Investigating the Epigenetic State of Young and Aged Mouse Hematopoietic Stem Cells

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Abstract

Hematopoietic stem cells produce all types of blood cells and are known for their self-renewal capacity, but are nevertheless prone to the detrimental effects of aging, such as a deteriorated immune functioning and a higher incidence of myeloid malignancies. Histone posttranslational modifications (PTMs) play a role in gene expression and silencing. One form of PTMs, epigenetic modifications, is being studied for their possible contribution to the aging of the hematopoietic system. A protocol for intracellular FACS staining was optimized to quantify the amount of H3K4me3, H3K27me3, H3K27ac and H3K9ac marks in LT-HSCs isolated from young and old mice.

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Introduction

Hematopoiesis

Hematopoiesis is the process in which all blood components are formed from one single cell type: the Long Term-Hematopoietic Stem Cell (LT-HSC). (Hematopoietic) stem cells are unique in their capability to not only differentiate into more mature cell types, but also maintain their own stem cell pool. The choice between self-renewal and differentiation is directed by various cell-intrinsic and -extrinsic factors, such as signals coming from the HSC-niche, an hypoxic microenvironment in the bone marrow where the HSCs reside in a quiescent state (Geiger et al, 2013).

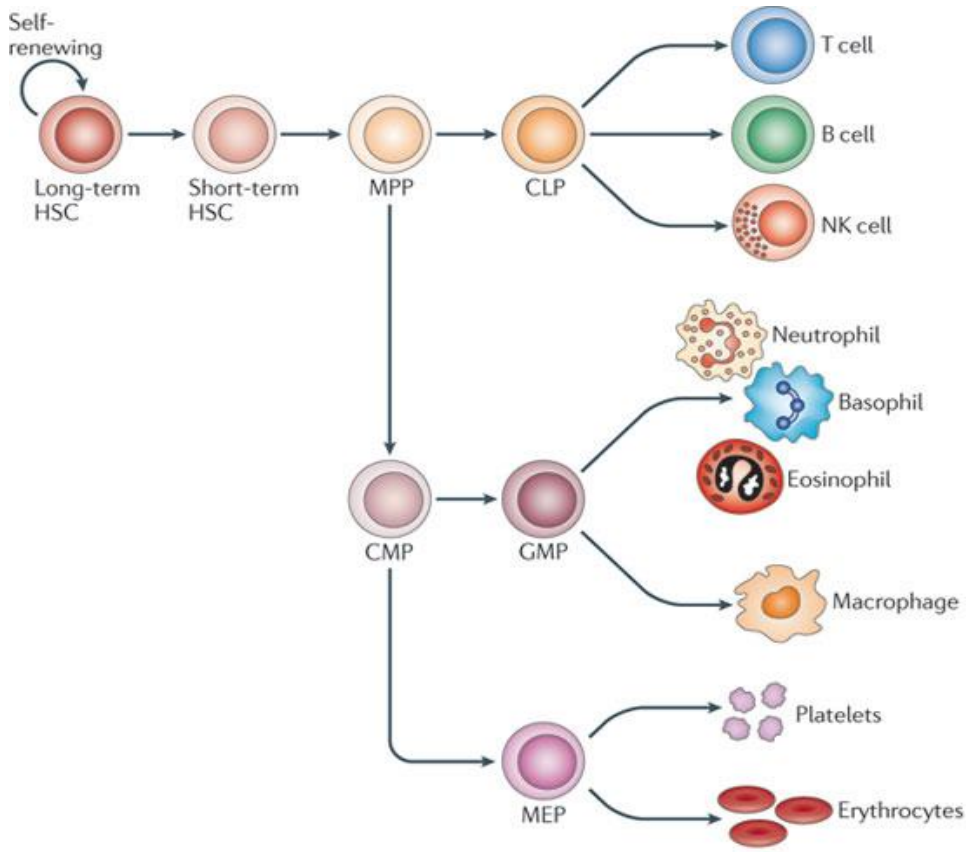

***Figure 1: Hematopoietic tree**. King* et al. *(2004)*

The stem cell hierarchy is depicted in *figure 1*. Up in the hierarchy is the LT-HSC itself, which is capable of either resupplying the HSC pool or differentiating into common myeloid or lymphoid progenitors.

The HSC maintains its position in the HSC niche in the bone marrow, as do many of the cells further down in the hierarchy. All myeloblast descendants, as well as thrombocytes, can be found in the blood circulation. The maturation of certain common lymphoid progenitors takes a slightly different path: T lymphocytes are maturing in the thymus. But all other descendants of the common lymphoid progenitor are produced in the bone marrow and will eventually be found in the blood.

Epigenetics

All cells in an organism have the same DNA, but it depends on epigenetic mechanisms exactly which genes are being transcribed. Epigenetics is considered to function as gene switches. Gene silencing can be accomplished by posttranslationally modifying histone tails, DNA or the regulation of RNA (Goldberg *et al.*, 2007). This is how the difference in the number of genes between some higher and lower organisms is explained: both humans and mice have approximately 20-25X10^3 genes. But some lower organisms or plants have much more genes. Epigenetic mechanisms are facilitating the production of many proteins with relatively few genes (Guenet, 2005).

