroma cells and then further subdivide the CD45+ fraction into different differentiation stages (Figure 14). Independent of cytokine expression, the percentage of BFP2+ cells in culture dropped from an average of 4.0% of viable cells after one week to only 0.1% after three weeks. This correlated with the light microscopic appearance of the cultures, which showed stromal cells entering apoptosis, while hematopoietic cells proliferated.



**Figure 16:** FACS gating strategy for the examination of co-cultures of CD34<sup>+</sup> on the HS5 stromal feeder layer. Representative analysis after one week of co-culture on HS5 BFP2.

CD34<sup>+</sup> cells cultured with THPO had the highest percentage CD34<sup>+</sup>CD38<sup>+</sup> myeloid progenitors in the lineage negative (Lin<sup>-</sup>) fraction. After one week, 43.7% of Lin<sup>-</sup> cells were progenitors, which further decreased to 7.5% after three weeks. GM-CSF stimulation produced the lowest percentages of progenitors with 28.9% after one week and 2.6% after three weeks (Figure 17). The CD123<sup>lo/+</sup>CD45RA<sup>-</sup> CMPs were the most considerable progenitor fraction during weeks one and two, independently of cytokine stimulation, but decreased to only the second largest after CD123<sup>-</sup>CD45RA<sup>-</sup> MEPs after three weeks of co-culture. CD123<sup>lo/+</sup>CD45RA<sup>+</sup> GMPs were the least frequent progenitor population, with the highest percentages found after GM-CSF stimulation.

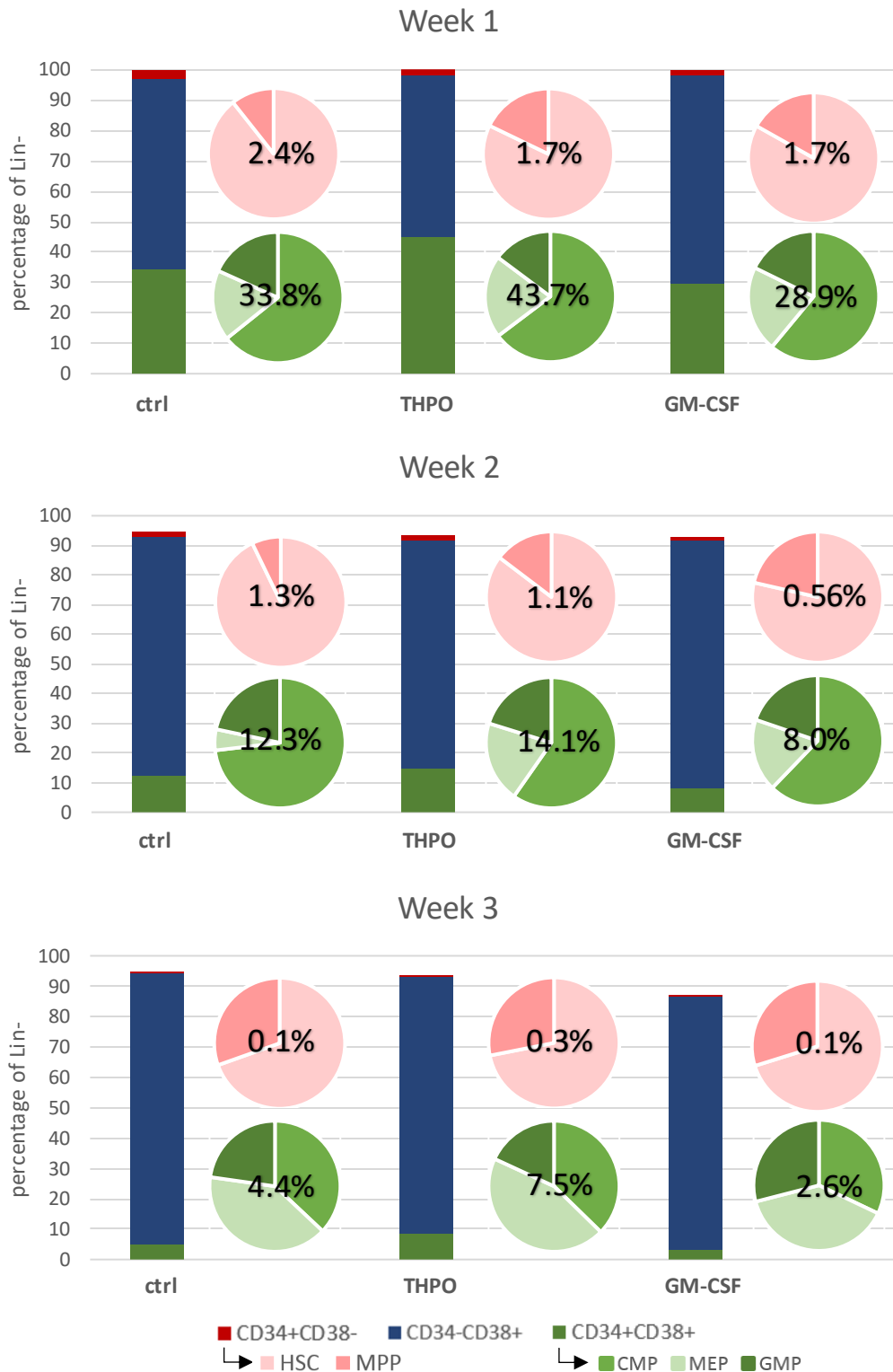
The stem cell enriched Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup> fraction also consistently decreased over the 21 days of culture. After one week, a maximum of 2.4% of CD34<sup>+</sup>CD38<sup>-</sup> cells were found in the Lin<sup>-</sup> fraction in the control without cytokines and 1.7% with cytokine stimulation. After three weeks, these early cells were barely detectable at only 0.1-0.3% of Lin<sup>-</sup> cells. Over 75% of CD34<sup>+</sup>CD38<sup>-</sup> cells were also CD90<sup>+</sup>CD45RA<sup>-</sup> and were thus categorized as HSCs in the first two weeks of culture. The proportion of CD90<sup>-</sup>CD45RA<sup>-</sup> MPPs only increased slightly after three weeks, as the mean percentage of early cells decreased.

This phenotypic analysis is in agreement with the clonogenic potential of sorted populations described above, showing that THPO preserved stem and progenitor fractions



better than GM-CSF or no cytokine stimulation. Furthermore, GM-CSF clearly pushed differentiation toward the granulocytic/macrophage lineages, whereas the addition of THPO did not significantly change the differentiation capacity of the cultures, as compared to control cultures, with similar percentages of MEP and GMP.

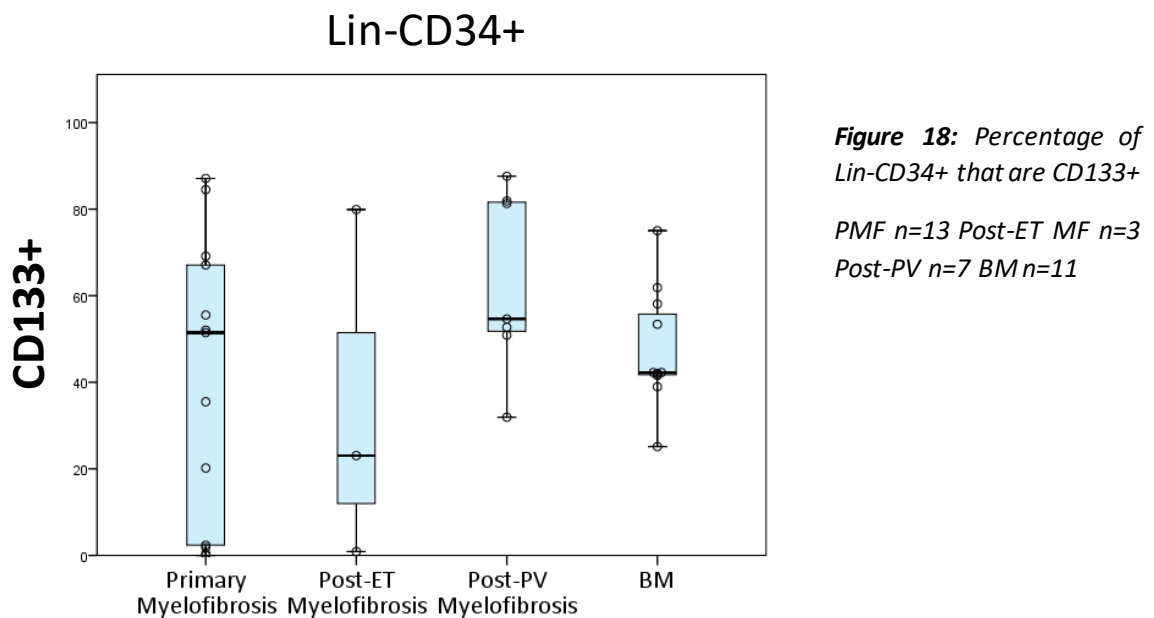
Unfortunately, to further investigate the maintenance of HSC levels in the THPO cultures would require an examination of long-term cultures (>6-weeks), which is not feasible in this system. The original aim of these long-term co-cultures could not be achieved with the established co-culture system. In the course of this work, members of our group established a culture system with primary mesenchymal stromal cells (MSC), which may more closely approximate the situation in patient samples. For these two reasons, this work was not continued.



**Figure 17:** flow cytometric analysis of Lin<sup>-</sup> hematopoietic cells co-cultured without cytokines (left) or with THPO (middle) or GM-CSF (right) after one, two or three weeks from top to bottom. Shown are the sub-fractions of CD34<sup>-</sup>CD38<sup>+</sup> (dark blue), CD34<sup>+</sup>CD38<sup>-</sup> (red) and CD34<sup>+</sup>CD38<sup>+</sup> (green) cells. The CD34<sup>+</sup>CD38<sup>-</sup> cells are further subdivided into HSC and MPPs (top pie chart) and the CD34<sup>+</sup>CD38<sup>+</sup> fraction into the three progenitor populations CMP, MEP and GMP (bottom pie charts), as well as the total percentage of Lin<sup>-</sup> cells indicated.

## Characterization of Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup>CD41<sup>+</sup>

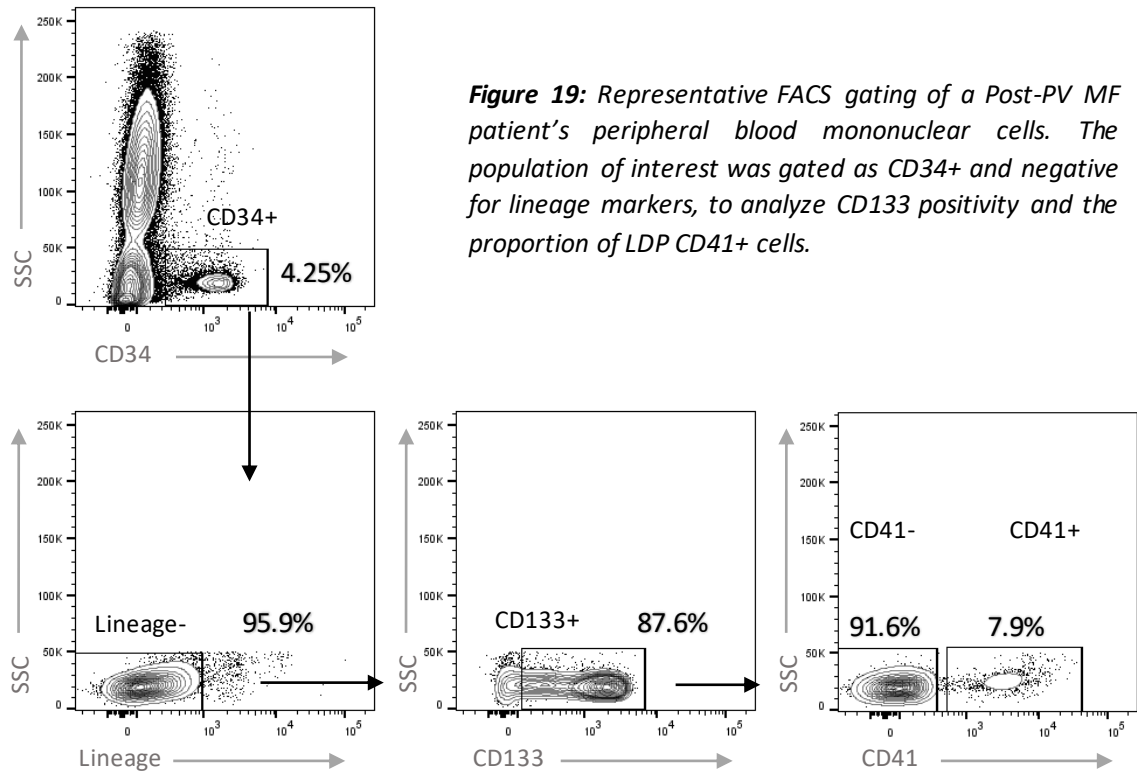
To investigate the existence of a possible megakaryocyte-primed population in the neoplastic stem cell population, defined as Lin<sup>-</sup>CD133<sup>+</sup>CD34<sup>+</sup> (Lineage<sup>neg</sup> double positive; LDP) cells, we analyzed CD41 positivity in this cell fraction by flow cytometry. As our group has published in the past, the percentage of CD133<sup>+</sup>CD34<sup>+</sup> found in MF patients' PB (peripheral blood) varied considerably between samples. In Figure 18, the range of percentages of CD133<sup>+</sup> cells observed in the Lin-CD34<sup>+</sup> compartment found in the cohorts is shown. This population is not found in normal PB but is found in healthy donor BM, which was also examined.



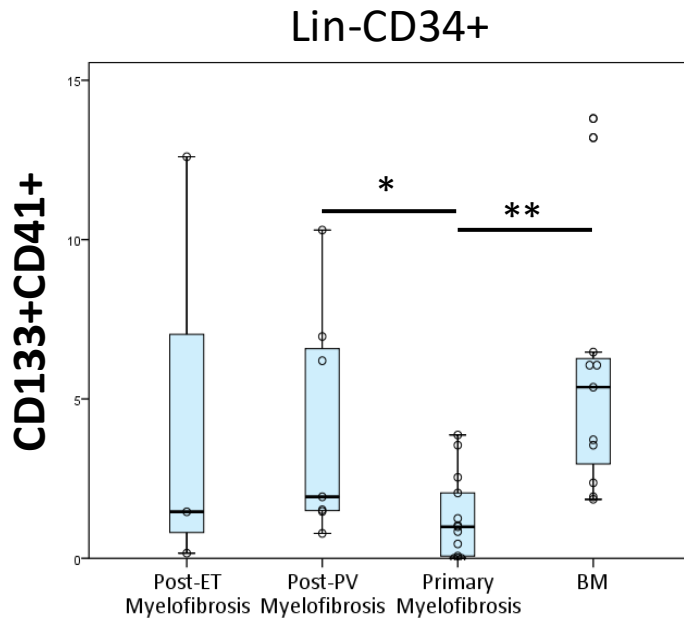
In the analyzed cohorts, the highest percentage of CD133<sup>+</sup> at 87.6% was found in a post-PV MF patient. The lowest value at 0.91% was detected in a post-ET MF patient, as well as in one PMF patient, which did not have any CD133<sup>+</sup> cells. The mean percentages across all patient groups (PMF 41.6%; Post-ET MF 34.6%; Post-PV MF 59.8%) were not significantly different compared to the mean percentage found in BM (43.6%). Nor were there any significant differences between the different diagnosis groups. An expansion of LDP in MF patient PB compared to healthy BM was not observed.

Flow cytometric analysis of MF patient PB and healthy donor BM confirmed the existence of a distinct subtype of Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> (LDP), which express CD41, as seen in Figure 19. The overall percentage of LDP CD41<sup>+</sup> cells was low (Figure 20). Two PMF patients did not have any LDP CD41<sup>+</sup> cells, and the LDP CD41<sup>+</sup> made up less than 1% of total Lin<sup>-</sup> cells in six

PMF patients. Several Post-ET and Post-PV MF patients had high percentages of LDP CD41<sup>+</sup> cells of up to 12.6%.



