ASSESSMENT OF THE ROLE OF NEUROPEPTIDE Y IN THE REGULATION OF HEMATOPOIETIC STEM CELLS

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ABSTRACT

ASSESSMENT OF THE ROLE OF NEUROPEPTIDE Y IN THE REGULATION OF HEMATOPOIETIC STEM CELLS

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Differentiation, self-renewal and quiescence of Hematopoietic stem cells (HSCs) is tightly regulated and the majority of the immature stem cells is quiescent. This protects the HSCs from the strain of constant cell division and depletion of the stem cell pool. Neuropeptide Y (NPY) is released from sympathetic nerves in the bone marrow (BM) and by MSCs, and has been shown to indirectly affect HSC function. However, the direct effects of NPY on HSCs has never been assessed.

In the framework of this thesis, we explored the effect of NPY on the regulation of HSCs by performing a detailed analysis of the effects of NPY on HSC cell cycling and gene expression. The NPY receptors Y1-Y5 were highly expressed on both immature and mature hematopoietic cell subsets. In culture, expression of Y1, Y2, Y4 and Y5 by HSCs was shown to decrease in time, whereas a significant increase in the expression of NPY-Y3 was observed. NPY suppressed HSC proliferation, as confirmed by an increase of HSCs in G0 phase and an increase in the gene expression levels of *FOXO3, DICER1, PCNA, SMARCA2* and *PDK1*, which all have been shown to play an important role in the regulation of cell proliferation and quiescence. Using RNASeq we detected two differentially expressed genes in NPY treated HSCs compared to untreated control HSCs. These data provide an indication that NPY plays

a direct effect on the regulation of HSC fate by modulating cell proliferation and quiescence.

Keywords: Hibernation, Neuropeptide Y, Hematopoietic Stem Cells

ÖZ

HEMATOPOETİK KÖK HÜCRE REGÜLASYONUNDA NÖROPEPTİD Y (NPY)´NİN ROLÜNÜN İNCELENMESİ

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Hematopoetik kök hücrelerin (HKH) farklılaşması, kendini yenilemesi ve sessizliği sıkı bir şekilde düzenlenir ve olgunlaşmamış kök hücrelerin çoğunluğu sesizdir. Bu, HKH'leri sürekli hücre bölünmesinden ve kök hücre havuzunun tükenmesinden korur. Bir nörotransmitter olan Neuropeptide Y (NPY) merkezi veya periferik sinir sistemindeki sempatik sinirlerden salınır. Nöropeptid Y'nin (NPY), MKH'ler tarafından kemik iliği nişinde salgılandığı ve dolaylı olarak HKH fonksiyonunu etkilediği gösterilmiştir. Bununla birlikte, NPY'nin HKH'ler üzerindeki doğrudan etkisi bugüne kadar araştırılmamıştır.

Bu tez çerçevesinde, NPY'nin HKH hücre döngüsü ve gen ekspresyonu üzerindeki etkilerini araştırarak NPY'nin HKH'lerin düzenlenmesi üzerindeki etkisinin açığa çıkarılması amaçlanmıştır. NPY reseptörleri Y1-Y5'in, çoğu HKH'de ve olgun hematopoetik hücre alt gruplarında yüksek oranda ifade edildiği gösterilmiştir. Kültürde Y1, Y2, Y4 ve Y5 ifadelerinin zamanla azaldığı, buna karşın NPY-Y3 ifadesinde belirgin bir artış olduğu gözlenmiştir. 300 nM NPY'nin HKH çoğalmasını baskıladığı, G0 fazındaki HKH miktarında ve hücre çoğalması ve sessizliğinde görevli FOXO3, DICER1, PCNA, SMARCA2 ve PDK1'in gen ekspresyon seviyelerinde artışa neden olduğu gösterilmştir. RNAseq dizileme sonuçlarına göre NPY

uygulanmış HKH’lerde kontrol grubuna göre ifadesi değişen iki gen olduğunu göstermiştir. Bu bulgular, NPY'nin, hücre çoğalmasını ve sessizliğini modüle ederek, HKH kaderinin düzenlenmesinde doğrudan bir etkisi olduğunu işaret etmektedir.

Anahtar Kelimeler: Hibernasyon, Nöropeptid Y, Hematopoetik Kök Hücre

To my father…

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ABBREVIATIONS	
AGM	Aorta-Gonad Mesonephros
Akt	Protein Kinase B
Angptl3	Angiopoietin-like 3
ACTB	Beta actin
BSA	Bovine serum albümin
BAF	Brg1/Brm-associated factor
BM	Bone marrow
BFU-E	Burst forming unit-erythroblast
BrdU	Bromodeoxyuridine
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
CLP	Common lymphoid progenitors
CK	Cytokine
c-Kit	Tyrosine kinase receptor
CNS	Central nervous system
CAR	CXCL12-abundant reticular
CXCR-4	CXC-chemokine receptor-4
CI	Cell Index
cAMP	Cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid

DC	Dendritic cells
DNA	Deoxyribonucleic acid
DAPI	4′,6-diamidino-2-phenylindole
ERK	Extracellular-signal-regulated kinase
ECM	Extra cellular matrix
EPO	Erythropoietin
ECs	Endothelial cells
emPZR	Emulsion polymerase chain reaction
Flt3-L	Fms-like tyrosine kinase 3 ligand
FGF	Fibroblast growth factor
FBS	Fetal bovine serum
FN	Fibronectin
GvHD	Graft versus-host disease
GM-CSF	Granulocyte-macrophage colony stimulating factor
G-CSF	Granulocyte-colony stimulating factor
GPCR	G-protein coupled receptor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GPCR	G-protein coupled receptor
GIRK	G protein-coupled inwardly-rectifying potassium channels
HSCs	Hematopoietic stem cells