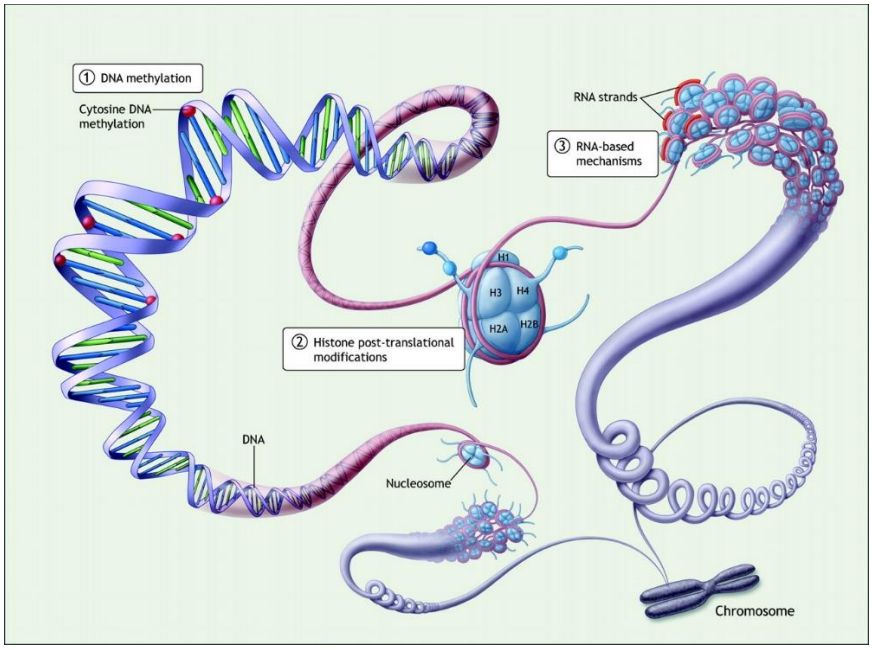

Figure 2: Posttranslational modifications*. Matouk and Marsden (2008)*

Figure 2 shows three epigenetic methods of adding an additional "layer" of information to the DNA. One method, DNA methylation, is the addition of methyl groups to specific nucleotides of the DNA. In somatic cells, methylation occurs at CpG sites: Methyl groups are added to cytosines which are next to a guanine. DNA methylation is known to inhibit gene expression. The general principle behind the other method is that RNA based mechanisms are involved in chromosome stability and gene expression via RNA interference pathways. And yet another method, histone modifications, is part of the focus of this report. Histones consists of 2 times four core protein subunits: H2A, H2B, H3 and H4. These four core subunits are highly conserved among eukaryotes (Cutter and Hayes, 2015). Each histone subunit consists of 2 parts: the histone fold and the tail. The C-terminal domain of the tail consists of alpha helices and the N-terminal tails are involved in chromatin formation and contribute to nucleosome stability. The tails undergo internucleosomal interactions leading to higher order chromatin structures. The tails, as compared to the globular part of the histones, are modified more frequently, because they are readily accessible (Iwasaki *et al.*, 2013).

The tails of the histones are subjected to modifications, which affect the expression of the genes on the DNA that is wrapped around those particular histones. Some modifications are known to be stimulating gene transcription, while other modifications are repressing. Several modifications of histones are possible: acetylation, methylation, ubiquitylation, phosphorylation,

sumoylation, ribosylation and citrullination. Among the various modifications, histone methylations at lysine and arginine residues are relatively stable and are therefore considered potential marks for carrying the epigenetic information that is stable through cell divisions (Barski *et al*., 2007).

In the case of acetylation, an acetyl group is transferred to the tail, which causes the DNA to "loosen up". This happens because the newly added acetyl group removes positive charge, so there is less interaction between the histones and the negatively charged DNA. This loosened configuration of the DNA enables it to be easier transcribed. In other words: acetylation of the histones has an activating effect on DNA transcription. Another example of histone modification is methylation. This is only possible on lysine or arginine, but mostly found at lysine. Arginine has an N-terminus with two N2H groups and a maximum total of two hydrogen atoms can be replaced by a methyl group, either symmetrically or asymmetrically. Lysine has a NH3 terminus, from which all hydrogen atoms can be exchanged by a methyl group. Very specifically, for example genes with histone 3 trimethylation at lysine 4 (H3K4me3) are expressed. The same trimethylation, but on a different lysine (H3K27me3) has the opposite effect: this is a marker for repression of the gene in question, as reviewed by Rando and Chang (2012).

Aging of the hematopoietic system

The higher in the hematopoietic hierarchy a cell is, the more multipotency and self-renewal capacity it has. The further along in the hierarchy, the more mature and differentiated a cell is (see *figure 1*). Upon maturation and differentiation of the cells in the hematopoietic system, more and more genes are silenced via epigenetic mechanisms; they become less multipotent (Lunyak and Rosenfeld, 2008). It is known that intrinsic factors, such as Reactive Oxygen Species can have an effect on epigenetics, and the same holds true for extrinsic factors, such as radiation (Geiger *et al.*, 2013).