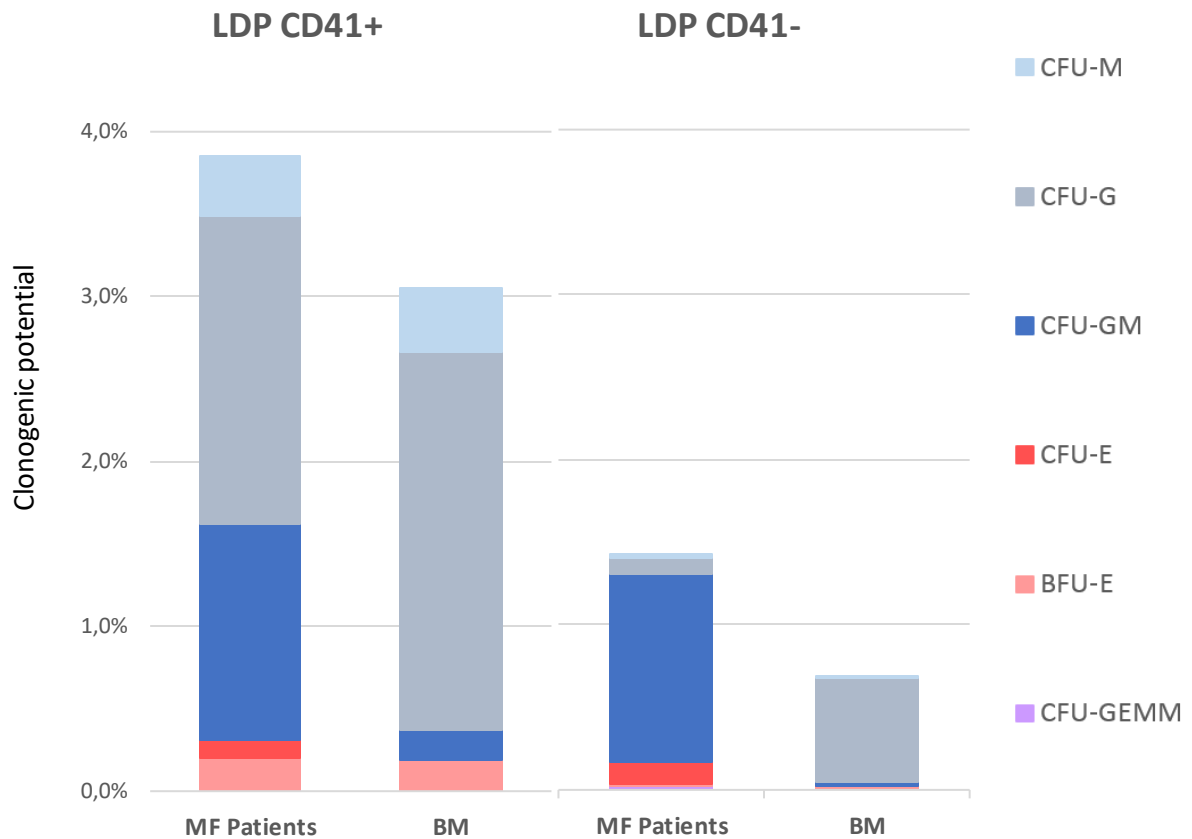
PMF patients had the lowest overall percentage of LDP CD41<sup>+</sup> cells (1.3%), significantly lower than in Post-PV MF patients (4.2%). The mean percentage in healthy BM was 5.9% and thus also significantly higher than in PMF PB. No significant difference between the mean percentage of LDP CD41<sup>+</sup> cells in Post-ET MF patients (4.7%) compared to the mean percentages found in other patient groups or BM was observed. This patient group was the smallest, so a possible difference cannot be excluded based on this result.



**Figure 20:** Percentage of CD133+CD41+ in the CD34+Lin- population. Post-ET MF n=3 Post-PV MF n=7 PMF n=13 BM n=11. Significance calculated with Mann-Whitney Test, \*= p<0.05 \*\*=p<0.01

Healthy LDP CD41<sup>+</sup> cells show priming towards megakaryopoiesis *in vitro*

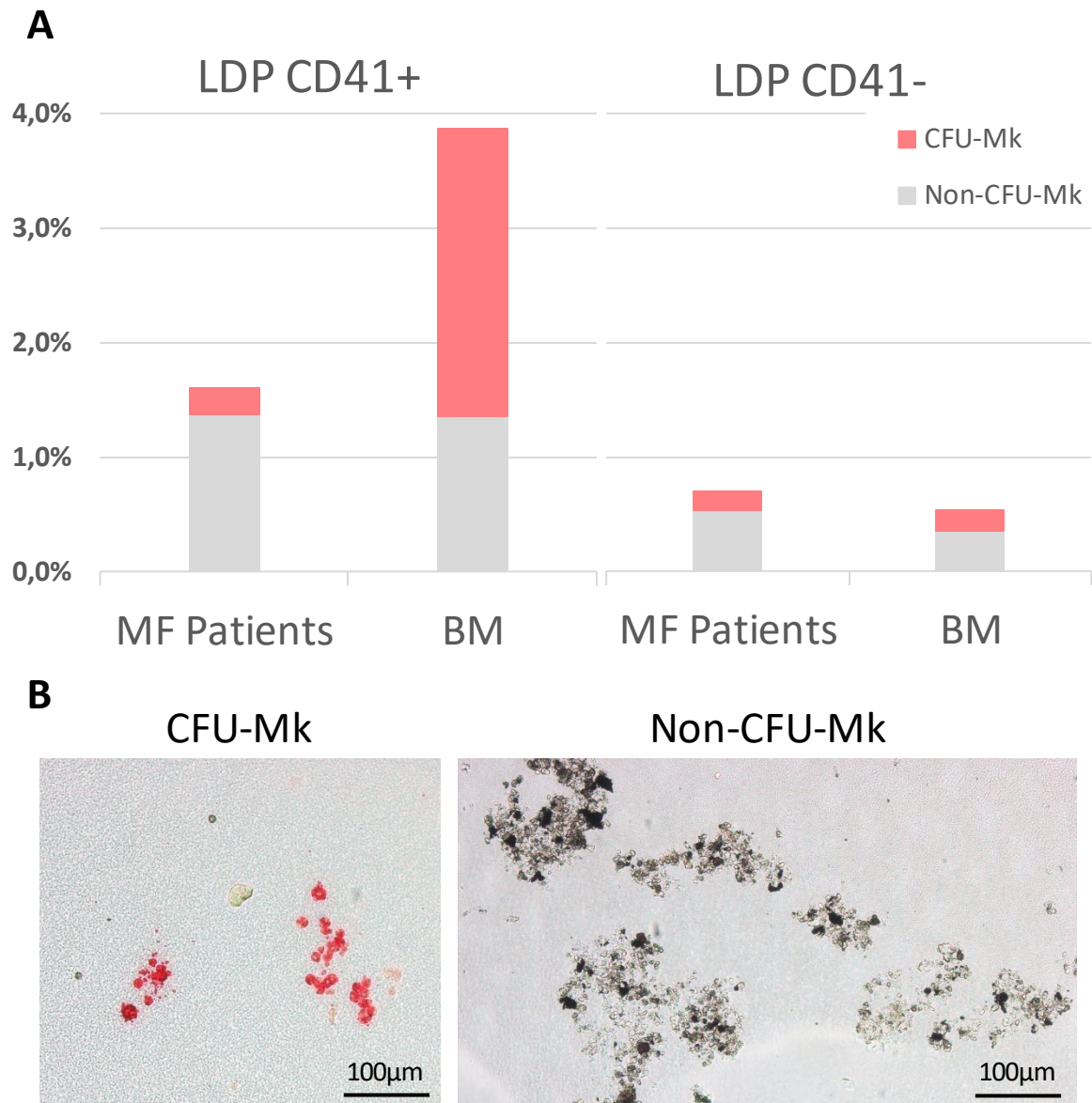
After identifying the LDP CD41<sup>+</sup> population by flow cytometry, we tested the *in vitro* cloning potential of sorted LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> in semi-solid colony assays using either methylcellulose or collagen, the latter of which supports megakaryocytic colonies. If this population is indeed primed towards megakaryopoiesis, a higher clonogenic output of this lineage would be expected.



**Figure 21:** Clonogenic potential of LDP CD41<sup>+</sup> (left) and LDP CD41<sup>-</sup> (right) in Methocult™. MF patients n=6, BM n=5.

Both LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> cells formed colonies in *in vitro* Methocult™ assays (Figure 21). The overall clonogenic potential was low (0-14.5%). All LDP CD41<sup>+</sup> samples formed colonies, whereas some LDP CD41<sup>-</sup> did not. In the patient cohort, the lowest clonogenic potential of 0.01% was found in an unclassifiable MPN patient and the highest of 14.5% in a post-PV MF patient. The majority of colonies were either CFU-GM or CFU-G; however, bi-lineage potential was observed in both LDP CD41<sup>+</sup> and CD41<sup>-</sup>. CFU-GEMM were the rarest type of colony and found only in one PMF patient. The mean clonogenic potential of LDP CD41<sup>+</sup> from patients was 3.9% and, therefore, higher than of LDP CD41<sup>-</sup> (̄ 1.4%) from MF patients. Patient LDP CD41<sup>+</sup> had the highest mean clonogenic potential out of all the samples tested. The highest clonogenic potential of LDP CD41<sup>-</sup> was 3.8% in a PMF patient, and the lowest was one post-ET MF patient that did not form any colonies.

In samples from healthy BM, LDP CD41<sup>+</sup> also formed more colonies than CD41<sup>-</sup> cells (3.0% vs. 0.7%). Both fractions also had multi-lineage potential, with a dominance of the G/M-lineage. This first analysis of the potential of LDP CD41<sup>+</sup> points toward a higher cloning efficiency than their non-CD41 expressing counterparts.



**Figure 22:** Clonogenic potential of LDP CD41<sup>-</sup> and LDP CD41<sup>+</sup> in SCF and EPO supplemented Megacult™. MF patients n=4, BM n=6. **A.** Overview of mean percentages of clonogenic potential of LDP CD41<sup>+</sup> (left) and LDP CD41<sup>-</sup> (right) from BM and MF patients. **B.** Examples of colonies formed by LDP CD41<sup>+</sup> cells. A colony stained CD41<sup>+</sup> CFU-Mk is shown on the left and a non-CFU-Mk on the right.

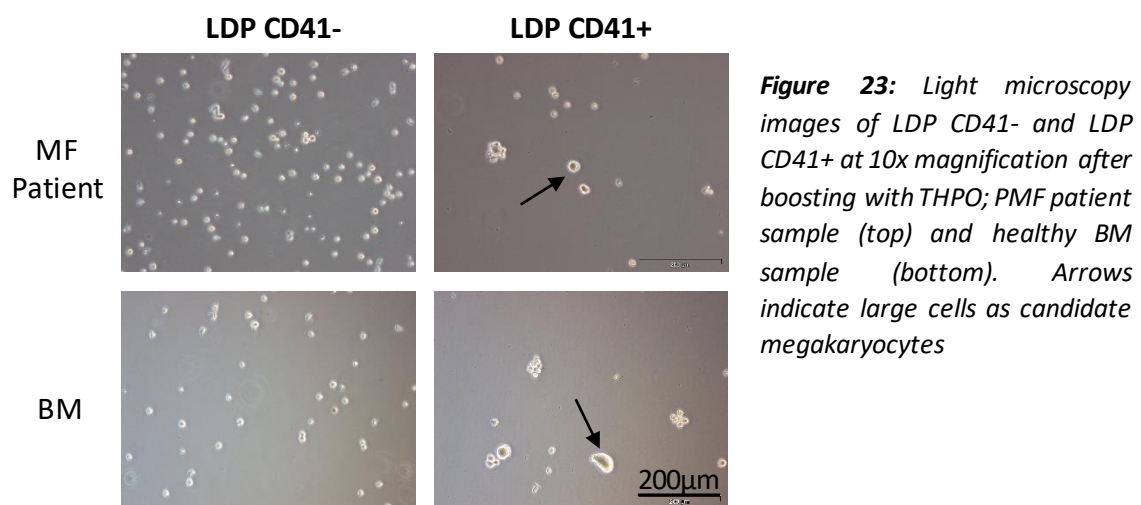
To test for megakaryocytic potential, in particular, collagen-based semi-solid colony assays were performed. Overall, the clonogenic potential in this assay was low and, as can be expected, lower than in Methocult™ (compare Figure 21 and Figure 22). The only exception to this finding was the higher mean clonogenic potential of BM LDP CD41<sup>+</sup> observed in Megacult™ (3.9%) as compared to Methocult™ CFU assays (3.0%). In Megacult™, LDP CD41<sup>+</sup> cells from MF patients had a clonogenic potential of 1.7%, which was higher than in the LDP CD41<sup>-</sup> fraction (0.7%). However, and somewhat surprisingly, the LDP CD41<sup>-</sup> had a higher proportion of CFU-Mk within the clonogenic population (24%) as compared to 13%

for the CD41<sup>+</sup> fraction. Nevertheless, the total CFU-Mk potential of the CD41 expressing group was slightly higher at 0.23% vs. 0.17% for the LDP CD41<sup>-</sup> population.

In healthy BM, the overall clonogenic potential of LDP CD41<sup>+</sup> was also higher than of LDP CD41<sup>-</sup> cells with a mean clonogenic potential of 3.8 % vs. 0.5%. In comparison to LDP CD41<sup>+</sup> from patients, healthy LDP CD41<sup>+</sup> did not only form more colonies, but the majority of these colonies were CFU-Mk (63%). Healthy LDP CD41<sup>-</sup> had a similar cloning behavior as seen from patients with a total CFU-Mk potential of 0.17%, which was equal to 33% of all colonies formed by this fraction. Thus, the hypothesis that CD41 marks a progenitor primed towards the megakaryocytic lineage could be confirmed in healthy LDP CD41<sup>+</sup>, but not in patient samples.

#### Boosting with THPO can induce megakaryocytic differentiation of Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> cells

One explanation for the low cloning efficiencies observed in the assay is that early HSC/MPP are not proliferating and generating progenitors, which will form colonies. To test if the clonogenic potential can be boosted and the formation of megakaryocytic colonies promoted in the LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> fractions, the cells were cultured in StemSpan SFEM II medium with megakaryocyte differentiation supplement containing THPO for one week. During the assay period, cells were observed via light microscopy for morphological changes (Figure 23).

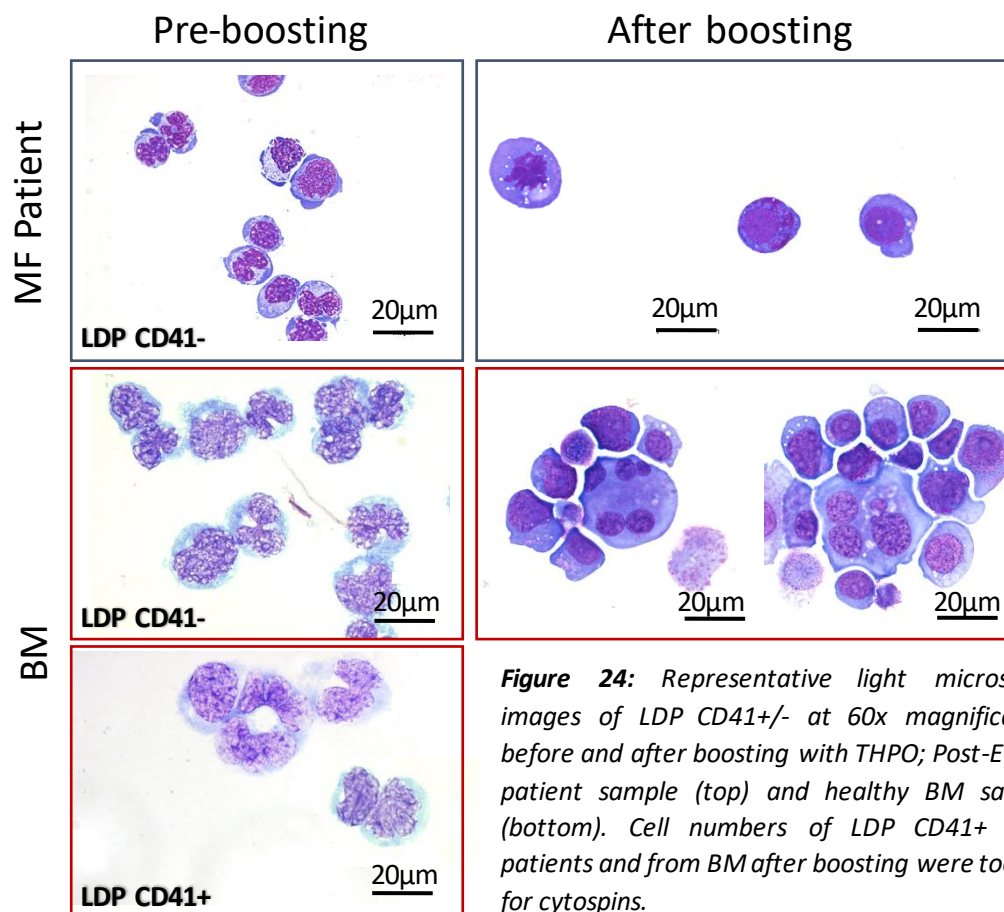


LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> cells behaved differently in co-culture. LDP CD41<sup>+</sup> cultures from healthy BM significantly decreased in cell numbers during the boosting period, but larger



cells indicating megakaryopoiesis were observed. Such larger cells could also be seen after boosting in LDP CD41<sup>+</sup> cultures from MF patients (Figure 23).