HSPCs	Hematopoietic stem and progenitor cell
HPC	Hematopoietic progenitor cell
HGFs	Hematopoietic growth factors
HLA	Human Leucocyte Antigen
IL-1	Interleukin-1
IGF	Insulin-like growth factor
IGF-BP2	IGF-binding protein 2 hormones
IFN	Interferon
LFA-1	Leukocyte function antigen 1
MNCs	Mononuclear cells
MPP	Multipotent progenitors
MSCs	Mesenchymal stem cells
MMP9	Matrix metallopeptidase 9
M-CSF	Macrophage colony-stimulating factor
mTOR	Mammalian Target of Rapamycin
NK	Natural killer
NPY	Neuropeptide Y
NaN_3	Sodium azide
OBs	Osteoblasts
Opn	Osteopontin
PLGF	Placental growth factor
PI3-K	Phosphatidylinositol-3-kinase

PKA	Protein kinase A
PLC	Phospholipase C
PBS	Phosphate buffered saline
PBN	PBS/BSA/NaN_3
PCNA	Proliferative cell nuclear antigen
PDK1	3-phosphoinositide-dependent protein kinase 1
qPCR	Quantitative real time polymerase chain reaction
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
SCF	Stem Cell Factor
SNS	Sympathetic nervous system
SDF-1 (CXCL12)	Stromal cell-derived factor-1
SNPs	Single nucleotide changes
TPO	Thrombopoietin
TNF-α	Tumor necrosis factor-alpha
TGF-β	Transforming growth factor beta
TNCs	Total nucleated cells
TAC	Transcriptome Analysis Console
UCB	Umbilical cord blood
VLA-4	Very late antigen 4
VEGF	Vascular endothelial growth factor
VCAM-1	Vascular cellular adhesion molecule-1

CHAPTER 1

INTRODUCTION

1.1. Hematopoiesis

Hematopoiesis includes the development, self-renewal and differentiation processes of hematopoietic stem cells (HSCs), an important type of adult stems, capable of differentiation into all blood cell lineages (Doulatov et al., 2012). It also describes the process during which hematopoietic stem and progenitor cell (HSPCs) give rise to progressively more committed progenitors and create all types of mature blood and immune cells needed by the organism throughout its life (Majeti et al., 2007). Whereas primitive hematopoiesis is found predominantly in the Aorta-Gonad Mesonephros (AGM) and Yolk Sac regions during early embryonic development, adult or definitive hematopoiesis in healthy individuals occurs under physiologic conditions in the bone marrow (BM), fetal liver (FL) and in mice, spleen (Müller et al., 1994, Huang and Auerbach, 1993, Kiel et al., 2008). During hematopoietic stress, toxic injury, low oxygen levels, infection, exposure to chemotherapeutic agents, the balance between the maturation of blood cells and proliferation is rapidly adapted according to needs. HSCs maintain themselves through replication to maintain the HSCs pool (Wilson et al., 2008). Growth factors and interleukins within the marrow microenvironment orchestrate this balance between differentiation, proliferation and self-renewal of HSCs. The cell cycle regulates proliferation, quiescence and self-renewal of HSCs (Calvi and Link, 2015). Hence, the fate of these HSCs is tightly regulated by intrinsic and extrinsic signals originating from the niche and other homeostatic control mechanisms (Figure 1.1.) (Mendelson and Frenette, 2014). The process of differentiation is under normal physiological conditions irreversible. A small fraction of HSCs remains quiescent and rarely divides during homeostasis. Preservation of the

small quiescence fraction is necessary for the maintenance of the hematopoietic stem cells pool (Wilson et al., 2008, Kumar and Geiger, 2017).