There are indications that the epigenome changes upon aging, which might have detrimental effects. For example, studies in *C. elegans* indicate that too many trimethylations at of lysine 4 at histone 3 is harmful for an organism (Greer *et al.*, 2010). Although these examples are indications for aging related epigenetic changes, it is still unknown whether consistent aging related changes do exist.

There are functional differences between young and old mouse HSCs: the ratio myeloid and lymphoid precursors is increasing (myeloid skewing). Also, the potential to regenerate and home is decreasing upon aging, although the total stem cell number is increasing, as first described by De Haan and Van Zant in 1999. A higher incidence of myeloid malignancies (due to an accumulation of DNA mutations) is observed as well as a change in innate and acquired immune system. This compromised immune function is possibly partially compensated by a higher number of HSCs in aged mice.

Aim of the project

In this project, the aim is to investigate the epigenetic contribution to the aging of HSCs. The scope of this project is to screen 4 histone marks (H3K4me3, H3K27me3, H3K27ac and H3K9ac) and see if they change upon aging. In order to do so, we optimized a technique to quantify epigenetic changes in a limited amount of purified stem cells that enabled us to use both intracellular and surface markers to identify the very rare LT-HSC population and to quantify the histone marks. The method that was used, intracellular FACS staining, requires very few cells

and, in contrast with most methods nowadays, provides true quantitative information on the single cell level.
Additional experiments concerning H3K27me3 were performed in which the functionality of the LT-HSCs was monitored when treated with the methyltransferase inhibitor GSK 126. Since these experiments are ongoing, only trends are shown in this report and no conclusive results from this particular experiment.

Materials & Methods

Cell culturing

32D cell line

The 32D cell line was cultured in RPMI medium 1640 (Gibco) supplemented with 10% FCS, Pen/strep and 0,1% mouse IL-3 cytokine. The cells were passaged at 70% confluence (every 2-3 days) and incubated at 37°C.

32D with inhibitor GSK 126

To validate the sensitivity of FACS, 32D cells were incubated overnight with the methyltransferase inhibitor GSK 126. The downregulation of methylation in the cells caused a shift which was measured and detected with intracellular FACS staining (intracellular staining for H3k27me3). The 32D cells were incubated at 37°C in RPMI medium 1640 (Gibco) supplemented with 10% FCS, Pen/strep and 0,1% mouse IL-3 cytokine, with 0,5, 1 and 3 µM GSK 126.

32D with inhibitor Trichostatin (TSA)

32D cells were incubated overnight at 37°C in RPMI medium medium 1640 (Gibco) supplemented with 10% FCS, Pen/strep and 0,1% mouse IL-3 cytokine, with 4 µM deacetylase inhibitor TSA. Upon inhibition with TSA, a state of hyperacetylation occurred, which created an upward shift when intracellular FACS staining for H3K27ac was applied.

LT-HSCs

The LT-HSCs used to test the effect GSK 126 on stem cells were incubated in Stemspan (Stemcell Technologies, Vancouver, BC, Canada) medium, for 48 hours at 34°C.

Intracellular FACS staining

Collection and processing of bone marrow

With exception of the skull, all bones from C57BL/6 mice were collected. After removing excess tissue, the bones were mechanically crushed and the red blood cells were lysed with a total volume of 25 mL erylysis buffer at room temperature. After 5-10 minutes, an equal volume of PBS 0,2% BSA was added to stop the lysis. The cells were pelleted by centrifugation at 300 x g for 5 minutes. The pellet was then resuspended in 1 mL PBS 0,2% BSA and the cell number was determined.

Surface staining

In order to identify LT-HSC, bone marrow cells isolated from C57Bl/6 mice were stained with a cocktail of antibodies against Sca1, c-Kit, CD150, CD48, and lineage markers (Ter119, CD11b, CD3, B220 and Gr1, BioLegend, San Diego, CA).

After 30-40 minutes incubation in the dark at 4°C, the cells were washed with 0,2% BSA PBS to remove any residual antibodies. The cells were then centrifuged at 300 x g for 5 min and for the control samples that ware not going to be permeabilized, the pellets were resuspended in 0,2% BSA PBS and stored at 4°C in the dark until FACS analysis, together with one set of single stained controls.

Intracellular staining
In order to access the histones for staining, the cell and nuclear membrane were permeabilized. Two different agents were used for this purpose: formaldehyde/triton and BD buffer.

Formaldehyde/triton
To all samples and single stained controls, 100 µL of 0,1% formaldehyde was added and after incubation for 10 min, 100 µL of 0,1% triton in PBS was added and incubated for another 15 min. The cells were washed twice with 0,1% triton in PBS and resuspended in the remaining volume.

BD buffer
100 µL BD Cytofix/cytoperm was added to the single stained controls and 500 µL BD Cytofix/cytoperm was added to the samples. After 15 minute incubation at 4°C, 1 mL 0,1% triton in PBS was added and centrifuged for 5 minutes at 300 x g. Pellets were resuspended in the remaining volume.