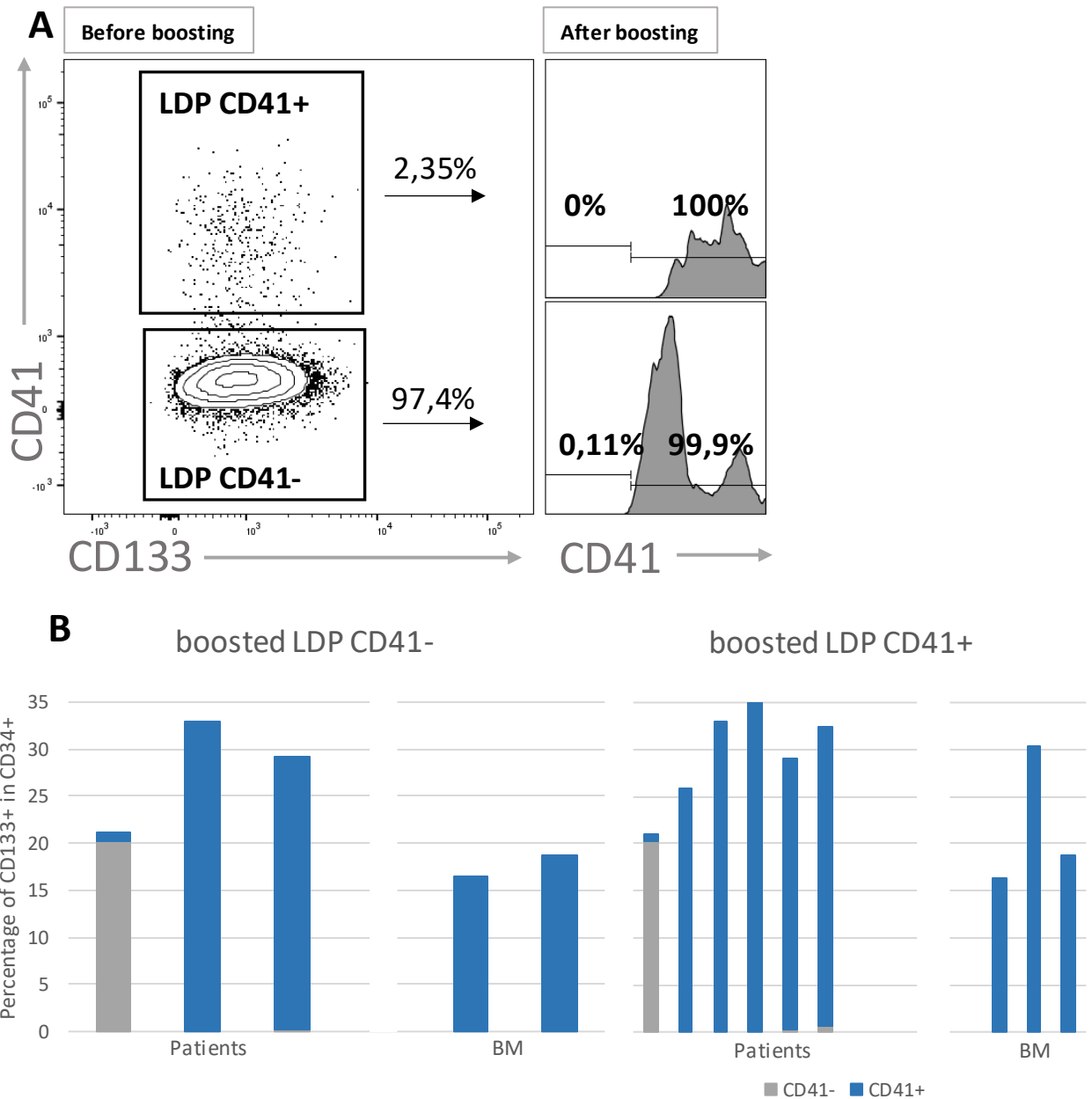
Larger cells were also observed via light microscopy in cultures of LDP CD41<sup>-</sup> cells, albeit at a lower frequency. As seen in Figure 24 below, cytopspins from healthy BM LDP CD41<sup>-</sup> after boosting showed differentiation into multi-nucleated megakaryocytes after one week of culture. LDP CD41<sup>-</sup> from MF patients were morphologically similar to BM cells before boosting; however, the large cells observed in cytopspins in these samples were not multi-nucleated megakaryocytes. Their phenotype more closely resembled the more primitive megakaryoblast, which may be a sign of the aberrant megakaryopoiesis found in MF. Due to the lower cell numbers in LDP CD41<sup>+</sup> cultures, cytopspins of these samples were not possible.



**Figure 24:** Representative light microscopy images of LDP CD41<sup>+/-</sup> at 60x magnification before and after boosting with THPO; Post-ET MF patient sample (top) and healthy BM sample (bottom). Cell numbers of LDP CD41<sup>+</sup> from patients and from BM after boosting were too low for cytopspins.

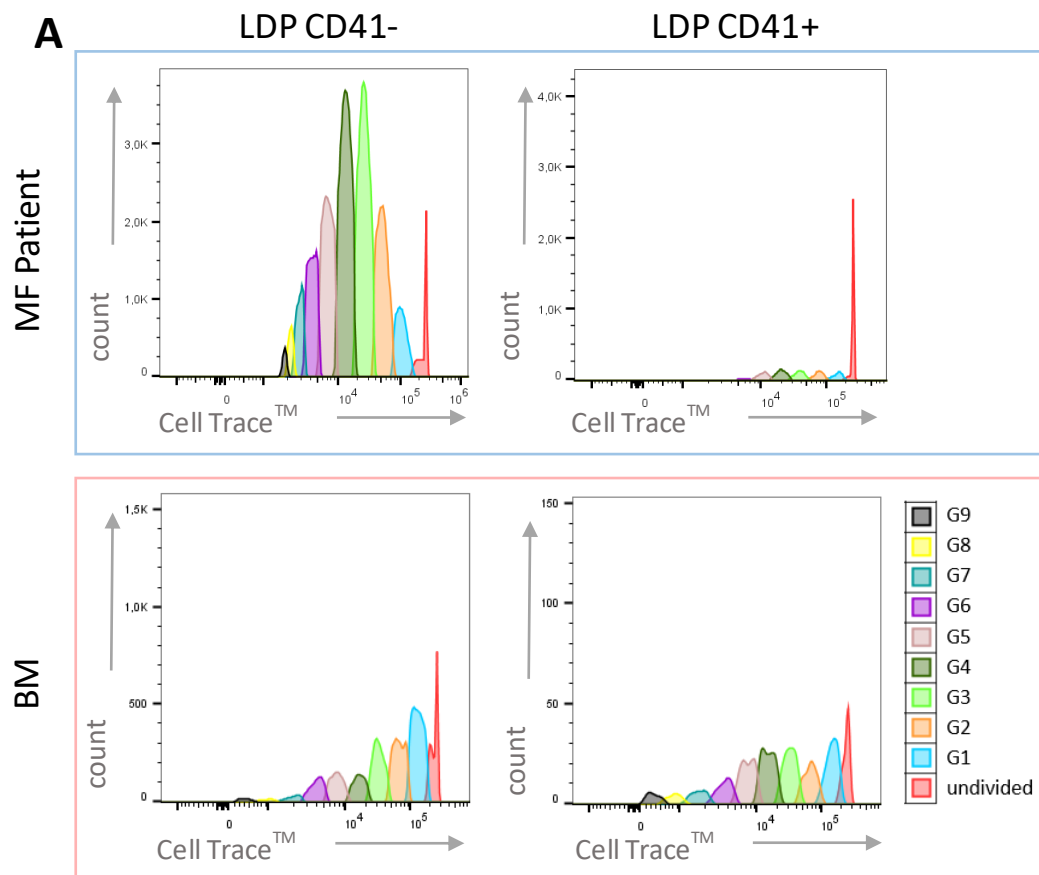
The percentage of CD41<sup>+</sup> cells changed during the culturing. While only an average of 28% of CD34<sup>+</sup> cells of cultured LDP CD41<sup>-</sup> and 31% of LDP CD41<sup>+</sup> cells still stained positive for CD133 after THPO stimulation in patients, the majority of these cells in two out of three

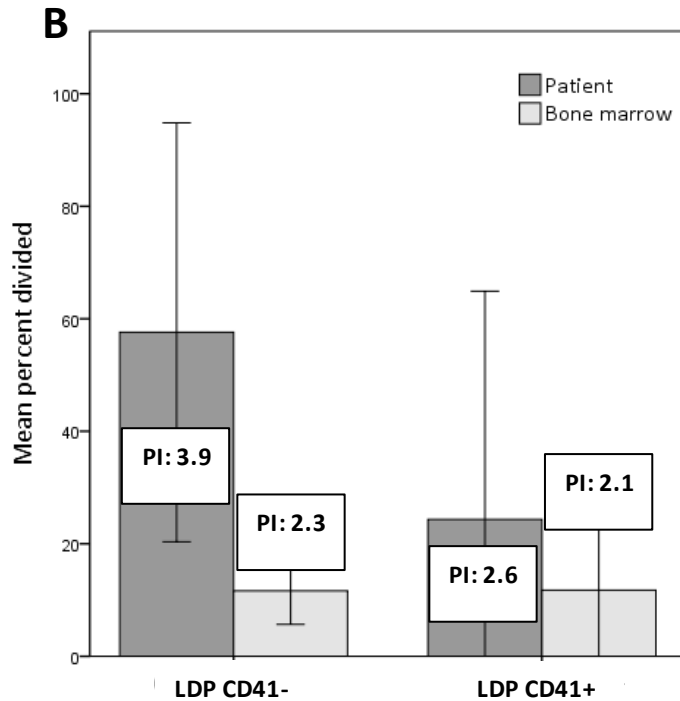
patients were positive for CD41 after the boosting period, even if previously negative (i.e., from LDP CD41<sup>-</sup> cultures) (Figure 25). In BM samples, all cultured LDP CD41<sup>-</sup> samples were positive for CD41 after boosting. Thus, LDP CD41<sup>-</sup> cells can either become CD41 positive or produce CD41<sup>+</sup> progeny, indicating that LDP CD41<sup>+</sup> cells lie downstream and are less primitive than LDP CD41<sup>-</sup> cells.



**Figure 25: Changes in CD41 expression after THPO boosting.** **A.** Representative flow cytometric analysis of a MF patient's PB. Only 2.35% of Lin-CD34+CD133+ cells expressed CD41 before boosting, but both LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> populations were overwhelmingly CD41<sup>+</sup> after boosting. **B.** Overview of cumulative CD133+CD41<sup>±</sup> populations within the CD34 fraction of boosted LDP CD41<sup>±</sup>.

To track the proliferation of the populations, the cells were stained with the cytoplasmic Cell Trace Violet™ dye and assessed by flow cytometry after the culture period. Distinct differences in the proliferation of the surviving cells were observed (Figure 26). Out of the five patient samples tested, only two showed proliferation in the LDP CD41<sup>+</sup> population, compared to four out of five in the CD41<sup>-</sup> population. In the CD41<sup>-</sup> samples, a mean of 68.6% had divided at least once after one week of stimulation, while only a mean of 18.8% of the CD41<sup>+</sup> cells had divided. If the cells had divided at least once, the average number of divisions (proliferation index) was 3.9 for CD41<sup>-</sup> cells and 2.6 for the CD41<sup>+</sup> fraction. LDP CD41<sup>-</sup> from BM (n=8) also proliferated more than LDP CD41<sup>+</sup> cells, with less proliferation overall compared to patient samples. 30% of LDP CD41<sup>-</sup> had divided after boosting, with an average number of 2.3 divisions. In comparison, 17% of LDP CD41<sup>+</sup> had divided with a mean of 2.1 divisions. None of these differences were statistically significant. This data confirmed the subjective observations with light microscopy of the boosting cultures that LDP CD41<sup>-</sup> proliferated more than CD41<sup>+</sup> cells.



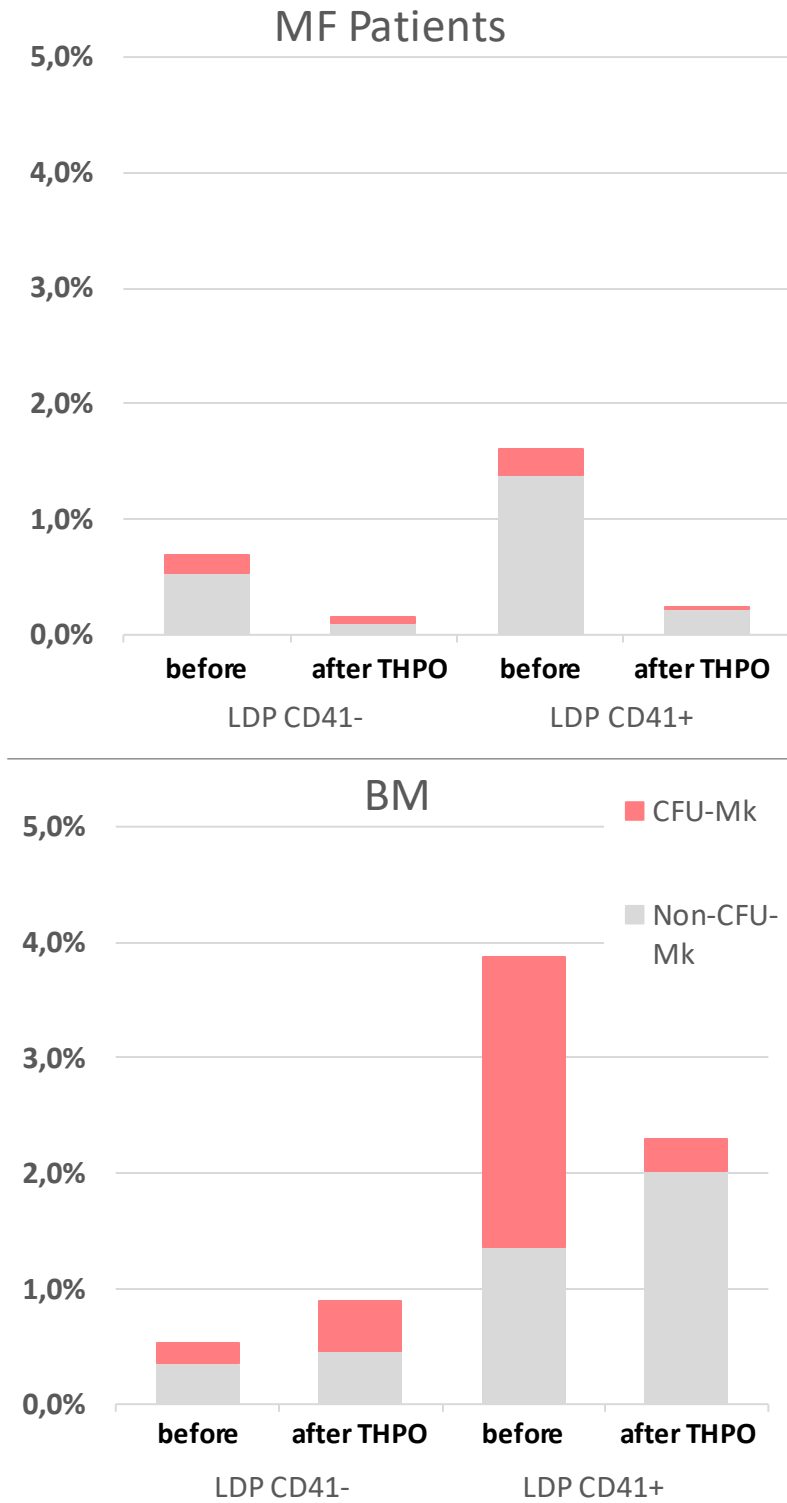


**Figure 26: Proliferation tracking of LDP CD41+ and LDP CD41- cells after boosting with THPO.** **A.** Representative generations found in LDP CD41- (left column) and LDP CD41+ (right column) from MF patients (top box) and from healthy donor BM (bottom box). Generations 1 through 9 (G1-G9) are shown from right to left in accordance with the loss of fluorochrome intensity after each division. **B.** Mean percentages of divided LDP CD41- (left) and CD41+ (right) from either MF patients (n=5) or healthy donor BM (n=8). Proliferation indices are indicated on the corresponding bar. 90% confidence intervals shown.