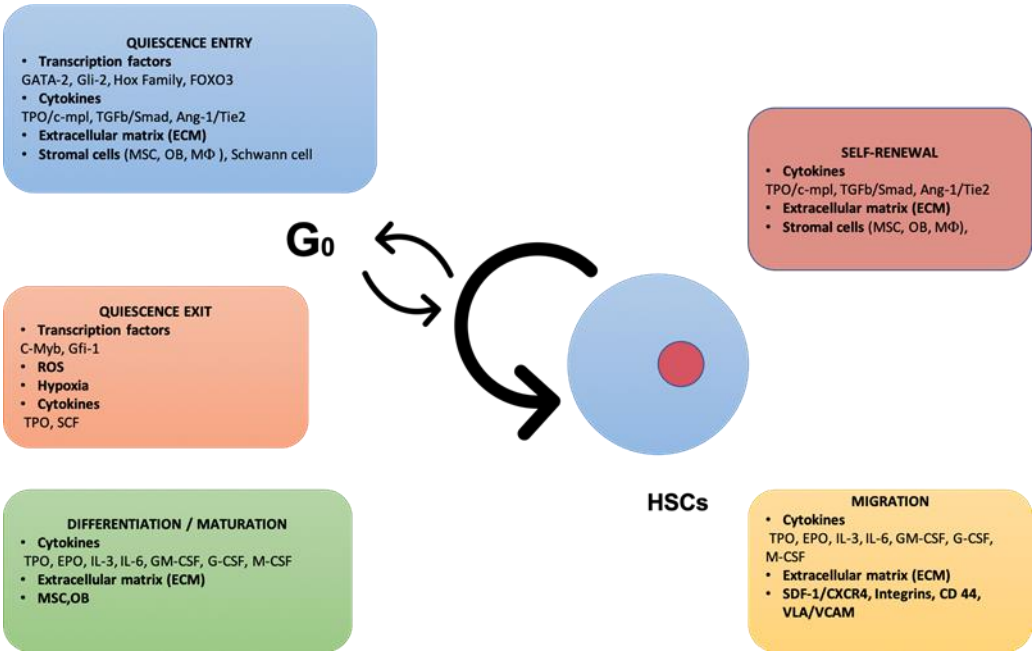

Figure 1. 1. Molecular mechanisms playing a role in the regulation of HSC maintenance.Self-renewal versus quiescence, proliferation, differentiation, maturation and migration of HSCs are tightly regulated by intrinsic and extrinsic signals emanating from the HSC niche (Adapted from Nakamura et al., 2014).

Because of the high daily turnover of blood cells, the blood cell production is a constantly ongoing process with an estimated production of 1.5 x 10^6 blood cells per second in adults (Catlin et al., 2011). Hierarchically, the multipotent progenitors (MPP), which are directly derived from HSCs maintain their potential for differentiation into cells of different lineages. However, during maturation these cells typically show a decreased capacity for self-renewal. Cells derived from the MPPs are then branched into common lymphoid progenitors (CLP) and common myeloid

progenitors (CMP) (Figure 1.2.) (Akashi et.al., 2000). The common lymphoid progenitors (CLP) give rise to mature lymphoid cells, such as B, T, dendritic, and natural killer (NK) cells, whereas myeloid progenitor subsets give rise to mature myeloid and erythroid terminally differentiated cells (Kondo et al., 1997). Direction of differentiation towards myeloid/erythroid or lymphoid direction occurs in response to hematopoietic growth factors (HGFs) and cytokines (CKs), as well as other clues from the microenvironment (Zhao and Baltimore, 2015; Möhle et al., 2007).

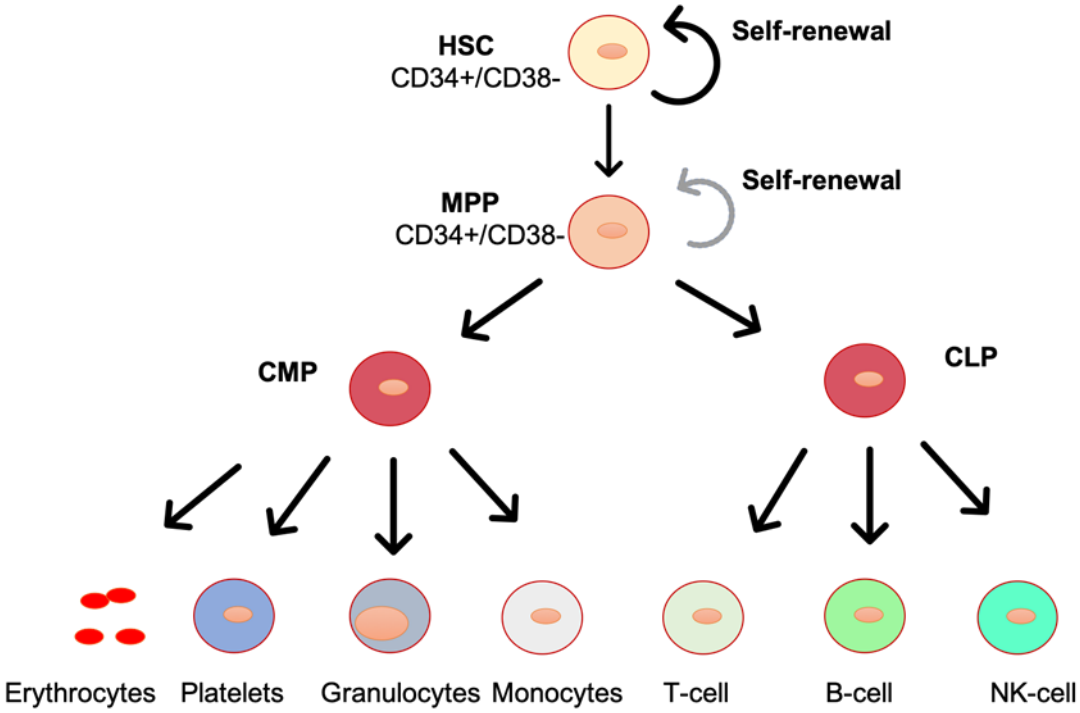

Figure 1. 2. Schematic overview of the hematopoietic hierarchy. At the top of the chart, HSCs with self-renewing capacity are found. These cells give further rise to multipotent progenitors (MPP), which still have some capacity for self-renewal. These cells then further branch into two lineages, of which the earliest detectable progenitors are known as the common lymphoid progenitor (CLP) and common myeloid progenitor (CMP). These progenitors together give rise to all types of blood cells (Luis et al., 2012).