Antibody incubation and fluorescence measurement
From this point on, the protocol for permeabilization with formaldehyde/triton and BD buffer was identical, with exception of the 3% BSA/PBS/0,1% triton that was used with the BD buffer to keep the cells permeable. With formaldehyde/triton, 3% BSA/PBS was used.
The permeabilized single stained controls went through the same protocol, but were not incubated with the primary and secondary antibody.
The samples were incubated for 30 min at 4°C with 10 µg/mL of the primary antibody (K4me3, H3K27me3, H3K27ac or H3K9ac, diluted with 3% BSA/PBS/0,1% triton). Next, the cells were washed with 3% BSA/PBS/0,1% triton and incubated for 20-30 min at 4°C with 100 µL of the secondary antibody (AF 488, GαR, diluted 1:1000 in 3% BSA/PBS/0,1% triton).
After the final incubation, the cells were washed three times with 3% BSA/PBS and eventually resuspended in 0,2% BSA/PBS after which the fluorescence was determined with FACS Canto.

Quantification of the FACS data
Samples were acquired with FACS Canto and analyzed with Kaluza (Beckman Coulter). Single cell fluorescence data for each histone mark per sample was exported to Excel. All samples were normalized to the average fluorescence of the young mice, which enabled to quantify and compare data acquired in different days. Statistical analysis was performed with PRISM.

CAFC (Cobblestone Area Forming Cell assay)
A 96 wells microtiter plate was seeded with a stromal feeder layer consisting of FBMD cells. The IMDM medium (Gibco) used contained the following substitutes: β-mercaptoethanol, Pen/Strep, hydrocortisone and 20% horse serum. LT-HSCs from both young and old mice were sorted onto the microtiter plate in limiting dilutions. The sorting was based on the same antibody mix as described in the previous section *Surface staining*.
For both the young and old mice, two different concentrations of the EZH2 methyltransferase inhibitor GSK 126 were used: 1 µM and 3 µM, as well as a control plate with no inhibitors.
Plates were incubated for 5 weeks at 34°C, with 5% CO2 and scored weekly for the presence of cobblestones, using a phase contrast microscope. The medium was refreshed weekly.

Results

32D cell line

In order to quantify histone marks in young and old HSCs, a protocol for intracellular staining and FACS quantification had to be optimized. The 32D bone marrow-like cell line was used to validate and optimize the protocol for intracellular FACS staining.

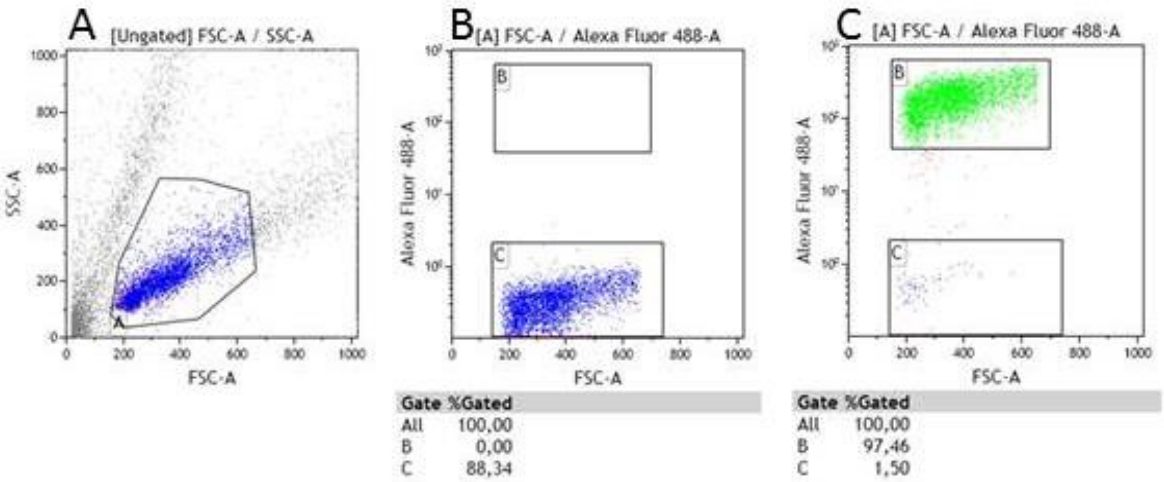

Figure 3: 32 cells. *A: Ungated. B: Blank control stained with Alexa Fluor 488. C: H3K4me3 stained with AF 488.*

A formaldehyde/triton permeabilization protocol has been used at first. Histone mark H3K4me3 was stained with Alexa Fluor 488. As shown in figure 3, a positive signal is acquired in the 488 channel (*figure 3C*) compared to the unstained control (*figure 3B*), confirming a successful intracellular staining.

32D with methyltransferase inhibitor GSK 126

Next, in order to answer the question whether there are differences in the amount of epigenetic marks between young and old mouse hematopoietic stem cells, we needed to ensure that FACS staining was capable to detect a decrease or increase in histone methylation or acetylation. To this end, the EZH2 inhibitor GSK 126 was used to inhibit H3k27me3.