To test how the clonogenic potential and, in particular, how the potential to form CFU-Mk changed after boosting with THPO, collagen-based colony assays were performed (Figure 27). Prior to boosting, only healthy LDP CD41<sup>+</sup> had shown a preference for forming CFU-Mk, suggesting priming towards megakaryopoiesis. After the boosting period, the majority of colonies formed were non-CFU-Mk from both patient and BM samples. LDP CD41<sup>-</sup> from BM had the highest percentage of CFU-Mk at 47% of total colonies formed, with an overall higher clonogenic potential than before boosting having increased from 0.5% to 0.9% in this fraction. This finding would suggest a boosting of the megakaryocytic potential of healthy LDP CD41<sup>-</sup> following the culturing with THPO without loss of clonogenic potential. By contrast, the healthy LDP CD41<sup>+</sup> appear to have differentiated further down the megakaryocytic lineage and thus retained less cloning potential, resulting in a decreased number of colonies overall, including a lower proportion of CFU-Mk (12.7%).

In patients, the cloning potential of both LDP CD41<sup>+</sup> and CD41<sup>-</sup> markedly decreased after THPO boosting. LDP CD41<sup>+</sup> previously had a slightly higher potential of 1.7%, which fell to 0.2% with a similar decrease of megakaryocytic potential, as seen in BM. Before boosting, 13% of colonies were CFU-Mk, while after boosting, this percentage dropped to 3%. Patient LDP CD41<sup>-</sup> also lost cloning potential after boosting (0.7% to 0.2%). In addition to this decrease, the proportion of CFU-Mk increased from 24% to 32%. This increase could be interpreted as a boosting of megakaryocytic potential in the LDP CD41<sup>-</sup> population,

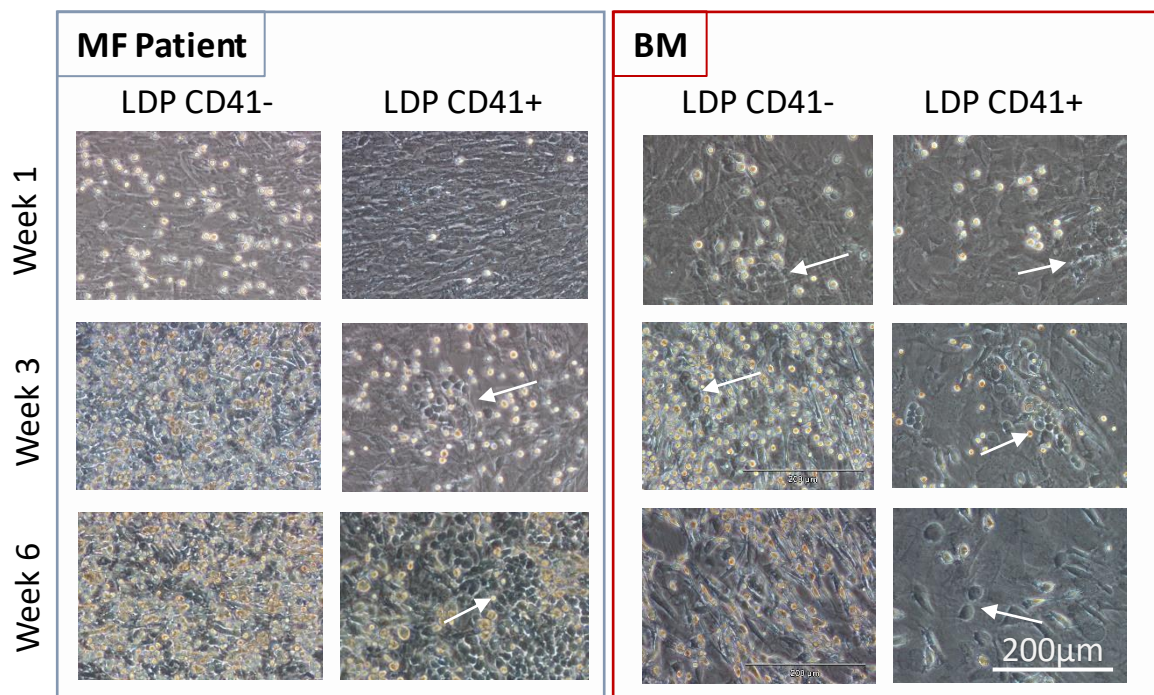
although overall patient LDP appear “burnt-out” after THPO boosting, most likely reflecting an end-point differentiation stage.



**Figure 27: changes in clonogenic potential after THPO boosting for one week.** Shown are the CFU-Mk and Non-CFU-Mk of LDP CD41+ and LDP CD41- from MF patients PB (before n=4, after boosting n=2) and healthy donor BM (before n=6, after boosting n=4) before and after boosting with THPO in Megacult™

Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup>CD41<sup>+</sup> contain long-term colony-initiating cells

The best *in vitro* assay to evaluate stem cells, which, by definition, has the long-term potential to self-renew is the Long-term colony-initiating cell assay (LTC-IC). Thus, to assess if our target populations contained stem cells with long-term potential, we bulk sorted LDP CD41<sup>-</sup> and CD41<sup>+</sup> HSPC isolated from MF patients' PB or healthy donor BM and seeded them onto sub-confluent primary human MSC differentiated from BM in Myelocult™ supplemented with the hydrocortisone, THPO, SCF and IL-3 for a co-culture period of six weeks. During the incubation period, half-medium changes were performed every week, and the culture assessed for the formation of cobblestones. Cobblestone-formation defined as “colonies of at least five small, non-refractile cells that grow underneath the stromal layer” (van Os, Dethmers-Ausema et al. 2008) was used as the first indicator of primitive long-term potential. After the end of the assay, cultures were trypsinized and analyzed by flow cytometry.

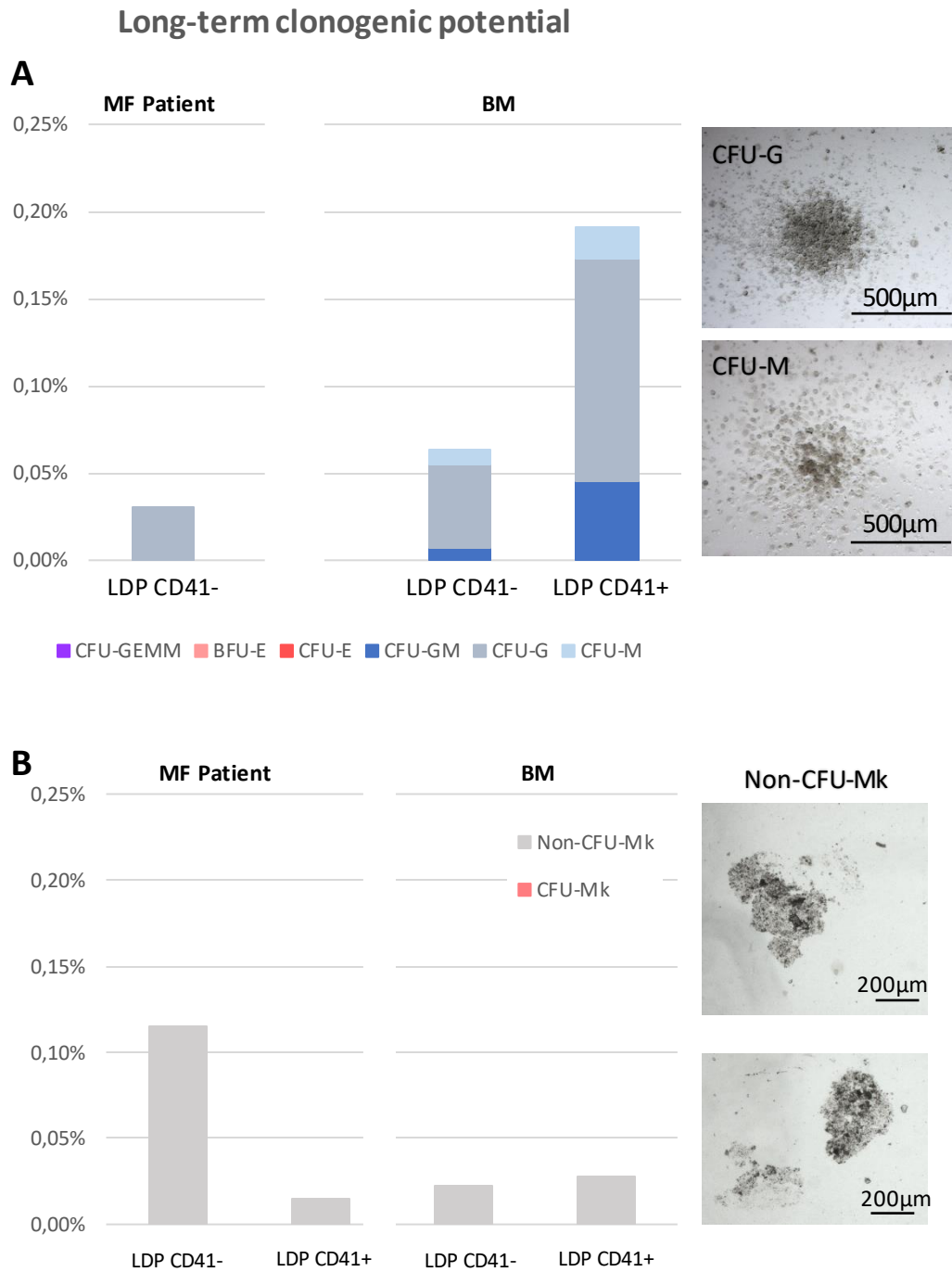


**Figure 28:** Representative light microscopy images of LDP CD41<sup>-</sup> and CD41<sup>+</sup> at 10x magnification during LTC-IC assay taken after one, three and six weeks of co-culture; Post-PV MF patient sample (blue panel) and healthy BM sample (red panel). Cobblestone areas indicated with white arrows.

LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> cells from both MF patient PB and healthy BM showed proliferation and formation of cobblestone areas in co-culture. LDP CD41<sup>+</sup> from healthy BM proliferated the least, but a subset did migrate beneath the stromal feeder layer to form cobblestone areas. LDP CD41<sup>-</sup> proliferated the most, and output cells covered large areas

of the culture well by the end of the culture period (Figure 28). The MSC feeder layer remained healthy throughout the co-culture. Notably, all wells supplemented with hydrocortisone showed signs of adipocyte differentiation with the formation of fat vacuoles, consistent with MSC potential.

After six weeks of co-culture, the MSCs were separated from the hematopoietic cells by FACS and their clonogenic potential tested in Metho- and Megacult™.

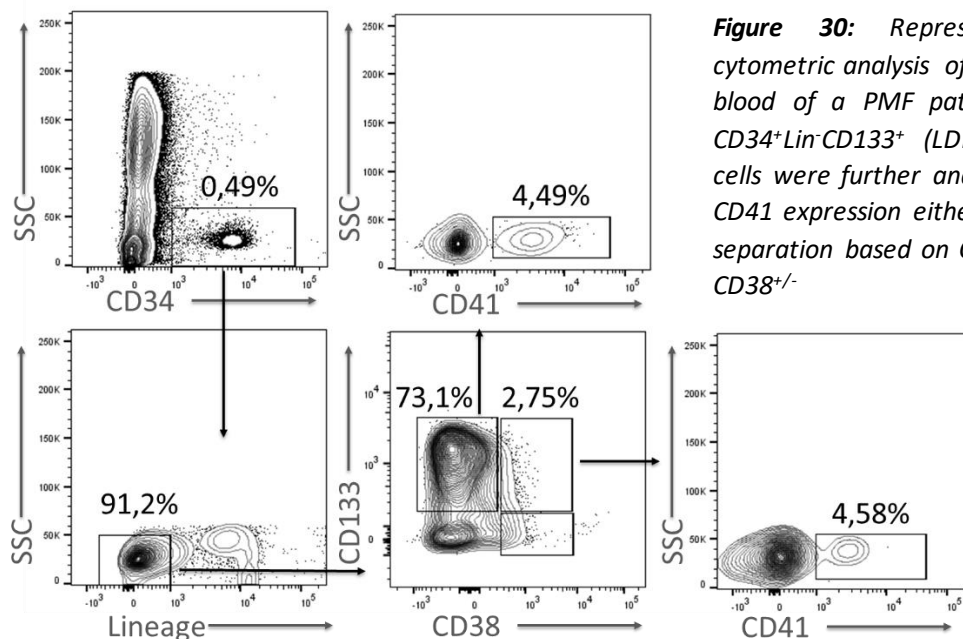


**Figure 29:** Long-term clonogenic potential of LDP CD41<sup>±</sup> from MF patients (left columns) and BM (right columns) after the LTC-IC assay. **A.** Clonogenic potential of assayed populations in Methocult™; colonies depicted on the right. **B.** Clonogenic potential in Megacult™; colonies are shown on the right.

Only a few of the assayed samples formed colonies after the co-culture, confirming that long-term potential is a rare event (Figure 29). Both LDP CD41<sup>+</sup> and CD41<sup>-</sup> cells from one BM and one patient PB formed colonies in both types of colony assays. Overall, the clonogenic potential of the remaining cells after co-culture was very low. In Methocult™, all colonies formed belonged to the G/M lineage. No megakaryocytic potential could be detected in any of the samples after co-culture. Overall, both the ability of LDP CD41<sup>+</sup> and CD41<sup>-</sup> to form cobblestone areas in co-culture and colonies in CFU assays point towards possible long-term potential within these populations. However, the rarity of these colonies and several assays without colony formation speaks against a high enrichment of stem cells in the LDP CD41<sup>+</sup> and CD41<sup>-</sup> fractions.