1.2. Hematopoietic Stem cells

In the adults the production of mature blood cells from the HSCs takes place in the BM. Exhaustion of stem cell reserves is prevented by the capacity of HSCs for self-renewal (Kiel et al., 2008). In addition to the BM, HSCs can be isolated from alternative sources, such as umbilical cord blood (UCB) and G-CSF mobilized peripheral blood (PB). Self-renewal of HSCs is very important for the continuity of the stem cell pool and may occur symmetrically or asymmetrically (Park et al., 2016; Lo Celso et al., 2011; Seshadri et al., 2016; Blank et al., 2015). Factors that shift the balance between symmetric and asymmetric divisions may cause depletion of the HSC pool and result in BM failure (Park et al., 2016; Lo Celso et al., 2011; Seshadri et al., 2016; Blank et al., 2015). Therefore, quiescence of HSCs serves to preserve a stationary HSC reservoir of cells that maintains the ability to spontaneously participate actively to cell cycling when needed, in order to regenerate and restore all blood lineages (Blank et al., 2015, Ayako et al., 2014). Mammalian cells exit the cell cycle in response to environmental factors, such as specific growth factors or an increased cell count. Quiescent HSCs (G_0 phase) are often located near the trabecular endosteum and are considered metabolically inactive. G_0 protects stem cells from the mutagenic hazards of DNA replication, accumulating metabolic side products and induced reactive oxygen species (ROS) (Arai et al., 2009). Quiescent cells are able to return to the cell cycle according to need and in response to stress (inflammation) or trauma (hemorrhagia), HSCs answer with an increase in proliferation, maturation and migration (Heldt et al., 2018, Batsivari et al., 2017). HSCs are maintained in quiescence by interactions with specific cytokines (TNFα, TGFβ), in response to TLR signaling, binding to extracellular matrix (ECM) molecules and as a result of the cell-cell interactions. In contrast, mature hematopoietic cells exit from the vascular niche to the peripheral blood circulation. Thus, the regulation of self-renewal, proliferation, maturation and differentiation processes are kept in a tight balance (Seshadri et al., 2016; Park et al., 2015; Winkler et al., 2012, Ayako et al., 2014).

Many mechanisms involved in the regulation of HSCs have been described and molecules such as TGF-β, p21, N-cadherin, Notch ligand Jagged-1 and Ang-1 are thought to control quiescence of HSCs (Yamazaki et al., 2006; Yamazaki et al., 2007; Laurenti et al., 2015). The decision regarding whether HSCs enter and exit from the cell cycle by HSCs is also regulated by a variety of transcription factors, including c-Myb, GATA-2, Gli-1 and the HOX family. HSCs have been shown to express lineage-specific transcripts in mature and lineage-committed cell types. Some of these transcripts are responsible for specifying the cell fate and others are lineage specification regulators (Figure 1.1.) (Hao et al., 2016; Aggarwal et al., 2012).

The CD34 antigen is a glycosylated transmembrane protein expressed by hematopoietic cells, vascular endothelium, and embryonic fibroblasts (Matsuoka et al., 2001). CD34 is one of the most important markers of HSCs (Majeti et al., 2007) and is expressed on 1-3% of human BM cells, 0.1-0.5% of UCB and 0.001-0.01% of peripheral blood cells. It is not expressed by the more mature progenitors. However, different early HSPC subpopulations, such as MPPs may express CD34 (Majeti et al., 2007, Matsuoka et al., 2001). A possible role for CD34 may be cytoadhesion, proliferation and regulation of cell differentiation. CD34 numbers in HSC grafts are directly related to the time to engraftment HSC recipients (Aerts, F., 2010). Most of the CD34 antigen positive (CD34+) fraction remains in a quiescent state until activated (Ivanovic et al., 2010). CD34 expression on HSCs has been shown to decrease with age (Matsuoka et al., 2001). Clinically, HSC transplantation has been successfully used for the treatment of inherited or acquired hematopoietic and non-hematopoietic, malignant and non-malignant diseases (Gandy and Weissman,1998, Bouchlaka et al., 2010). CD34 enumeration can be typically used for selection and enrichment of HSCs for transplantation purposes (Wojciechowski et al., 2008). HSC grafts can consist of full blood grafts, containing both CD34+ cells and more differentiated cells. These grafts typically allow for a relatively rapid engraftment and hematopoietic reconstitution. However, in some cases prospective isolation of pure CD34+ cells may be preferred due to HLA or blood cell type incompatibility

(Michallet et al., 2000, Vose et al., 2001). Graft processing using immunomagnetic selection can greatly affect the clinical outcomes of transplanted patients. Graft versus-host disease (GvHD) is more often observed after use of HLA-mismatched HSPC grafts and can be prevented by the removal of the cytotoxic T-cells (Ferrara et al.,1999, Shernan et al., 2014). Both positive selection using the CD34 antigen and negative selection using anti-CD3 or TCRα/β T-cell microbeads have been used in the clinic to obtain T-cell-reduced transplants, and to prevent the occurrence or decrease the severity of GvHD. The reduction of T cells in the HSC transplants resulted in significantly reduced incidence of GvHD but an increase in disease relapse, and prolonged impaired immune reconstitution (Li et al., 2009). In absence of a matching BM or mobilized PB donor, utilization of UCB may be used as an alternative source of HSCs. However, the use of UCB is often dependent on the number of HSCs per collected unit, and unfortunately most UCB units do not contain sufficient numbers of stem cells for adult recipients (Barker and Wagner, 2003, Koh and Chao, 2004, Majhail et al., 2009).