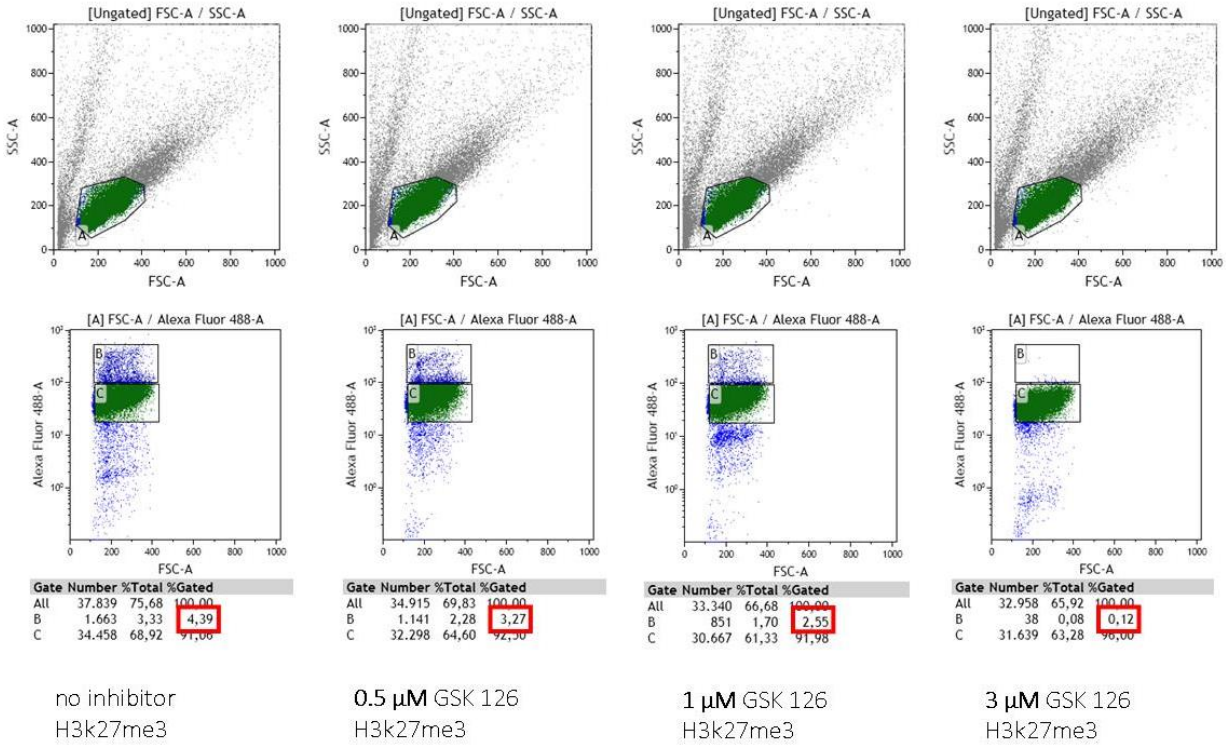

Figure 4: 32D cells treated with GSK 126 to inhibit methylation. *Upon increased concentration of GSK 126, a downward shift in fluorescence is visible.*

Figure 4 shows that the 32D cells that were treated with GSK 126, in fact have a lower fluorescence intensity as compared to the untreated cells, indicating that the shift is indeed detectable with FACS.

32D with deacetylation inhibitor Trichostatin (TSA)

In addition to the treatment of 32D cells with the EZH2 inhibitor GSK, which caused a downward shift of the fluorescence signal, 32D cells were also incubated with 4 µM of a deacetylation inhibitor TSA.

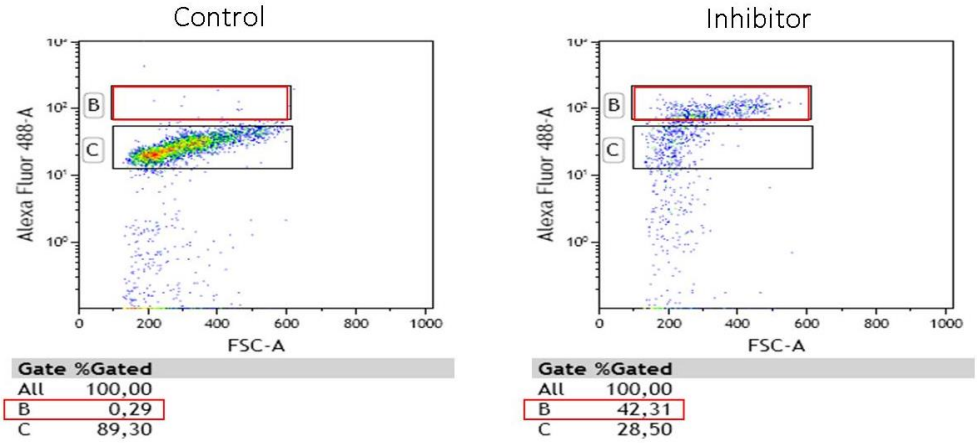

Figure 5: 32D cells treated with TSA to inhibit deacetylation. *The 32D cells incubated with TSA display a higher fluorescence intensity.*

Figure 5 displays a higher fluorescence intensity of the 32D cells that were treated with TSA, when comparing them to the untreated control. So also this shift is detectable with FACS, which further validates this method.

Mouse HSCs
Mouse HSCs are a rare population defined by the LSK SLAM code, which include a combination of 8 surface markers. Thus, after optimizing the intracellular staining, I worked on a protocol for simultaneous detection of surface and intracellular proteins. *Table 1* shows the membrane markers that were used and the conjugated fluorochromes.