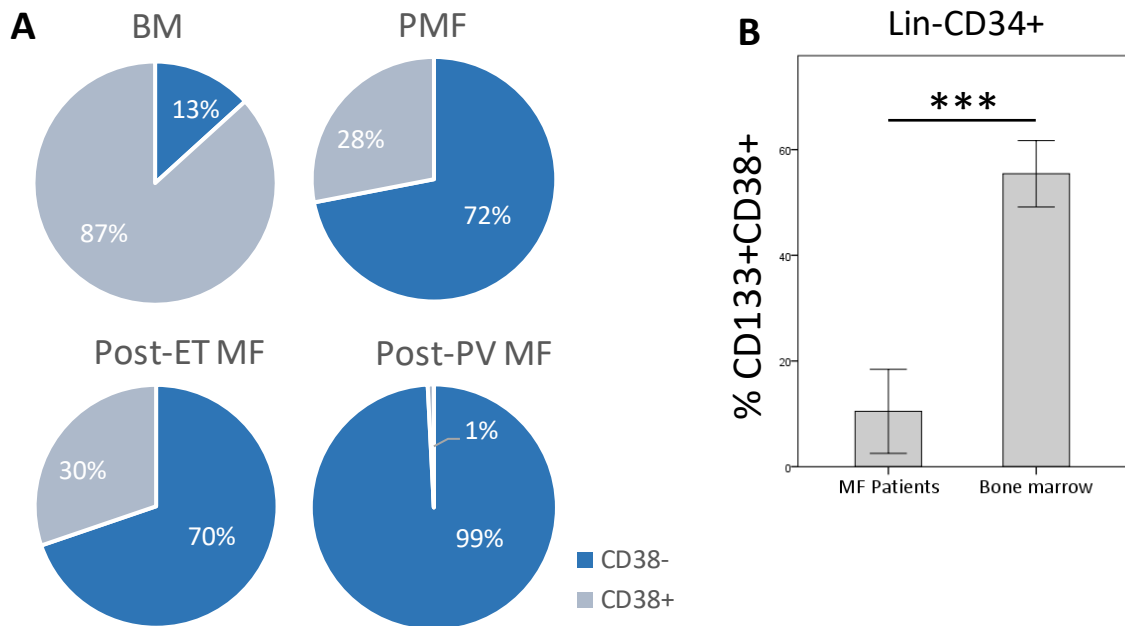
#### LDP from MF patients do not show CD38<sup>+</sup> myeloid progenitor phenotype

The overall higher clonogenic potential of LDP CD41<sup>+</sup> compared to CD41<sup>-</sup> raises the question if perhaps these cells could be a progenitor population. To investigate this hypothesis, we stained for CD38 as a positive marker of myeloid progenitors in the Lin<sup>-</sup>CD34<sup>+</sup> fraction. If LDP CD41<sup>+</sup> cells are myeloid progenitors, we would expect CD41<sup>+</sup> to be more frequently found among LDP CD38<sup>+</sup> compared to LDP CD38<sup>-</sup>. The percentage of LDP that expressed CD38 varied greatly between BM and MF patients PB.



**Figure 30:** Representative flow cytometric analysis of the peripheral blood of a PMF patient gated for CD34<sup>+</sup>Lin<sup>-</sup>CD133<sup>+</sup> (LDP) cells. These cells were further analyzed for their CD41 expression either without prior separation based on CD38 or as LDP CD38<sup>+</sup>/<sup>-</sup>.

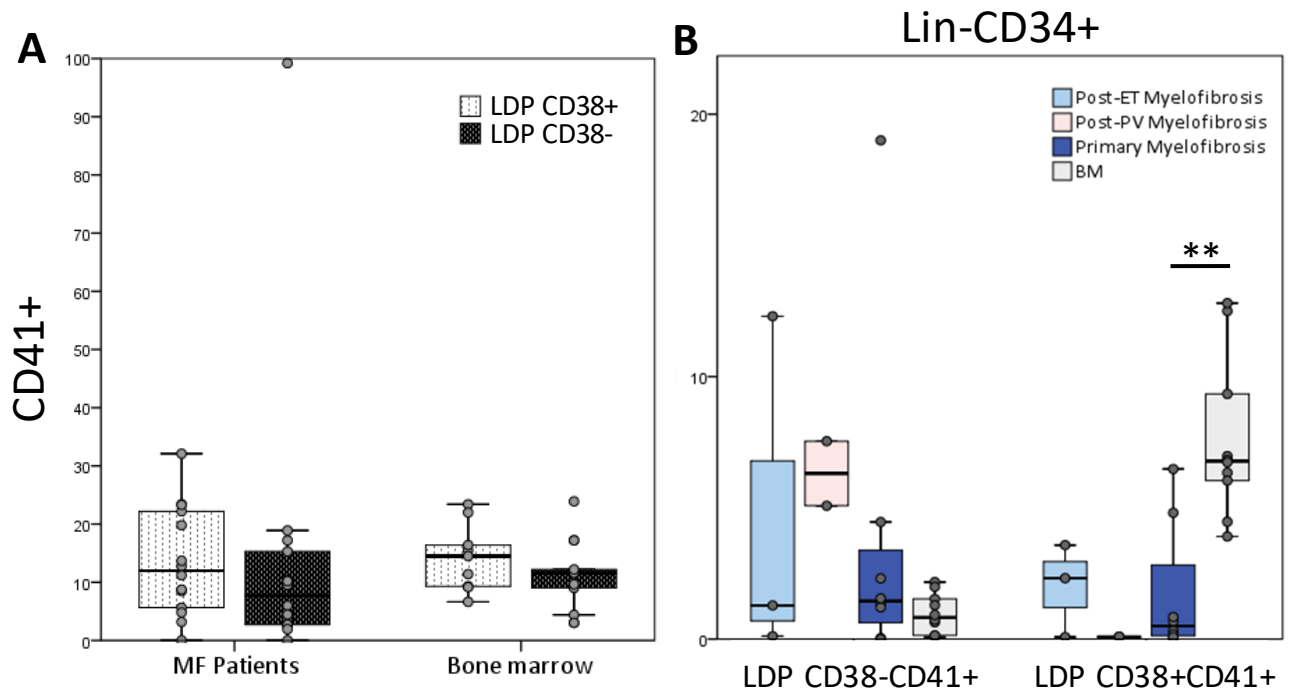




**Figure 31:** CD38 positivity of Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup>. **A.** Mean distribution of CD38<sup>+</sup> in the LDP fraction. **B.** Percentage of LDP CD38<sup>+</sup> cells found in total Lin<sup>-</sup>CD34<sup>+</sup>, 95% confidence interval shown. Significance calculated using Mann-Whitney test (\*\*\*=p<0.001) [BM n=9 PMF n=7 Post-ET MF n=3 Post-PV MF n=2]

LDP from BM overwhelmingly express CD38, whereas the majority of these cells from patients are CD38 negative (Figure 31A). Cells from post-PV MF patients showed the lowest percentages of CD38<sup>+</sup> with a mean of 1%, while PMF and Post-ET MF samples still had a mean percentage of 28-30% positivity. The percentage of LDP CD38<sup>+</sup> cells was significantly higher in the BM Lin<sup>-</sup>CD34<sup>+</sup> fraction than in the patient Lin<sup>-</sup>CD34<sup>+</sup> fraction as calculated by Mann-Whitney Test (p<0.000) (Figure 31B).

While CD41<sup>+</sup> could be found in both LDP CD38<sup>+</sup> and LDP CD38<sup>-</sup> fractions, as shown in figure 30, the proportion of CD41<sup>+</sup> cells differed in the LDP CD38<sup>-</sup> and LDP CD38<sup>+</sup> populations. A slightly higher percentage of CD41<sup>+</sup> can be found in the LDP CD38<sup>+</sup> fraction in both BM as well as in MF patients' PB (Figure 32A). This finding would support that LDP CD41<sup>+</sup> are composed of more progenitors than CD41<sup>-</sup> and thus represent a less primitive population. Among the MF patient cohorts, the highest mean percentage of LDP CD38<sup>+</sup>CD41<sup>+</sup> was found in Post-ET MF patient PB (ø 2.0%). Similar to the significantly lower number of overall LDP CD41<sup>+</sup>, the number of LDP CD38<sup>+</sup>CD41<sup>+</sup> was also significantly lower in PMF patient PB (ø 1.7%) compared to healthy BM (ø 7.1%). Despite the low mean percentage of LDP CD38<sup>+</sup>CD41<sup>+</sup>, some PMF patients did show higher levels of this population, perhaps indicating heterogeneity in this patient cohort (Figure 32B).



**Figure 32: percentage of CD41+ in the Lin-CD34+CD133+38<sup>+/-</sup> fractions. A.** Percentage of CD41+ within the Lin-CD34+CD133+CD38+ and CD38- populations (BM n=9, MF patients n=15). **B.** Percentage of LDP CD38-CD41+ and LDP CD38+CD41+ in the Lin-CD34+ population in the different MF diagnosis groups and healthy donor BM (Post-ET MF n=3, Post-PV MF n=2, PMF n=8, BM n=9). Significance calculated using Mann-Whitney test (\*\*=p<0.01).

Taken together, it seems highly likely that LDP CD41<sup>+</sup> in healthy BM represent a fraction enriched for progenitors. This phenotype is found less often in MF patient PB.

## Discussion

### Transgenic stromal cell lines are suboptimal for long-term co-culture systems

The first aim of this study was to determine if a co-culture cell system could be established that would allow testing the impact of abnormal cytokine production on the survival, proliferation or differential potential of normal HSPC or, eventually, neoplastic stem cells of PMF patients. For this system, a human stromal cell line (HS5) was manipulated to produce either THPO or GM-CSF and then co-cultured with isolated CD34+ HSPC. HS5 were transduced with a vector expressing the BFP2 fluorochrome, in combination with the cytokines, allowing reliable detection and identification of the stromal cell population by flow cytometry. High levels of both GM-CSF and THPO could be detected in the condition media of transduced HS5 by ELISA. However, validating the biological functionality of the cytokines proved more difficult. The TF-1 cell line was isolated from a patient with

erythroleukemia in 1989 and shown to be dependent on either GM-CSF, IL-3 or EPO for its survival (Kitamura, Tange et al. 1989). Today, it is used by manufacturers of recombinant human GM-CSF to determine its biological activity (PeproTech 2020). Condition media of HS5 BFP2 GM-CSF were able to support TF-1, thus proving the biological functionality of the synthesized cytokine. Applying the same principle to confirm the biological activity of THPO proved more difficult. The UT-7/TPO subline of the megakaryoblastic leukemia cell line UT-7 is dependent on the presence of THPO to survive and proliferate (Komatsu, Kunitama et al. 1996). UT-7/TPO stimulated with condition media from HS5 BFP2 THPO, however, showed lower proliferation rates than when stimulated with human recombinant THPO. Cell growth was only slightly better with condition media from HS5 BFP2 THPO as compared to HS5 BFP2, raising the question if HS5 cells produce other cytokines to sustain UT-7/TPO. A previous study demonstrated that HS5 produce measurable levels of GM-CSF and IL-6, and the production of GM-CSF by HS5 BFP2 was confirmed by ELISA in our experiments (Roecklein and Torok-Storb 1995). Considering GM-CSF and IL-6 can support UT-7/TPO *in vitro*, this cell line may, in retrospect, not be optimal for determining THPO biological activity (Komatsu, Kunitama et al. 1996). The possibility that the combined effects of IL-6 and GM-CSF may also be able to sustain UT-7/TPO as well as THPO alone has not been previously examined and can therefore not be excluded.

To inhibit proliferation and prevent the overgrowth of the feeder stromal layer in long-term culture, stromal cells were irradiated with 40Gy. While the cells did continue to grow during the first week of the assay, the viability of the culture thereafter rapidly deteriorated. By the end of the assay period, many cells had died. The surviving cells continued to stain positive for BFP, which would enable a clear separation of stromal cells and other co-cultured cells in the further. GM-CSF and THPO could still be detected in the condition media of irradiated stromal cells, but the concentration decreased proportionally to the lower cell counts. For long-term culture, it would be ideal if the stromal feeder layer were to remain stable for at least six weeks. In light of the high rate of apoptosis already observed after three weeks, using the transduced HS5 cell lines for long-term assays seemed questionable.

To analyze how HSPC would behave in co-culture with the stromal feeder layer, healthy CD34<sup>+</sup> from BM were co-cultured on HS5 GM-CSF and THPO for three weeks. The CD34<sup>+</sup> cells behaved differently depending on the cytokine in the set-up: CD34<sup>+</sup> cells cultured in

the presence of high levels of THPO maintained their multi-lineage potential longer and entered into cell cycle less frequently than identical cells cultured with GM-CSF. This is consistent with the known functions of THPO in steady-state hematopoiesis as a promotor of quiescence and preserver of multi-lineage potential. The loss of multi-lineage potential may be related to the decreasing levels of THPO following irradiation.

CD34<sup>+</sup> cells cultured with GM-CSF produced the fewest colonies and were more often in cell cycle. These results can be interpreted as a direct result of GM-CSF stimulation. GM-CSF mobilizes stem and progenitor cells from the BM niche in humans, an effect that can be used to harvest CD34<sup>+</sup> from the PB of stem cell transplantation donors. It also induces maturation of G/M lineage, influences lineage commitment and boosts proliferation (Metcalf 2013). While the presence of cytokines produced by the transgenic HS5 influenced the co-cultured CD34<sup>+</sup>, it is impossible to determine how much the changes in concentration influenced the assay outcome. Furthermore, as cells entering into apoptosis excrete additional cytokines, an effect of the loss of viability of the stromal feeder layer cannot be excluded (Szondy, Sarang et al. 2017). The established co-culture system, while providing high levels of cytokines over a short period of time, is suboptimal to simulate the conditions in the hematopoietic niche for long-term culture of a minimum of 6 weeks. The conditions are too unstable to draw reliable conclusions in light of the high number of confounding factors.

During the establishment of transgenic HS5 cell lines, our lab introduced protocols for the isolation of MSC from donor BM, which provided a sufficiently contact-inhibited stable feeder layer for long-term culture of six weeks without irradiation. A feeder layer of MSCs was therefore used in subsequent long-term co-culture assays, and this work was discontinued.

#### [Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup>CD41<sup>+</sup> represent a rare population in MF patient PB](#)

Published reports during the course of this work, demonstrating that CD41 expression could mark early megakaryocytic progenitors or HSC-derived, prompted us to investigate this marker further. We were able to identify a rare and novel Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> (LDP) CD41<sup>+</sup> population. Overall, LDP CD41<sup>+</sup> cells were found at a lower frequency in MF patient PB than in healthy BM, although some patients did exhibit larger fractions of LDP CD41<sup>+</sup>. In PMF

patients, this fraction was significantly less frequent in comparison to healthy BM, as well as compared to Post-PV MF samples within the CD34+ population.

It was somewhat unexpected that lower frequencies of the novel LDP CD41+ cells were found in CD34+ fractions of MF patients as compared to healthy BM. A likely explanation is that the analysis in MF was performed on PB and not BM. Of course, it would be ideal to compare patient BM to healthy BM samples. Considering, the patients used in this study all had an MF grade of II or higher, this was not possible. The aspiration of BM becomes increasingly difficult to impossible in correlation to the degree of myelofibrosis. Therefore, analyzing BM samples would only be feasible for patients in the early stages of the disease. Such an analysis would be interesting to conduct in the future.

Nevertheless, a previous publication from our lab had shown an expansion of CD133+ HSPC in the PBMC of PMF patients as compared to healthy BM (Trivaii, Stubig et al. 2015). To more narrowly define this population as an HSPC, the samples used in this study were enriched for the Lin<sup>-</sup> population. This makes a direct comparison to the previous data reporting CD133+ as a total percentage of PB mononuclear cells (PBMC) difficult. However, calculating Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> (LDP) as a percentage of MF patient PBMC after enrichment showed a mean of 1.1% of LDP with a variance of 1.7 (n=27), which is lower than the median of 4.8% of PMF PBMC reported previously without enrichment using lineage markers. This contradicts the expectation that the percentage of LDP would be higher in an enriched population if the frequency were similar. In contrast, the larger BM control group in this study (n=13 vs. n=4) showed a mean percentage of LDP of 0.9% of the total MNC with a variance of 1.1, which is circa fourfold higher than the 0.2% of CD133+ originally found in BM MNC samples. This higher percentage could be explained by the enrichment process. Closer analysis of the original publication revealed a few discrepancies that may explain the lower frequencies of CD133+ cells in the CD34+ compartment of MF patients. First of all, similarly to the earlier study, our cohorts of MF patients showed a wide range of CD133+ within the Lin<sup>-</sup>CD34<sup>+</sup> population. In contrast, however, samples with less than 1% of HSPC of PBMC were excluded from the analysis from the previous study, which led to the removal of up to 25% of the patient samples from the calculation. Moreover, Lin<sup>-</sup>CD34<sup>-</sup>CD133<sup>+</sup> and Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>-</sup> were also included in the previous study, which may also account for some of the differences seen in MF patient samples. Another distinction is that the cohort reported in 2015 only included PMF patients, while we studied PMF patients alongside

Post-ET and Post-PV MF patients. However, the latter two patient cohorts did not show significantly lower numbers of LDP to explain the lower overall percentage of LDP. In summary, this analysis of 27 PB samples from MF patients and 13 BM samples from healthy controls, the percentage of Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> in MF patient PB was similar to that found in BM. Despite the low variance of our cohort in this comparative analysis, it may be advisable to increase the number of patient samples to increase the significance of this data.