1.2.1. Regulation of Cell Cycle in HSCs

In the adult, HSCs must actively participate in the cell cycle to maintain a constant number for equilibrium between self-renewal and differentiation. HSCs infrequently enter the cycle and most of them stay in G_0. Regulation of the cell cycle is crucial for hematopoiesis, stemness and self-renewal of HSCs. Decision of quiescent state or proliferative state is under control by extrinsic and intrinsic mechanisms. Cyclin-dependent kinases (CDKs) are core molecules involved in the regulation of the cell cycle. The catalytic activity of CDKs is regulated through interactions with CDK inhibitors (CKIs) and cylins (Lim and Kaldis, 2013). CKIs plays a crucial role in the arrest of cells in G_1 through interaction with the CDK/cyclin complex and blockage of activity of the kinases. CDKs, cyclins and CKIs play crucial roles in processes, such as transcription and stem cell self-renewal. Many transcription factors have been shown to regulate the cell cycle, such as Forkhead box O (FOXOs), Smarca2, Gli-1, c-Myb and GATA-2 (Orford et al., 2008, Cheng and Scadden, 2002). Comparison of

microarray data sets revealed a gene signature common in quiescent HSCs, muscle stem cells (MuSCs) and hair follicle stem cells (HFSCs) (Cheung and Rando, 2013). The authors specifically compared differentially expressed genes, that function during distinct regulatory processes of the cell cycle, including cell cycle progression and checkpoint control, DNA replication and chromosome segragation, mitochondrial function, chromatin and nuleosome assembly, regulation of transcription and RNA processing. Among the common genes found to be particularly upregulated during quiescence, the authors found significant differences in expression of *PDK1, SMARCA2, FOXO3* and *DICER1*. *PCNA* was found to be one of the common genes downregulated during quiescence of stem cells (Cheung and Rando, 2013).

The FOXO family consists of FOXO1, FOXO3, FOXO4 and FOXO6 and has been shown to play an important role in quiescence through modulation of activity of the PI3K/AKT pathway. This pathway also regulates metabolism, self-renewal, differentiation, longevity and apoptosis (Bakker et al., 2004). The PI3K/AKT/FOXO pathway arrests the cell cycle at G_1 (Medema et al., 2000). AKT is inactive in quiescent HSCs. However, when AKT is activated by cytokines, it causes the export of FOXO3 to the cytoplasma (Yamazaki et al., 2006). FOXO3 is a transcription factor that plays an important role in transcriptional regulation and direction of stem cell fate decisions and has been shown to be upregulated in quiescent HSCs (Aggarwal et al., 2012). By regulating quiescence, FOXO3 has been shown to play a role in survival mechanisms employed by HSCs in response to stress factors, such as protection from oxidative stress (Kops et al., 2002; Tothova and Gilliland, 2007). FOXO3 also modulates expression of p57, p27 and many negative cell cycle regulators (Miyamoto et al., 2007).

SMARCA2 (also called BRAHMA, BRM) is an ATPase and functions as a chromatin regulator, which plays a crucial role in gene transcription by managing structure and modifications of chromatin. SMARCA2 has been shown to exert its effects through cooperation with other factors. Whereas cooperation with TopBP1 is essential for the regulation of DNA replication, interaction with cyclin D3 causes inhibition of

proliferation (Liu et al., 2004). The expression pattern of catalytic and complex subunits in long-term quiescence HSC (LT-HSC) suggest that LT-HSCs have a BAF (Brg1/Brm-associated factor or mSWI/SNF) complex containing SMARCA2 as the catalytic subunit dominant. Smarca2 (Brm) also functions as an alternative subunit in a subfamily of BAF (Guerrero-Martínez and Reyes, 2018).

Post-transcriptional regulation plays an important role in regulation of quiescence in stem cells. Quiescent stem cells can be identified by their relatively low RNA content. miRNAs play important roles in diverse cellular processes. miRNAs bind to the 3'untranslated region (3'UTR) of target mRNAs and result in their cleavage or translational repression. In HSCs, specifically miR-126 has been shown to control stem cell quiescence by attenuating multiple components of the PI3K/AKT signaling pathway (Lechman et al., 2012). Dicer1 is a RNase III endonuclease essential for miRNA biogenesis and RNA processing. It is a processor of miRNA and regulates the spontaneous reactivation of quiescent stem cells (Cheung and Rando, 2013). Dicer1 deletion results in altered hematopoiesis and development of myelodysplasia (Kumar et al., 2007). Conditional knockout of Dicer further triggers the spontaneous activation of quiescent MuSCs and HSCs, that subsequently undergo apoptosis. Loss of DICER promotes induction of quiescence in stem cells (Fukada et al., 2007, Forsberg et. al., 2010).

Proliferative cell nuclear antigen (PCNA) has been described as a `sliding clamp' and forms a ring around DNA. PCNA act as the processivity clamp of DNA polymerase δ, which is required for DNA synthesis during replication (Li et al., 2009). It plays a role in linking Cdk2 to its substrates. PCNA furthermore is involved in DNA replication, repairing, cell cycle control, chromatin remodeling and apoptosis. Together with Ki-67, PCNA can be used to monitor cell proliferation. It intereacts with p21, which plays a role in cell cycle arrest via inhibition of the activity of CDK. A high level of p21 causes cell cycle arrest, which in turn reduces the level of PCNA. Interaction of p21 and PCNA causes inhibition of DNA synthesis. Cell proliferation correlates with increased *PCNA* gene expression (Strzalka and Ziemienowicz, 2011).