Table 1: Antigen and fluorochrome combination

Antigen	Fluorophore
Sca1	PB
c-Kit	PE
CD150	PE/Cy7
CD48	AF 647
B220	AF 700
CD11b	AF 700
CD3	AF 700
Gr1	AF 700
Ter119	AF 700

In order to access the histones and stain them, the cells needed to be permeablized. The primary and secondary antibodies are then able to enter the nucleus.
With the method for fixation and permeabilization we initially used (formaldehyde and triton), we observed a substantial loss of intensity in the PE, PB and PE/Cy7 channels (*figure 6*).

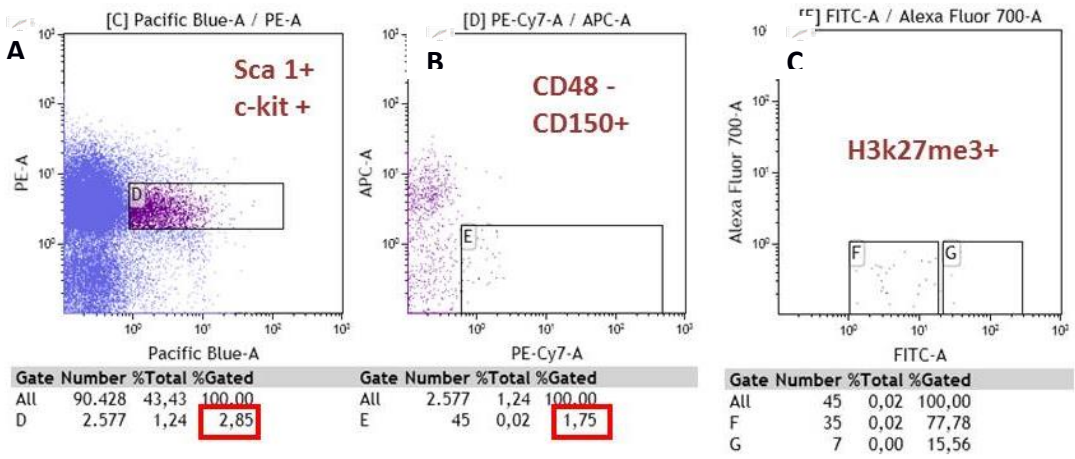

Figure 6: Loss of signal. *Fluorescence in the CD150 and the subsequent channels is significantly reduced.*

In *figure 6B* the signal in the gate for CD150+ is very low. As a consequence, populations gated on this population are also greatly reduced, leading to an almost undetectable histone mark signal.
When using a buffer, specifically designed for the same purpose, the signal was maintained when comparing the permeabilized with the non-permeabilized cells (*figure 7*).

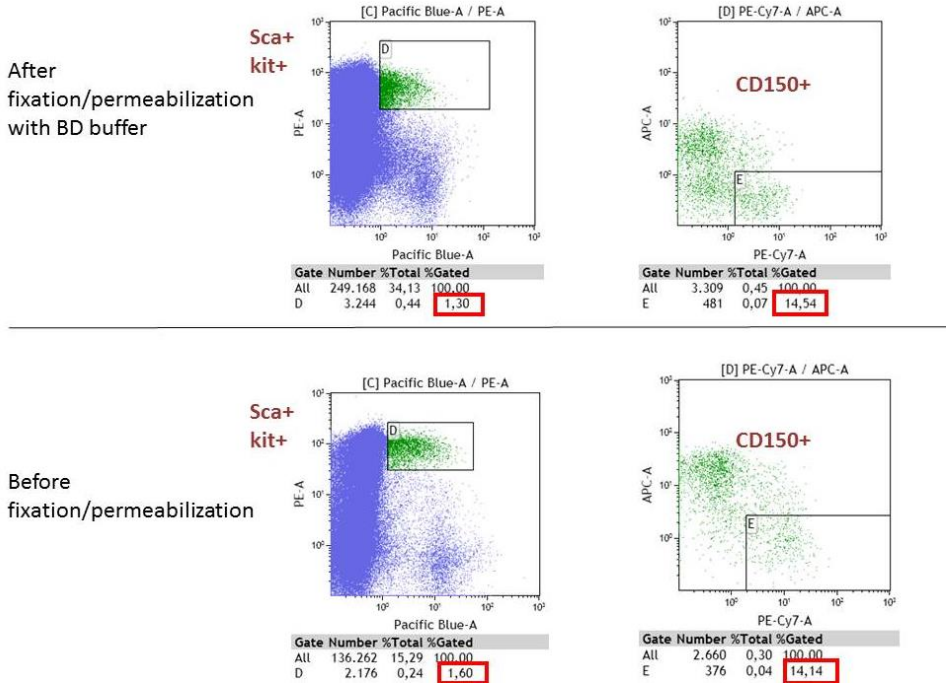

Figure 7: Comparison between permeabilized and non-permeabilized samples.
The percentage gated cells is the same between both treatments.

Figure 7 displays the permeabilized en non-permeabilized HSCs and as can be seen, the percentage of cells in the gates is the same. This indicates that permeabilization with this buffer has no negative effect on the intracellular staining.