#### LDP CD41<sup>+</sup> show multi-lineage potential with priming for megakaryopoiesis

LDP have been characterized to be a heterogeneous population of multi-, bi- and uni-potent potential (Trivai, Stubig et al. 2015). We hypothesized that CD41 expression in the LDP fraction marks a neoplastic HSPC primed for megakaryocytic differentiation in MF patients. CFU-assays confirmed the multi-potency of both LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> to form colonies of the G/M-, erythroid and megakaryocyte lineages. However, only LDP CD41<sup>+</sup> from healthy BM showed a preference to form megakaryocytic colonies over other colony types. LDP CD41<sup>+</sup> from MF patient PB had higher clonogenic potential than LDP CD41<sup>-</sup>, but the proportion of CFU-Mk was not significantly increased. This finding does not confirm our initial hypothesis. Generally, the sample size used to assess clonogenic potential was too small to calculate any significant statistical differences. Increasing the sample size is advisable to confirm if the tendency to form more CFU-Mk would be significant if more samples are tested.

The priming towards megakaryopoiesis was only observed in healthy BM, which may indicate that this pathway of megakaryopoiesis is impaired in MF. Considering the relatively lower levels of LDP CD41<sup>+</sup> in MF patient PB, a contribution to the increased megakaryopoiesis characteristic for this disease seems unlikely. If this were the case, an expansion of this population or another population with megakaryocytic potential would be more plausible.

Boosting with THPO confirmed that LDP CD41<sup>+</sup> lie downstream of LDP CD41<sup>-</sup> cells. Both populations proliferated, as visualized with Cell Trace Violet™ and formed mature megakaryocytes. LDP CD41<sup>-</sup> overwhelmingly either started expressing CD41 or more likely considering the greater percentage of dividing cells, produced LDP CD41<sup>+</sup> progeny. The lower proliferation rates of LDP CD41<sup>+</sup> agree with their hypothesized progenitor status to

confirm their shorter pathway to end-stage maturation. Samples from MF Patient PB proliferated more than healthy BM samples, possibly reflecting the aberrant proliferation capacity of the disease. It is important to note, that the use of the cytoplasmic proliferation tracking dye Cell Trace Violet™ for a one-week assay is not optimal, as the manufacturer only recommends a maximum tracking period of 72h. The dye can be used to track up to 8-9 generations and can, therefore, be used to visualize proliferation or lack thereof, but it is highly likely that many cells will have exceeded this number of divisions after one week, influencing the accuracy of any further conclusions.

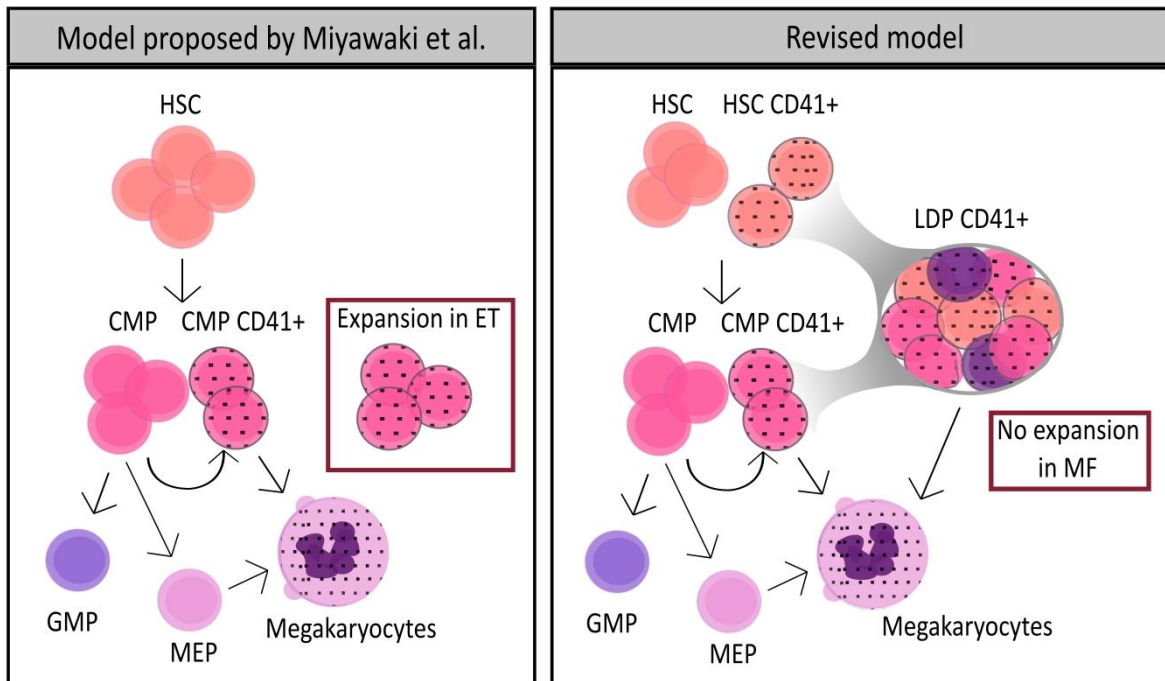
The cloning potential of samples from MF patient PB was greatly diminished after one week, re-affirming that these populations had matured. Healthy BM LDP CD41<sup>-</sup> cells showed an increase in cloning potential after boosting. If these cells are more primitive than LDP CD41<sup>+</sup>, this increase could be explained by fewer quiescent cells after the boosting period contributing to the formation of colonies. Overall, boosting with THPO revealed the LDP CD41<sup>+</sup> to be distinctly different cell populations in MF patient PB and healthy BM.

[Heterogenous LDP CD41<sup>+</sup> population challenges a previous model limiting CD41 expression to megakaryocytic progenitors](#)

The differences observed between the clonogenic potential of LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> prompted us to investigate if LDP CD41<sup>+</sup> included a higher percentage of cells positive for CD38, a marker of myeloid progenitors. Although CD133 has been described as a stem cell marker, it is known that CD133 and CD38 expression is not mutually exclusive (Yin, Miraglia et al. 1997). Analysis of CD38 positivity among LDP showed that a significantly higher number of LDP from BM express CD38 compared to MF patient PB. Furthermore, the fraction of CD41<sup>+</sup> cells was higher in LDP CD38<sup>+</sup> from both MF patient PB and healthy BM. This would support the hypothesis that LDP CD41<sup>+</sup> include more myeloid progenitor compared to LDP CD41<sup>-</sup> cells.

Recently, Miyawaki and colleagues identified a CD41<sup>+</sup> common myeloid progenitor (CMP; CD34<sup>+</sup>CD38<sup>+</sup>IL-3α<sup>dim</sup>CD45RA<sup>-</sup>) with uni-potent megakaryocytic potential in human BM and CB. In their work, CD41 expression was limited almost exclusively to this population and was not found in more primitive fractions such as the HSC (Miyawaki, Iwasaki et al. 2017). It is possible that the LDP CD41<sup>+</sup> population found in healthy BM, which overwhelmingly

also stained positive for CD38, could include the CD41<sup>+</sup>CMP reported by Miyawaki et al., as shown in Figure 33. If this were the case, the priming towards megakaryopoiesis found in healthy BM LDP CD41<sup>+</sup> could be contributed to the presence of uni-potent cloning potential CD41<sup>+</sup>CMP within a heterogeneous LDP CD41<sup>+</sup> population.



**Figure 33: Revised model of CD41 positive populations in human HSPC.** The model proposed by Miyawaki and colleagues is shown on the left, and a new and revised model based on the results of this work is shown on the right. Populations with black pattern are CD41<sup>+</sup>.

However, our new data disagrees with the previous report that CD41 expression is unique to megakaryocytic progenitors in humans. LDP CD41<sup>+</sup> showed multi-lineage potential with only a slight increase of megakaryocytic potential in healthy BM. The latter could be explained by an enrichment of CD41<sup>+</sup>CMP in LDP CD41<sup>+</sup> but would continue to contradict the report of CD41 as an exclusive marker for MKP within the Lin<sup>-</sup>CD34<sup>+</sup> fraction. While it is unlikely that the previously described bi-potent MEP could contribute to the megakaryocytic potential observed in LDP cells, it is possible that uni-potent megakaryocyte progenitors (MKP) could. Sanada and colleagues recently identified adult human progenitors with either megakaryocyte-erythroid potential (MEP) or uni-potent MKP in the CD41<sup>-</sup> or CD41<sup>+</sup> fractions of Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>mid</sup>CD45RA<sup>-</sup>Flt3<sup>-</sup>CD110<sup>+</sup>CD36<sup>-</sup> cells, respectively. MEP gave rise to megakaryocyte-erythroid colonies and were excluded from being CMP by seeding the same population in Methocult™ CFU assays to test their myeloid potential. In contrast, human MKP formed almost exclusively CFU-Mk (Sanada, Xavier-Ferruccio et al. 2016). Their work used many additional markers of myeloid progenitors that



were not included in our analysis; thus, it cannot be directly confirmed or denied that the MKP progenitors described by Sanada et al. are found in the LDP population. However, although these CD41<sup>+</sup> MKP may contribute to their increased megakaryocytic potential, they do not explain the myeloid potential observed here. CD41<sup>-</sup> MEP are possibly found in the LDP CD41<sup>-</sup> fraction, which showed the potential to form all three lineages *in vitro*.

The idea that CD41 can be expressed on primitive stem and progenitor cells is not new (Fraser, Leahy et al. 1986). Other studies using CD41 as a marker on early populations have previously been shown to contain LTC-IC (Debili, Robin et al. 2001). Debili and colleagues determined the presence of LTC-IC in the CD34<sup>+</sup>CD41<sup>-</sup>CD42<sup>-</sup> population isolated from cord blood or leukapheresis samples. The LSKCD34<sup>-</sup>CD150<sup>+</sup>CD41<sup>+</sup> fraction in mice investigated by Yamamoto and colleagues was also composed of ST-HSC, megakaryocyte-erythroid and megakaryocyte repopulating progenitors (Yamamoto, Morita et al. 2013). The LTC-IC assay was used to screen for cells with long-term potential in the LDP CD41<sup>+</sup> fraction. Both the formation of cobblestone-areas during the assay and the colonies formed after six weeks in co-culture indicate that LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> cells from MF patient PB and healthy BM include LTC-IC. These results, taken together with the colony assays showing differentiation in both GM and Meg lineages, demonstrate that the newly identified LDP41<sup>+</sup> fraction contains cells with multi-lineage and long-term potential. This is consistent with studies in mice (Gekas and Graf 2013, Miyawaki, Arinobu et al. 2015).

With this data, we would propose that the heterogeneous LDP CD41<sup>+</sup> population studied in this work comprises several different subsets of CD41<sup>+</sup> HSPC, which may very well include the populations previously described by Miyawaki et al. and Debili et al. (Figure 33). A revised model of CD41 expression found in human HSPC would indicate that CD41 is expressed by various HSPC fractions and not restricted to a uni-potent megakaryocyte progenitor, as suggested by Yamamoto et al.

A number of differences in the experimental approaches may also explain the discrepancy in results. LDP CD41<sup>+</sup> were not double sorted like the CD41<sup>+</sup>CMP, as the cell counts were already quite low. To exclude cross-contamination completely, it may be advisable to double sort LDP populations and assess their functionality. Assays at the single-cell level would also eliminate this confounder.

Results from the LTC-IC assay can only be interpreted as an indicator of cells with long-term potential in the LDP population, rather than definitive confirmation of a LT-HSC. Very few

colonies of uni-potent potential formed after the assay, and some samples did not form any colonies at all. Limiting dilution assays were not performed to calculate LTC-IC frequency of bulk LTC-IC assays as recommended (Stemcell Technologies). Even without limiting dilution assays, it seems reasonable to assume that the frequency of LTC-IC is very low, considering very few of the sorted cells formed any colonies at all after the assay period. LTC-IC are an inherently rare population, and to precisely calculate the frequency of LTC-IC in the LDP CD41<sup>+</sup> and CD41<sup>-</sup> populations, the assay would need to be repeated and limiting dilutions implemented. Furthermore, all cultures were sorted by FACS to eliminate the MSC prior to seeding for CFU assays after the six-week culture period. The handling of the cells, including the cell sorting itself, is known to put stress on the HSPC, which may further inhibit their cloning potential. To evaluate this effect, it could be useful to perform LTC-IC assays without prior cell sorting in the future.

The gold standard for the identification of cells with long-term repopulating potential continues to be xenotransplantation. Therefore, it would be necessary to transplant LDP CD41<sup>+</sup> and LDP CD41<sup>-</sup> from MF patient PB and healthy BM in the future to study their reconstitution potential and possible contributions to megakaryopoiesis *in vivo*.

#### Contribution of the CD41<sup>+</sup> population to disease

In the setting of MPN, the CD41<sup>+</sup>CMP population was found to be significantly expanded within the CD34<sup>+</sup> fraction of BM samples from ET patients by Miyawaki and colleagues. This finding could indicate that it may contribute to aberrant megakaryopoiesis. In agreement with this hypothesis, the allele burden of the *JAK2V617F* driver mutation was also the highest in CD41<sup>+</sup>CMP compared to other progenitors or HSC in ET patients (Miyawaki, Iwasaki et al. 2017). Due to the reasons stated above, it was not possible to analyze MF patient BM in this study. In addition, samples of patients in the early stages of ET, PV and PMF were not available for analysis. The analysis performed here did not differentiate between CMP, MEP and GMP, but including CD38 in our analysis reliably detects the myeloid progenitors, so it can be compared with this study. This analysis demonstrated a highly significant reduction in the LDP CD38<sup>+</sup>CD41<sup>+</sup> fraction in CD34<sup>+</sup> cells in PMF patient samples as compared to BM clearly indicating that the CD41<sup>+</sup>CMP fraction is not expanded in circulating HSCP.

Further analysis of the possible myeloid progenitors in the LDP CD41<sup>+</sup> compartment in BM is necessary to evaluate which populations make up this heterogeneous fraction and contribute to the increased megakaryocytic potential. The depleted numbers of LDP CD38<sup>+</sup>CD41<sup>+</sup>, as well as the absence of megakaryocytic priming in LDP CD41<sup>+</sup> from MF patient PB, make it highly unlikely that this population contributes significantly to the aberrant megakaryopoiesis of myelofibrosis. The lower frequency of CD38 positivity in the LDP could serve to explain the increased clonogenic potential of this fraction in patients, but the possibility of aberrant CD41 expression in the setting of disease that is without any influence on the functionality of the population cannot be excluded.

## Summary

The exact mechanisms contributing to aberrant megakaryopoiesis in Myelofibrosis (MF) are still unclear but may involve either extrinsic interactions with the niche or disruption of normal differentiation programs. In the first part of this thesis, a co-culture system using transgenic human stromal cells expressing either THPO or GM-CSF was established to monitor the impact of abnormal cytokine production within the BM niche environment on normal and neoplastic HSPC. High levels of the cytokines were detected after transduction, and the biological activity confirmed by functional assays. The aim to use these stroma cells to investigate the long-term effects of local overexpression of the cytokines on hematopoietic stem and progenitor cells was not reached, as the stromal feeder layer did not remain stable over the minimum period of six weeks.