PDK1 (3-phosphoinositide-dependent protein kinase 1) plays a critical role in cell cycle control through PI3K/Akt/FOXO signaling pathways and maintenance of hematopoiesis. It mainly regulates cell proliferation by controlling G0/G1 to S and G2/M transition (Nakamura et al., 2008). PDK1-deficient HSCs showed decreased numbers of cells in G0-phase, which led to reduced HSC reconstitution ability. PDK1 deletion further resulted in a significant loss of progenitor cells, mature B cells and T cells (Hu et al., 2017; Wang et al., 2018).

1.3. Hematopoietic Growth Factors

HSCs and progenitors are regulated by interactions with locally produced hematopoietic growth factors (HGFs). Erythropoietin (EPO), Thrombopoietin (TPO), Granulocyte-macrophage colony stimulating factor (GM-CSF), Granulocyte-colony stimulating factor (G-CSF), Stem Cell Factor (SCF), Fms-related tyrosine kinase 3 ligand (Flt3-ligand) and the cytokines Interleukin-1 (IL-1), IL-2, IL-3, IL-6 and IL-7 are among the most well-characterized and well-known HGFs. Levels of HGFs may increase in response to infection/inflammation or trauma (Möhle et al., 2007). Most HGFs are multifunctional and may function at different stages of hematopoietic development to ensure the continued survival and proliferation of hematopoietic cells. They also regulate the fate of progenitor cells through modulation of their differentiation and maturation and are responsible for the functional activities of differentiated cells. HGFs and cytokines function in concert to achieve a high level of biological response, although they are present only at a picograms/nanograms per milliliter. The HGF and cytokine (CK) receptors that provide signal transduction in the cells are present at low density on the cell surface. Therefore, the expression-degradation of cytokines and their receptors is strictly controlled.

TPO is considered to be the main regulatory growth factor for thrombocyte formation, which regulates the development and differentiation of megakaryocytes and platelets. It is also one of the most important HGFs to support maintenance and expansion of HSCs *in vitro* (Möhle et al., 2007). TPO is mainly produced in the liver and kidney,

but is also secreted by locally by stromal cells in response to hematopoietic stress (Shimada et al., 2008).

Fms-like tyrosine kinase 3 ligand (Flt3-L) is an important HGF that affects the development of in particular the lymphoid lineages. It is able to stimulate amplification of primitive hematopoietic cells *in vitro* and synergistically supports hematopoietic progenitor and stem cell mobilization *in vivo* when used in combination with G-CSF (Audet et al., 2002; Aerts F., 2010). Flt3-L expands immature B cells, natural killer (NK) cells and dendritic cells (DC) *in vivo*. Flt3-L is expressed on the surface of cells and secreted after proteolytic cleavage into the soluble protein. The membrane-bound and soluble forms of Flt3-L have been shown to be both biologically active (Hilary et al., 2000).

Stem cell factor (SCF) is an HGF that exists as a soluble and as a transmembrane form. SCF regulates the migration of hematopoietic cells and maintenance of normal basal hematopoiesis. Endothelial cells and fibroblasts have been shown to constitutively produce SCF. SCF can be used to expand and promote the survival of HSCs. It can also modulate the adhesive behavior of hematopoietic cells and accelerate their entry into cell cycle. SCF binds to a tyrosine kinase receptor (c-Kit) that is highly expressed by HSCs (Zhang et al., 2008).

G-CSF stimulates the development of myeloid cells and in particular neutrophilic granulocytes, which produce several matrix-degrading enzymes, such as metalloproteinases, and cause the release of HSCs from the niche, making G-CSF the most commonly used mobilizing agent for the induction of HSPC mobilization. G-CSF also affects concentrations of Stromal cell-derived factor-1 (SDF-1, CXCL12) and promotes the release of a range of proteolytic enzymes, including cathepsin G, elastase, proteinase 3 and gelatinase B (MMP-9) from mature neutrophils (Möhle et al., 2007). Vascular cellular adhesion molecule-1 (VCAM-1) and CD26 are a target of these proteases (Möhle et al., 2007). G-CSF also works synergistically with IL-3 on stimulation of the development of early hematopoietic progenitors (Muench et al.,

1992). GM-CSF and IL-3 exhibit largely overlapping activities due to the fact that they share a common beta (βc) receptor subunit and promote activation of similar signaling pathways. However, the effects of GM-CSF and IL-3 are more general and extend to other myeloid cells, predominantly macrophages. Alternatively, mobilization of HSCs using GM-CSF allows collection of sufficient stem cell numbers for transplantation. GM-CSF also acts earlier during myeloid differentiation, whereas IL-3 may stimulate the development of myeloid, erythroid, as well as lymphoid series (Möhle et al., 2007). M-CSF supports viability, proliferation and differentiation of macrophages and monocytes. It works synergistically in combination with other HGS and CKs on proliferation and differentiation of hematopoietic progenitors.

Red blood cell formation and proliferation factor EPO was the first defined humoral regulator (Miyake et al.,1977, Lin et al., 1985). The main source of EPO is the kidney. Whereas EPO together with SCF stimulates the development of early red blood cell progenitors and BFU-E (Burst forming unit-erythroblast), EPO alone is sufficient to promote maturation of red blood cells (Dzierzak and Philipsen, 2013).