After optimizing the intracellular staining protocol for LT-HSCs, the amount of 4 different histone marks (H3K4me3, H3K27me3, H3K27ac and H3K9ac) was measured in stem cells isolated from young (4 months old) and aged (24 months old) mice. We then calculated the average fluorescence of the young mice and normalized all single cell data on this value. When looking at the mean fluorescent intensity at the single cell level, a comparison between young and old was made for each mark. As shown in *figure 8* old LT-HSCs are characterized by an increase in H3k27me3 and a difference in H3K27ac.

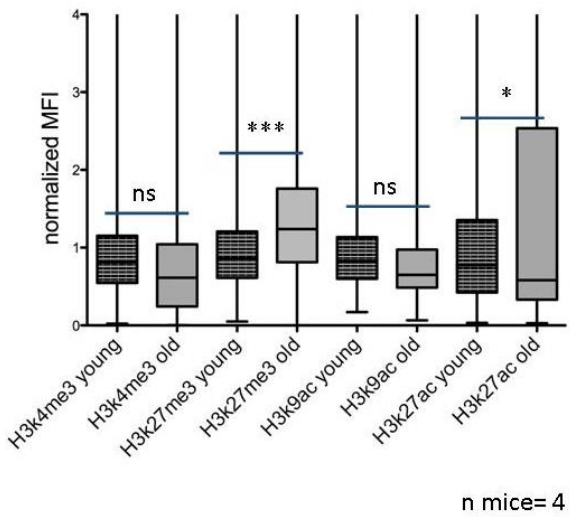

Figure 8: Normalized Mean Fluorescent Intensity. *H4k27me3 and H3k27ac are significantly different.*

The normalized Mean Fluorescent Intensities (MFI) of the individual marks are presented in *figure 8.* H3K27me3 and H3K27ac are significantly different. And when looking more closely to the numbers we can observe that the standard deviation of the old HSCs is higher than the SD of the young cells, indicating that old HSCs are more heterogeneous than young HSCs.

H3K27me3 was chosen as a candidate for further study and was subjected to further investigation via a EZH2 methyltransferase inhibitor, GSK 126.

Functional Assay
In the previous experiments, we detected a significant increase in H3k27me3 upon aging. Thus, we set out to investigate whether an inhibition of H3k27me3 has a functional effect in old LT-HSCs. To this end, we used the Cobblestone Area Forming Cell assay to assess and compare the repopulating capacity of young and old LT-HSCs with or without GSK 126 treatment. The scoring of the plates was based on the presence or absence of cobblestones: cells that manage to grow underneath the stromal feeder layer.

A decrease in H3k27me3 in LT-HSCs was confirmed by FACS analysis upon 48 h incubation with the inhibitor, the fluorescence of LT-HSCs has been measured to ensure that stem cells respond to the inhibitor the same way as the cell line, *figure 9.*

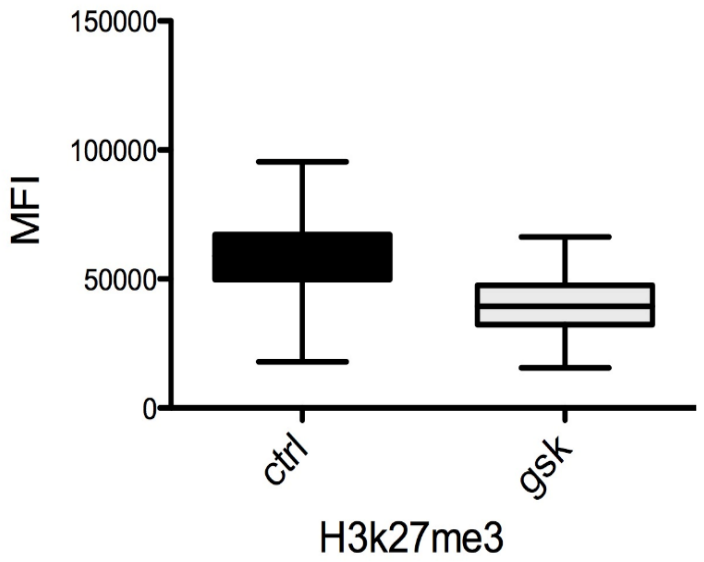

Figure 9: LT-HSCs treated with GSK 126. *The mean fluorescence intensity is decreased upon incubation with GSK 126.*

Figure 9 shows a decrease in fluorescence intensity when LT-HSCs were incubated with the methyltransferase inhibitor GSK 126.