In a second approach, changes in an intrinsic differentiation program were investigated by analysis of neoplastic HSPC in MF patients. A recent report suggested that an increase in megakaryocyte-primed CD41<sup>+</sup> myeloid progenitors in MPN patients may contribute to the malignant disease. Here we characterized a novel Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> (LDP) CD41<sup>+</sup> HSPC population primed towards megakaryopoiesis in healthy BM. Overall, LDP CD41<sup>+</sup> show multi-lineage potential and formed megakaryocytic colonies *in vitro*. LDP CD41<sup>+</sup> from BM were able to differentiate into megakaryocytes upon stimulation with THPO. However, the proliferative capacity of this fraction was lower than of LDP CD41<sup>-</sup>, with the latter being able to produce CD41<sup>+</sup> progeny. Thus, LDP CD41<sup>+</sup> cells lie downstream of the LDP CD41<sup>-</sup> population in the hematopoietic hierarchy. The presence of long-term colony-initiating cells was observed in both LDP CD41<sup>+</sup> and CD41<sup>-</sup> fractions. The majority of LDP cells

expressed the progenitor marker CD38 but are distinct to the unipotent megakaryocyte progenitor CD41<sup>+</sup>CMP described earlier in that they have maintained myeloid potential. Further purification is necessary to confirm that CD41<sup>+</sup> marks a long-term, multipotent HSCP, as has been described in the mouse.

Significantly, LDP CD41<sup>+</sup> were also detected in PB from MF patients; however, their levels were not increased within the CD34<sup>+</sup> compartment compared to healthy BM, in contrast to a previous study examining BM of ET patients. Functional assays confirmed megakaryocytic and myeloid differentiation capacity, although a pronounced preference for the meg lineage was not observed. The absence of priming towards megakaryopoiesis, as well as the reduction of this fraction in MF patient PB, does not support a contribution to aberrant megakaryopoiesis in Myelofibrosis.

## Zusammenfassung

Die genauen zugrundeliegenden Mechanismen der aberranten Megakaryopoese bei Myelofibrose (MF) sind bisher nur unzureichend verstanden. Als ursächlich diskutiert werden sowohl extrinsische Interaktionen in der Stammzellnische als auch Störungen der intrinsischen Zelldifferenzierung. Im ersten Teil dieser Arbeit wurde ein Ko-Kultur System mit transgenen humanen Stromazellen, welche entweder THPO oder GM-CSF produzieren, etabliert, mit dem Ziel den Einfluss stark erhöhter Zytokinkonzentrationen innerhalb der Knochenmarksnische auf gesunde und neoplastische hämatopoitische Stamm- und Vorläuferzellen (HSPC) zu untersuchen. Hohe Konzentrationen der gewünschten Zytokine konnten erfolgreich nach der Transduktion der Stromazellen nachgewiesen und ihre biologische Aktivität bestätigt werden. Das Ziel diese transgenen Stromazellen zu verwenden um den Langzeiteinfluss der Überexpression lokaler Zytokine auf HSPC zu untersuchen konnte nicht erreicht werden, da diese nicht über die minimal angesetzte Kulturzeit von sechs Wochen stabil geblieben sind.

Im zweiten Teil wurden Veränderungen innerhalb des intrinsischen Differenzierungsprogramms einer neoplastischen HSPC Population von MF Patienten untersucht. Neue Erkenntnisse deuteten darauf hin, dass ein Anstieg von präferenziell-megakaryozytischen CD41<sup>+</sup> myeloischen Vorläuferzellen ursächlich an der Entstehung von MPN beteiligt sein könnten. Wir konnten eine bisher unentdeckte Lin<sup>-</sup>CD34<sup>+</sup>CD133<sup>+</sup> (LDP) CD41<sup>+</sup> HSPC Population charakterisieren, welche im gesunden Knochenmark eine Präferenz

in Richtung der Megakaryopoese aufweist. Insgesamt besitzen LDP CD41<sup>+</sup> die Fähigkeit in mehrere Zellreihen zu differenzieren und konnten *in vitro* Megakaryozyten-Kolonien bilden. Nach Stimulation mit THPO zeigten gesunde LDP CD41<sup>+</sup> Zellen eine Differenzierung zu Megakaryozyten und deren Vorläufern. Insgesamt war das proliferative Potenzial dieser Fraktion jedoch geringer als bei LDP CD41<sup>-</sup>. Letztere waren zudem in der Lage LDP CD41<sup>+</sup> Zellen zu generieren. Daraus lässt sich ableiten, dass es sich bei der LDP CD41<sup>+</sup> Population um die weniger primitive Differenzierungsstufe handelt. Sowohl in Langzeitkulturen aus LDP CD41<sup>+</sup> als auch aus LDP CD41<sup>-</sup> wurden Langzeit-Koloniebildende Zellen (LTC-IC) gefunden. Jedoch fand sich der Vorläufermarker CD38 in einer Mehrheit von LDP CD41<sup>+</sup>. Im Vergleich zur vorbeschriebenen CD41<sup>+</sup>CMP Population verfügen LDP CD41<sup>+</sup> weiterhin über das Differenzierungspotenzial für die myeloische Reihe. Eine weitere Aufreinigung CD41<sup>+</sup> Zellen ist notwendig um zu bestätigen, dass CD41<sup>+</sup> eine multipotente-langzeit-Stamm- und Vorläuferzelle markiert wie es bereits in der Maus beschrieben wurde.

LDP CD41<sup>+</sup> konnten auch im peripheren Blut von MF Patienten nachgewiesen werden, allerdings war der Anteil dieser Zellen innerhalb des CD34<sup>+</sup> Kompartiments, im Gegensatz zu einer vorherigen Studie an gesundem Knochenmark und dem Knochenmark von ET Patienten, nicht erhöht. Funktionelle Assays konnten belegen, dass LDP CD41<sup>+</sup> aus Patienten zwar sowohl die megakaryozytäre als auch die myeloische Reihe bilden konnten, allerdings fand sich keine Neigung zur Bildung von Megakaryozyten oder deren Vorläufern. Das Fehlen dieser Präferenz als auch die insgesamt sehr niedrige Frequenz dieser Population unterstützt nicht die Hypothese, dass LDP CD41<sup>+</sup> zur aberranten Megakaryopoese im Rahmen der Myelofibrose beitragen.

## Supplementary material

### List of abbreviations

<b><math>\alpha</math>-MEM</b>	Alpha-Minimum Essential Medium
<b>bFGF</b>	$\beta$ -Fibroblast growth factor
<b>BM</b>	Bone marrow
<b>BFU</b>	Burst forming unit
<b>CALR</b>	Calreticulin
<b>CMP</b>	Common myeloid progenitor
<b>CFU</b>	Colony forming unit
<b>CFU-E</b>	Colony forming unit erythroid
<b>CFU-G</b>	Colony forming unit granulocyte
<b>CFU-GEMM</b>	Colony forming unit granulocyte, erythroid, macrophage, megakaryocyte
<b>CFU-GM</b>	Colony forming unit granulocyte/monocyte
<b>CFU-M</b>	Colony forming unit monocyte
<b>CFU-Mk</b>	Colony forming unit megakaryocyte
<b>c-kit</b>	Proto-oncogene c-kit / stem cell growth factor
<b>CLP</b>	Common lymphoid progenitor
<b>CMRP</b>	Common myeloid repopulating progenitor
<b>CXCL4</b>	C-X-C motif ligand 4
<b>EPO</b>	Erythropoietin
<b>FBS</b>	Fetal bovine serum
<b>Flt3</b>	Cytokine polypeptide deformylase-like tyrosine kinase receptor 3
<b>GM-CSF</b>	Granulocyte-Monocyte stimulating factor
<b>GMP</b>	Granulocyte monocyte progenitor
<b>HSC</b>	Hematopoietic stem cell
<b>IL-3</b>	Interleukin 3
<b>IL-6</b>	Interleukin 6
<b>IL-11</b>	Interleukin 11
<b>IMDM</b>	Isocove's minimum essential medium
<b>JAK2</b>	Janus kinase 2
<b>KuO</b>	Kusabira-Orange
<b>LeGO</b>	Lentiviral Gene Ontology Vector
<b>Lin</b>	Lineage
<b>LMPP</b>	Lymphoid-myeloid primed progenitor
<b>LTC-IC</b>	Long-term colony-initiating cell assay
<b>LT-HSC</b>	Long-term hematopoietic stem cell
<b>MEP</b>	Megakaryocyte Erythrocyte progenitor cell
<b>MERP</b>	Megakaryocyte-erythroid repopulating progenitor
<b>MDS</b>	Myelodysplastic syndrome
<b>MK</b>	Megakaryocyte

<b>MKP</b>	Megakaryocyte progenitor
<b>MkRP</b>	Megakaryocyte repopulating progenitor
<b>MLP</b>	Multilymphoid progenitor
<b>Mpl</b>	Myeloproliferative leukemia receptor
<b>MPP</b>	Multipotent progenitor
<b>PBS</b>	Phosphate buffered saline
<b>PDC</b>	Paired daughter cell assay
<b>PMF</b>	Primary myelofibrosis
<b>Post-ET MF</b>	Post-Essential Thrombocythemia Myelofibrosis
<b>Post-PV MF</b>	Post-Polycythaemia vera Myelofibrosis
<b>Propidium iodide</b>	PI
<b>SCF</b>	Stem cell factor
<b>Sca-1</b>	Stem cell antigen 1
<b>SLAM</b>	Signaling lymphocyte activation molecule
<b>SL-MkP</b>	Stem cell-like megakaryocyte progenitors
<b>ST-HSC</b>	Short-term hematopoietic stem cell
<b>THPO</b>	Thrombopoietin
<b>Thy-1</b>	Thymocyte antigen 1 (CD90)
<b>vWF</b>	Von Willebrand factor

Supplementary Table 1: Overview of patients

<b>Pat. Nr.</b>	<b>Diagnosis</b>	<b>Mutation</b>
<b>P122</b>	post-PV MF	JAK2
<b>P141</b>	PMF	JAK2
<b>P155</b>	unclassified MF	triple negative
<b>P158</b>	post-PV MF	EZH2
<b>P162</b>	post-PV MF	JAK2
<b>P163</b>	PMF	JAK2
<b>P195</b>	post-PV MF	JAK2
<b>P221</b>	PMF	JAK2
<b>P245</b>	PMF	JAK2
<b>P246</b>	Post PV MF	JAK2
<b>P249</b>	Post-ET MF	MPL
<b>P252</b>	PMF	JAK2
<b>P255</b>	PMF	CALR
<b>P258</b>	PMF	JAK2
<b>P260</b>	Post-ET MF	CALR
<b>P268</b>	PMF	JAK2
<b>P271</b>	post-PV MF	JAK2
<b>P272</b>	PMF	JAK2
<b>P273</b>	Post-ET MF	CALR
<b>P281</b>	post-ET MF	CALR
<b>P282</b>	post-PV MF	JAK2
<b>P283</b>	PMF	JAK2
<b>P284</b>	Post-ET MF	MPL
<b>P286</b>	PMF	JAK2
<b>P288</b>	PMF	CALR

<b>P290</b>	PMF	JAK2
<b>P291</b>	PMF	CALR
<b>P292</b>	PMF	JAK2 rearrangement
<b>P293</b>	MPN unclassified	JAK2
<b>P294</b>	post-PV MF	JAK2
<b>P295</b>	PMF	JAK2
<b>P300</b>	Post-ET MF	triple negative
<b>P303</b>	post-PV MF	n.a.
<b>P304</b>	MDS/MPN MF	JAK2
<b>P306</b>	PMF	JAK2

## References

Adamson, J. W., et al. (1976). "Polycythemia vera: stem-cell and probable clonal origin of the disease." N Engl J Med **295**(17): 913-916.

Adolfsson, J., et al. (2001). "Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity." Immunity **15**(4): 659-669.

Adolfsson, J., et al. (2005). "Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment." Cell **121**(2): 295-306.

Aivado, M., et al. (2007). "Serum proteome profiling detects myelodysplastic syndromes and identifies CXC chemokine ligands 4 and 7 as markers for advanced disease." Proc Natl Acad Sci U S A **104**(4): 1307-1312.

Akashi, K., et al. (2000). "A clonogenic common myeloid progenitor that gives rise to all myeloid lineages." Nature **404**(6774): 193-197.

Ansorge, S. and M. Täger (2014). Immunologie. Löffler/Petrides Biochemie und Pathobiochemie. P. C. Heinrich, M. Müller and L. Graeve. Berlin, Heidelberg, Springer Berlin Heidelberg: 893-930.

Ballmaier, M., et al. (2001). "c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia." Blood **97**(1): 139-146.

Barbui, T., et al. (2018). "The 2016 WHO classification and diagnostic criteria for myeloproliferative neoplasms: document summary and in-depth discussion." Blood Cancer J **8**(2): 15.

Baum, C. M., et al. (1992). "Isolation of a candidate human hematopoietic stem-cell population." Proc Natl Acad Sci U S A **89**(7): 2804-2808.

Baxter, E. J., et al. (2005). "Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders." Lancet **365**(9464): 1054-1061.

Becker, A. J., et al. (1963). "Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells." Nature **197**: 452-454.



- Bhatia, M. (2001). "AC133 expression in human stem cells." Leukemia **15**(11): 1685-1688.
- Bhatia, M., et al. (1997). "Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice." Proc Natl Acad Sci U S A **94**(10): 5320-5325.
- Bradley, T. R. and D. Metcalf (1966). "The growth of mouse bone marrow cells in vitro." Aust J Exp Biol Med Sci **44**(3): 287-299.
- Bruns, I., et al. (2014). "Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion." Nat Med **20**(11): 1315-1320.
- Burstein, S. A., et al. (1984). "Platelet factor-4 excretion in myeloproliferative disease: implications for the aetiology of myelofibrosis." British Journal of Haematology **57**(3): 383-392.
- Castro-Malaspina, H., et al. (1981). "Human megakaryocyte stimulation of proliferation of bone marrow fibroblasts." Blood **57**(4): 781-787.
- Christensen, J. L. and I. L. Weissman (2001). "Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells." Proc Natl Acad Sci U S A **98**(25): 14541-14546.
- Ciurea, S. O., et al. (2007). "Pivotal contributions of megakaryocytes to the biology of idiopathic myelofibrosis." Blood **110**(3): 986-993.
- Civin, C. I., et al. (1984). "Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells." J Immunol **133**(1): 157-165.
- Conneally, E., et al. (1997). "Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice." Proc Natl Acad Sci U S A **94**(18): 9836-9841.
- Corbeil, D., et al. (2000). "The human AC133 hematopoietic stem cell antigen is also expressed in epithelial cells and targeted to plasma membrane protrusions." J Biol Chem **275**(8): 5512-5520.
- Crane, G. M., et al. (2017). "Adult haematopoietic stem cell niches." Nat Rev Immunol **17**(9): 573-590.
- de Wynter, E. A., et al. (1998). "CD34+AC133+ cells isolated from cord blood are highly enriched in long-term culture-initiating cells, NOD/SCID-repopulating cells and dendritic cell progenitors." STEM CELLS **16**(6): 387-396.
- Debili, N., et al. (2001). "Different expression of CD41 on human lymphoid and myeloid progenitors from adults and neonates." Blood **97**(7): 2023-2030.
- Delhommeau, F., et al. (2009). "Mutation in TET2 in myeloid cancers." N Engl J Med **360**(22): 2289-2301.
- Doulatov, S., et al. (2010). "Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development." Nat Immunol **11**(7): 585-593.
- Doulatov, S., et al. (2012). "Hematopoiesis: a human perspective." Cell Stem Cell **10**(2): 120-136.