1.4. Hematopoietic Stem Cell Culture

The major bottle neck with HSC *ex vivo* cultures is that cells may lose their self-renewal capacity during prolonged culture. For this reason, maintenance of self-renewal capacity of HSCs and expansion of long-term repopulating (LTR) stem cells have become the main objective. Many different cytokines are used to promote the *ex vivo* propagation of human HSCs. The most important of these are SCF, Flt3-L and TPO (Wognum et al., 2000). Although SCF supports survival of HPCs rather than inducing expansion, it has been shown to particularly support expansion of myeloid-biased HSCs. Similarly, Flt3-L as a single agent does not support substantial proliferation of HPCs *in vitro* but it synergistically increases the effects of other HGFs on the expansion of HSPCs. However, it predominantly supports expansion of lymphoid-biased HSCs. Furthermore, it synergistically enhances the effects of G-CSF

on HSCs mobilization (Möhle et al., 2007; Aerts F., 2010). TPO was initially found to be a megakaryocyte and platelet development and differentiation supporting factor, acting all along the differentiation pathway from the earliest HSC to complete maturation of platelets. The stimulating role of TPO on primitive progenitors has now been recognized, and TPO has been shown to even recruit pluripotent, dormant progenitors into cell cycle, thus potentiating the proliferative response of HPCs to several HGFs (Möhle et al., 2007).

IL-1, IL-3 and IL-6 have been used less frequently for expansion of HSCs (Möhle et al., 2007, Wognum et al., 2000). Using these cytokine combinations, a maximum of 2-4 times HSC expansion can be obtained, and a gradual loss in the self-renewal capacity of HSC and the capacity of long-term reconstitution is observed after prolonged culture. Even though SCF, TPO and Flt3-L maintain cell viability in culture, these HGFs alone are not sufficient to results in clinically useful amplification of human HSC numbers. Therefore, in addition to these cytokines, when HSCs were cultured in serum-free culture conditions, the addition of Angiopoietin-like 3 (Angptl3)/Angptl5 or Insulin-like growth factor (IGF)/IGF-binding protein 2 hormones (IGF-BP2) has been shown to increase HSC expansion by 11-fold, which may be useful for clinical applications of HSCs (Zhang et al., 2004; Farahbakhshian et al., 2014; Zhang et al., 2006).

1.5. Hematopoietic Stem Cells Niche

The dynamic maintenance of HSCs occurs in a special microenvironment called the BM niche, which was first proposed by Schofield (Schofield, 1978). The niche contains all requirements necessary for the support of healthy hematopoiesis. HSCs functions and fate are determined by this microenvironment that regulates stem cell quiescence, self-renewal, proliferation, differentiation, migration and other biological functions. The BM niche is formed by many different types of supporting cells, signals transferred from these cells, cell-cell interactions, cell-ECM interactions, growth factors and cytokine interactions and gradients, adhesion molecules for anchoring or

release of HSCs (Figure 1.3.). The niche further provides a buffer to protect stem cells from environmental and physiological stress conditions and restrains the stem cells from differentiation and overproduction. Thus, the BM microenvironment is responsible for the dynamic equilibrium between self-renewal and differentiation of HSCs and it also regulates the activity of stem cells with respect to long-term maintenance and mobilization of the stem cells for tissue repair (Li et al., 2006, Nakada et al., 2011).

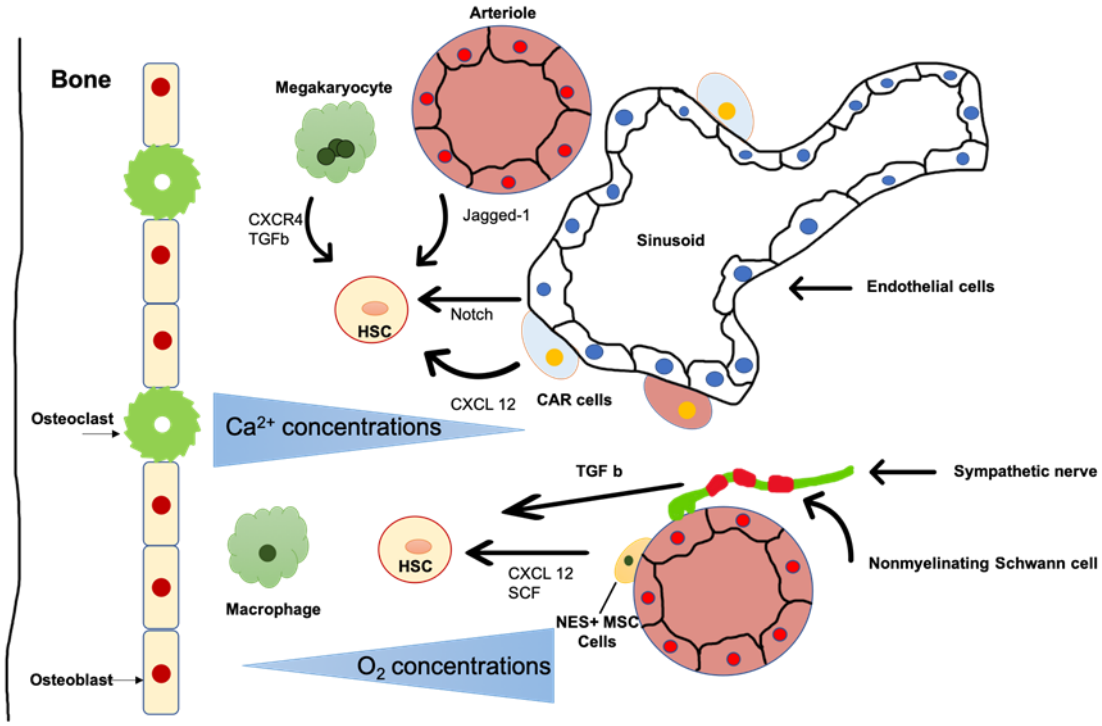

Figure 1. 3. The HSC niche contains all the requirements for the hematopoiesis. The niche is formed by many different types of supporting cells and signals.