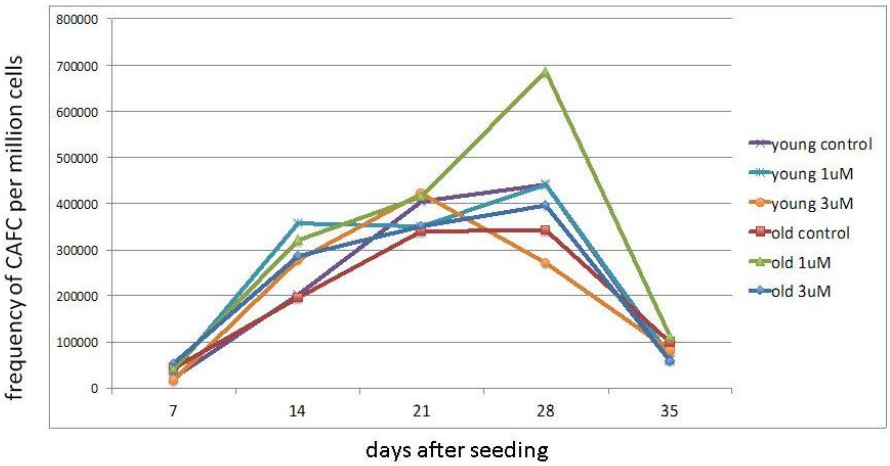

Figure 10: CAFC scoring. *LT-HSCs were treated with 1 µM or 3 µM GSK or untreated controls.*

Upon 5 weeks scoring of the CAFC assay, we observed that in the second week (day 14 after seeding) the 1 µM GSK appeared to have a positive effect on the repopulating ability of the cells for both the young and old LT-HSCs (with exception of day 21 for young sample), when compared to the controls (*figure 10).* This effect did not create a strong trend, since from the next scoring point onward (day 21), the frequency of cobblestones per million cells varies between treatments. Generally, however, the untreated old sample performs worse than the old samples with either 1 µM or 3 µM GSK, so there seems to be a positive effect of the inhibitor on the performance of the LT-HSCs. This effect is also visible in *figure 11*.

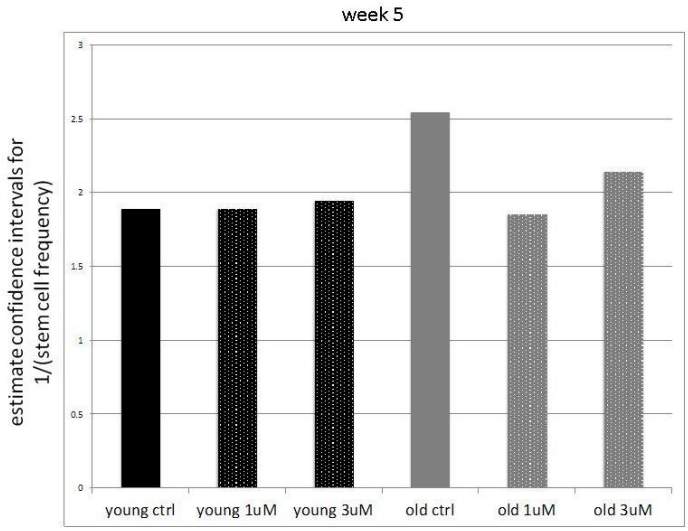

Figure 11: Confidence intervals of week 5. *For the old LT-HSCs, the frequency is higher in the samples treated with GSK.*

Figure 11 shows the confidence intervals for 1/(stem cell frequency) in week 5 of the assay. The treated and untreated young LT-HSCs appear to have the same frequency, but for the old LT-HSCs, there seems to be an effect of the inhibitor. Both the 1 µM and 3 µM GSK 126 treatment give a higher LT-HSC frequency than the control, and these frequencies are similar to all the young samples.
So far, however, this assay is only performed once, so these graphs display no conclusive data.

Discussion

It is known that the hematopoietic system is prone to aging, despite the self-renewal capacity of the hematopoietic stem cells. Aging-related changes of the hematopoietic system are detrimental to the organism and understanding the mechanisms behind aging is of great importance. Posttranslational modification of histones is suspected to be associated with the aging of the hematopoietic system and therefore it is extremely valuable to assess these epigenetic changes in a quantitative way.

The protocol that was optimized during this project enabled us to quantitatively compare epigenetic histone modifications between young and old hematopoietic stem cells and investigate the epigenetic contribution to the process of aging of the hematopoietic system. We did indeed manage to quantify the amount of histone marks and detect significant differences between young and aged mouse HSCs: Hematopoietic stem cells isolated from old mice harbor significantly more H3k27me3 and H3k27ac marks than HSCs originating from young mice.

The EZH2 methyltransferase inhibitor GSK 126 was used in a functional assay to study the effect of H3k27me3 on the repopulating ability of the LT-HSCs. Although we cannot conclude much from our current data yet, it seems very promising to continue this assay, because evidence from the field of psychiatry shows that epigenetic marks can be reversed pharmacologically in humans (Peckham, 2013). This can be promising to our field of interest as well.

Next steps can involve the expansion of this screening to more marks, because so far, we covered only 4 histone modifications. From these four marks, H3K27me3 and H3K27ac appeared to exhibit significant differences between young and old mice.

Also, to gain more solid evidence for epigenetic differences between young and old HSCs, different techniques can be used. One such technique is Wes. Since we are working with a very rare LT-HSC population, we can only use a very small cell number. Wes, in contrast to conventional western blotting, is capable of using these very low cell numbers and is also quantitative.

The functional Cobblestone Area Forming Cells assay was started with GSK 126 and should be continued to increase the significance of the acquired data. Also, other inhibitors or agents can be used to see the effect on the performance of LT-HSCs

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