- Dykstra, B., et al. (2007). "Long-term propagation of distinct hematopoietic differentiation programs in vivo." Cell Stem Cell **1**(2): 218-229.
- El Agha, E., et al. (2017). "Mesenchymal Stem Cells in Fibrotic Disease." Cell Stem Cell **21**(2): 166-177.
- Fargeas, C. A., et al. (2003). "AC133 antigen, CD133, prominin-1, prominin-2, etc.: prominin family gene products in need of a rational nomenclature." STEM CELLS **21**(4): 506-508.
- Florek, M., et al. (2005). "Prominin-1/CD133, a neural and hematopoietic stem cell marker, is expressed in adult human differentiated cells and certain types of kidney cancer." Cell Tissue Res **319**(1): 15-26.
- Forsberg, E. C., et al. (2006). "New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3+ multipotent hematopoietic progenitors." Cell **126**(2): 415-426.
- Fraser, J. K., et al. (1986). "Expression of antigens of the platelet glycoprotein IIb/IIIa complex on human hematopoietic stem cells." Blood **68**(3): 762-769.
- Fried, W. (1971). "Anemia of renal failure." Med Clin North Am **55**(1): 3-9.
- Gallacher, L., et al. (2000). "Isolation and characterization of human CD34(-)Lin(-) and CD34(+)Lin(-) hematopoietic stem cells using cell surface markers AC133 and CD7." Blood **95**(9): 2813-2820.
- Gangat, N. and A. Tefferi (2020). "Myelofibrosis biology and contemporary management." BrJ Haematol.
- Gekas, C. and T. Graf (2013). "CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age." Blood **121**(22): 4463-4472.
- Geyer, H. L., et al. (2014). "Distinct clustering of symptomatic burden among myeloproliferative neoplasm patients: retrospective assessment in 1470 patients." Blood **123**(24): 3803-3810.
- Gianelli, U., et al. (2012). "The European Consensus on grading of bone marrow fibrosis allows a better prognostication of patients with primary myelofibrosis." Modern Pathology **25**(9): 1193-1202.
- Greaves, M., et al. (1981). "Mapping cell surface antigen expression of haemopoietic progenitor cells using monoclonal antibodies." Ciba Found Symp **84**: 109-129.
- Haas, S., et al. (2015). "Inflammation-Induced Emergency Megakaryopoiesis Driven by Hematopoietic Stem Cell-like Megakaryocyte Progenitors." Cell Stem Cell **17**(4): 422-434.
- Heinrich, P. C., et al. (2014). Rezeptoren und ihre Signaltransduktion. Löffler/Petrides Biochemie und Pathobiochemie. P. C. Heinrich, M. Müller and L. Graeve. Berlin, Heidelberg, Springer Berlin Heidelberg: 411-441.
- Ikuta, K. and I. L. Weissman (1992). "Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation." Proc Natl Acad Sci U S A **89**(4): 1502-1506.
- Jacobson, R. J., et al. (1978). "Agnogenic myeloid metaplasia: a clonal proliferation of hematopoietic stem cells with secondary myelofibrosis." Blood **51**(2): 189-194.

James, C., et al. (2005). "A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera." Nature **434**(7037): 1144-1148.

Jamieson, C. H., et al. (2006). "The JAK2 V617F mutation occurs in hematopoietic stem cells in polycythemia vera and predisposes toward erythroid differentiation." Proc Natl Acad Sci U S A **103**(16): 6224-6229.

Kaushansky, K. (1997). "Thrombopoietin: understanding and manipulating platelet production." Annu Rev Med **48**: 1-11.

Kaushansky, K., et al. (1994). "Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin." Nature **369**(6481): 568-571.

Kawashima, I., et al. (1996). "CD34+ human marrow cells that express low levels of Kit protein are enriched for long-term marrow-engrafting cells." Blood **87**(10): 4136-4142.

Kitamura, T., et al. (1989). "Establishment and characterization of a unique human cell line that proliferates dependently on GM-CSF, IL-3, or erythropoietin." J Cell Physiol **140**(2): 323-334.

Klampfl, T., et al. (2013). "Somatic mutations of calreticulin in myeloproliferative neoplasms." N Engl J Med **369**(25): 2379-2390.

Komatsu, N., et al. (1996). "Establishment and characterization of the thrombopoietin-dependent megakaryocytic cell line, UT-7/TPO." Blood **87**(11): 4552-4560.

Komura, E., et al. (2003). "Spontaneous STAT5 activation induces growth factor independence in idiopathic myelofibrosis: possible relationship with FKBP51 overexpression." Exp Hematol **31**(7): 622-630.

Kondo, M., et al. (1997). "Identification of clonogenic common lymphoid progenitors in mouse bone marrow." Cell **91**(5): 661-672.

Krantz, S. B. and E. Goldwasser (1984). "Specific binding of erythropoietin to spleen cells infected with the anemia strain of Friend virus." Proc Natl Acad Sci U S A **81**(23): 7574-7578.

Kroger, N., et al. (2009). "Allogeneic stem cell transplantation after reduced-intensity conditioning in patients with myelofibrosis: a prospective, multicenter study of the Chronic Leukemia Working Party of the European Group for Blood and Marrow Transplantation." Blood **114**(26): 5264-5270.

Kuter, D. J. (2013). "The biology of thrombopoietin and thrombopoietin receptor agonists." International Journal of Hematology **98**(1): 10-23.

Lacout, C., et al. (2006). "JAK2V617F expression in murine hematopoietic cells leads to MPD mimicking human PV with secondary myelofibrosis." Blood **108**(5): 1652-1660.

Lansdorp, P. M., et al. (1990). "Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow." J Exp Med **172**(1): 363-366.

Larochelle, A., et al. (2011). "Human and rhesus macaque hematopoietic stem cells cannot be purified based only on SLAM family markers." Blood **117**(5): 1550-1554.

- Liu, Y. C., et al. (2020). "Clinicopathologic characterisation of myeloid neoplasms with concurrent spliceosome mutations and myeloproliferative-neoplasm-associated mutations." J Clin Pathol.
- Mansson, R., et al. (2007). "Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors." Immunity **26**(4): 407-419.
- Manz, M. G., et al. (2002). "Prospective isolation of human clonogenic common myeloid progenitors." Proc Natl Acad Sci U S A **99**(18): 11872-11877.
- Marty, C., et al. (2016). "Calreticulin mutants in mice induce an MPL-dependent thrombocytosis with frequent progression to myelofibrosis." Blood **127**(10): 1317-1324.
- Mead, A. J. and A. Mullally (2017). "Myeloproliferative neoplasm stem cells." Blood **129**(12): 1607-1616.
- Metcalf, D. (2013). "The Colony-Stimulating Factors and Cancer." Cancer immunology research **1**(6): 351-356.
- Milosevic Feenstra, J. D., et al. (2016). "Whole-exome sequencing identifies novel MPL and JAK2 mutations in triple-negative myeloproliferative neoplasms." Blood **127**(3): 325-332.
- Miraglia, S., et al. (1997). "A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning." Blood **90**(12): 5013-5021.
- Miyawaki, K., et al. (2015). "CD41 marks the initial myelo-erythroid lineage specification in adult mouse hematopoiesis: redefinition of murine common myeloid progenitor." STEM CELLS **33**(3): 976-987.
- Miyawaki, K., et al. (2017). "Identification of unipotent megakaryocyte progenitors in human hematopoiesis." Blood **129**(25): 3332-3343.
- Mizrak, D., et al. (2008). "CD133: molecule of the moment." J Pathol **214**(1): 3-9.
- Momen-Heravi, F., et al. (2013). "Current methods for the isolation of extracellular vesicles." Biol Chem **394**(10): 1253-1262.
- Morita, Y., et al. (2010). "Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment." J Exp Med **207**(6): 1173-1182.
- Morrison, S. J. and D. T. Scadden (2014). "The bone marrow niche for haematopoietic stem cells." Nature **505**(7483): 327-334.
- Morrison, S. J. and A. C. Spradling (2008). "Stem cells and niches: mechanisms that promote stem cell maintenance throughout life." Cell **132**(4): 598-611.
- Morrison, S. J., et al. (1997). "Identification of a lineage of multipotent hematopoietic progenitors." Development **124**(10): 1929-1939.
- Morrison, S. J. and I. L. Weissman (1994). "The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype." Immunity **1**(8): 661-673.
- Mulisch, M. W., Ulrich (2010). Färbungen. Romeis - Mikroskopische Technik, Springer Spektrum: 181-297.

- Nakamura-Ishizu, A., et al. (2014). "Megakaryocytes are essential for HSC quiescence through the production of thrombopoietin." Biochem Biophys Res Commun **454**(2): 353-357.
- Nangalia, J., et al. (2013). "Somatic CALR Mutations in Myeloproliferative Neoplasms with Nonmutated JAK2." N Engl J Med **369**(25): 2391-2405.
- Nishikii, H., et al. (2015). "Unipotent Megakaryopoietic Pathway Bridging Hematopoietic Stem Cells and Mature Megakaryocytes." STEM CELLS **33**(7): 2196-2207.
- Notta, F., et al. (2011). "Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment." Science **333**(6039): 218-221.
- Notta, F., et al. (2016). "Distinct routes of lineage development reshape the human blood hierarchy across ontogeny." Science **351**(6269): aab2116.
- Osawa, M., et al. (1996). "Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell." Science **273**(5272): 242-245.
- Papayannopoulou, T. and K. Kaushansky (2016). "Evolving insights into the synergy between erythropoietin and thrombopoietin and the bipotent erythroid/megakaryocytic progenitor cell." Exp Hematol **44**(8): 664-668.
- Pardanani, A., et al. (2011). "Primary myelofibrosis with or without mutant MPL: comparison of survival and clinical features involving 603 patients." Leukemia **25**(12): 1834-1839.
- Pardanani, A. D., et al. (2006). "MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients." Blood **108**(10): 3472-3476.
- Passamonti, F. and M. Maffioli (2016). "Update from the latest WHO classification of MPNs: a user's manual." Hematology Am Soc Hematol Educ Program **2016**(1): 534-542.
- Passamonti, F., et al. (2008). "Prognostic factors for thrombosis, myelofibrosis, and leukemia in essential thrombocythemia: a study of 605 patients." Haematologica **93**(11): 1645-1651.
- Passamonti, F., et al. (2005). "Leukemic transformation of polycythemia vera: a single center study of 23 patients." Cancer **104**(5): 1032-1036.
- Passamonti, F., et al. (2010). "A prospective study of 338 patients with polycythemia vera: the impact of JAK2 (V617F) allele burden and leukocytosis on fibrotic or leukemic disease transformation and vascular complications." Leukemia **24**(9): 1574-1579.
- PeproTech (2020). "Recombinant Human GM-CSF." Retrieved 18.02.2020, 2020, from <https://www.peprotech.com/en/recombinant-human-gm-csf>.
- Pikman, Y., et al. (2006). "MPLW515L is a novel somatic activating mutation in myelofibrosis with myeloid metaplasia." PLoS Med **3**(7): e270.
- Reya, T., et al. (2001). "Stem cells, cancer, and cancer stem cells." Nature **414**(6859): 105-111.
- Roecklein, B. A. and B. Torok-Storb (1995). "Functionally distinct human marrow stromal cell lines immortalized by transduction with the human papilloma virus E6/E7 genes." Blood **85**(4): 997-1005.

- Sanada, C., et al. (2016). "Adult human megakaryocyte-erythroid progenitors are in the CD34+CD38mid fraction." Blood **128**(7): 923-933.
- Sanjuan-Pla, A., et al. (2013). "Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy." Nature **502**(7470): 232-236.
- Schneider, R. K., et al. (2017). "Gli1+ Mesenchymal Stromal Cells Are a Key Driver of Bone Marrow Fibrosis and an Important Cellular Therapeutic Target." Cell Stem Cell **20**(6): 785-800 e788.
- Schneider, R. K., et al. (2017). "Gli1+ Mesenchymal Stromal Cells Are a Key Driver of Bone Marrow Fibrosis and an Important Cellular Therapeutic Target." Cell Stem Cell **20**(6): 785-800.e788.
- Schofield, R. (1978). "The relationship between the spleen colony-forming cell and the haemopoietic stem cell." Blood Cells **4**(1-2): 7-25.
- Scott, L. M., et al. (2007). "JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis." N Engl J Med **356**(5): 459-468.
- Shi, H., et al. (2016). "ASXL1 plays an important role in erythropoiesis." Sci Rep **6**: 28789.
- Sieburg, H. B., et al. (2006). "The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets." Blood **107**(6): 2311-2316.
- Singh, R., et al. (2020). Embryology, Hematopoiesis. StatPearls. Treasure Island (FL), StatPearls Publishing  
StatPearls Publishing LLC.
- Sitnicka, E., et al. (2003). "Human CD34+ hematopoietic stem cells capable of multilineage engrafting NOD/SCID mice express flt3: distinct flt3 and c-kit expression and response patterns on mouse and candidate human hematopoietic stem cells." Blood **102**(3): 881-886.
- Spangrude, G. J., et al. (1988). "Purification and characterization of mouse hematopoietic stem cells." Science **241**(4861): 58-62.
- Spivak, J. L. (1989). "Erythropoietin." Blood Rev **3**(2): 130-135.
- Sungaran, R., et al. (1997). "Localization and regulation of thrombopoietin mRNA expression in human kidney, liver, bone marrow, and spleen using in situ hybridization." Blood **89**(1): 101-107.
- Sutherland, H. J., et al. (1989). "Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro." Blood **74**(5): 1563-1570.
- Swerdlow, S. H., et al. (2017). WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues, International Agency for Research on Cancer.
- Szondy, Z., et al. (2017). "Anti-inflammatory Mechanisms Triggered by Apoptotic Cells during Their Clearance." Frontiers in immunology **8**: 909-909.
- Takano, H., et al. (2004). "Asymmetric division and lineage commitment at the level of hematopoietic stem cells: inference from differentiation in daughter cell and granddaughter cell pairs." J Exp Med **199**(3): 295-302.

- Tavian, M., et al. (2010). "Embryonic origin of human hematopoiesis." Int J Dev Biol **54**(6-7): 1061-1065.
- Tefferi, A. (2000). "Myelofibrosis with myeloid metaplasia." N Engl J Med **342**(17): 1255-1265.
- Tefferi, A. (2010). "Novel mutations and their functional and clinical relevance in myeloproliferative neoplasms: JAK2, MPL, TET2, ASXL1, CBL, IDH and IKZF1." Leukemia **24**(6): 1128-1138.
- Till, J. E. and E. A. McCulloch (1980). "Hemopoietic stem cell differentiation." Biochim Biophys Acta **605**(4): 431-459.
- Trivaii, I., et al. (2015). "CD133 marks a stem cell population that drives human primary myelofibrosis." Haematologica **100**(6): 768-779.
- Trivaii, I., et al. (2018). "ASXL1/EZH2 mutations promote clonal expansion of neoplastic HSC and impair erythropoiesis in PMF." Leukemia: 1-11.
- Ugo, V., et al. (2004). "Multiple signaling pathways are involved in erythropoietin-independent differentiation of erythroid progenitors in polycythemia vera." Exp Hematol **32**(2): 179-187.
- Ulrich Welsch, T. D., Wolfgang Kummer (2014). Lehrbuch Histologie/ Welsch. München, Elsevier, Urban & Fischer.
- Vainchenker, W. and R. Kralovics (2017). "Genetic basis and molecular pathophysiology of classical myeloproliferative neoplasms." Blood **129**(6): 667-679.
- van Os, R. P., et al. (2008). "In vitro assays for cobblestone area-forming cells, LTC-IC, and CFU-C." Methods Mol Biol **430**: 143-157.
- Wang, H., et al. (2019). "Prognostic value of U2AF1 mutant in patients with de novo myelodysplastic syndromes: a meta-analysis." Ann Hematol **98**(12): 2629-2639.
- Wang, X., et al. (2012). "Spleens of myelofibrosis patients contain malignant hematopoietic stem cells." J Clin Invest **122**(11): 3888-3899.
- Weber, K., et al. (2008). "A multicolor panel of novel lentiviral "gene ontology" (LeGO) vectors for functional gene analysis." Mol Ther **16**(4): 698-706.
- Xavier-Ferruccio, J. and D. S. Krause (2018). "Concise Review: Bipotent Megakaryocytic-Erythroid Progenitors: Concepts and Controversies." STEM CELLS.
- Xu, M., et al. (2005). "The constitutive mobilization of bone marrow-repopulating cells into the peripheral blood in idiopathic myelofibrosis." Blood **105**(4): 1699-1705.
- Yamamoto, R., et al. (2013). "Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells." Cell **154**(5): 1112-1126.
- Yan, X. Q., et al. (1996). "A model of myelofibrosis and osteosclerosis in mice induced by overexpressing thrombopoietin (mpl ligand): reversal of disease by bone marrow transplantation." Blood **88**(2): 402-409.

Yang, L., et al. (2005). "Identification of Lin(-)Sca1(+)kit(+)CD34(+)Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients." Blood **105**(7): 2717-2723.

Yin, A. H., et al. (1997). "AC133, a Novel Marker for Human Hematopoietic Stem and Progenitor Cells." Blood **90**(12): 5002-5012.



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## Curriculum vitae

Curriculum vitae was omitted in adherence to data-protection laws.

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