Hormones, CKs, ECM, paracrine and autocrine interactions between the distinct cell types, such as osteoblasts (OBs), adipocytes, reticular cells, mesenchymal stem cells

(MSCs) and fibroblasts within the BM contribute all to the determination of HSC fate (Wilson et al., 2006). Through interaction with the stromal cells and ECM molecules, including fibronectin (FN), collagen, laminin and proteoglycans present in the niche, HSCs respond with proliferation or differentiation. In the BM niche, HSCs are surrounded by endothelial cells, mesenchymal cells and MSCs that give rise to many different cell types including myocytes, adipocytes, chondrocytes, fibroblasts, and osteoblasts (Klamer and Voermans, 2014). HSCs are heavily affected by interactions with OBs, MSCs and sympathetic nerves in the BM (Seshadri et al., 2016; Park et al., 2015; Lucas et al., 2013; Boulais et al., 2015). The OBs produce the bone matrix and are found on the endosteal surface. However, OBs also secrete several factors that promote proliferation and maintenance of HSCs. OBs and HSCs interact directly through N-cadherin and β1-integrin, which are expressed by the OBs and anchor the HSCs firmly into the endosteal niche. OBs probably also regulate HSCs through cell-surface adhesion molecules and/or secretion of signaling molecules (Schepers et al., 2015). OBs secrete angiopoietin, TPO and SDF-1(CXCL12). SDF-1 regulates HSC migration and adherence to the BM niche (Yoshihara et al., 2007). Osteoclasts are also implicated in HSC maintenance and secrete Matrix metallopeptidase 9 (MMP-9), Cathepsin K and SDF-1 (Kollet et al., 2006). MSCs have been shown to regulate HSC maintenance by expressing factors, such as angiopoietin and SDF-1 (Xiaobing et al., 2016). Altogether, these CKs, HGFs, chemokines, cell-surface and adhesion molecules cooperate to provide an optimal niche for the HSCs and to ensure that these cells remain quiescent or migrate to the vascular niche. Signaling of angiopoietin-1 (Ang-1) and its receptor tyrosine kinase (RTK) Tie-2 are also required for HSC quiescence. Tie-2 has been shown to regulate long-term repopulating activity of HSCs and cell cycle progression through the phosphoinositide 3-kinase (PI3K)–Akt–p21 pathway, a target of Ang-1 signaling. OBs express the Ang-1 ligand, whereas HSCs express its receptor Tie-2. Megakaryocytes and perivascular cells may also secret angiopoietin (Li et al., 2006, Hsu et al., 2000). TPO/c-MPL activation also promotes HSC quiescence (De Graaf and Metcalf, 2011).

Up-regulation of c-Myc causes downregulation of N-cadherin and integrins in HSCs. As a result, they lose their capacity for self-renewal and differentiation. SDF-1 is secreted by endothelial cells, OBs, reticular cells and other stromal cells (Kiel et al., 2008). In contrast, the SDF-1 receptor CXCR4 is expressed by HSCs. SDF-1 promotes HSC trans-endothelial migration. CXCL12-abundant reticular (CAR) cells interact with HSCs in the BM niche. Activation of the adhesion molecules VLA-4 (very late antigen 4) and LFA-1 (leukocyte function antigen 1) is necessary for migration (Peled et al., 2000). Matrix metalloproteinase (MMP)-9, MMP-2 and other proteolytic enzymes released from granulocytes in response to G-CSF cause a decrease in SDF-1 levels and the subsequent egress of HSPCs from the BM into the PB (Heissig et al., 2002). Fibroblast growth factor (FGF)-4 promotes HSPC recruitment and adherence to the vascular niche. Multiple FGF receptors are expressed by HSCs. FGF signaling is involved in regulation of HSC proliferation *in vitro*. The gradient of FGF in the niche is important for the recruitment of HSPCs (Yoon et al., 2017). Vascular endothelial growth factor (VEGF) and placental growth factor (PLGF) are also important for HSC mobilization and/or recruitment (Carlos et al., 2012). Osteopontin (Opn) is expressed in osteoblasts and is mainly involved in HSC migration (Susan et al., 2005).

Intrinsic genetic programs support the balance between self-renewal, multipotency and differentiation. Hedgehog, Wnt, Notch, BMP, and FGF-signaling pathways are known to modulate HSC-niche activity. Wnt activation causes rapid HSC proliferation, as well as expansion of the HSC pool (Richter et al., 2017). Active Notch signaling produced by OBs increases the HSC population and regulates HSC maintenance and lymphoid-lineage commitment (Lampreia et al., 2017). Notch activation by Jagged1, which is expressed on OBs, also promotes HSC maintenance in culture (Duncan et al., 2005; Kumar and Geiger, 2017). At least two different niches have been described to co-exist in the BM and regulate different functions of HSCs: the endosteal niche (osteoblastic niche) near the trabecular bone surface and the endothelial (vascular) niche, which is formed by the low-pressure blood vessels in the

center of the BM. These two niches are not two physically separate entities, but rather function together and are connected to each other. HSC activity in these two niches is strongly affected by local conditions (Li et al., 2006; Morrison and Scadden, 2014).

The endosteal niche is generally associated with the presence of quiescent HSCs, whereas the endothelial niche is thought to facilitate the activities of more active HSPCs (Mendelson et al., 2014). The endosteal region, which is considered to be rich in calcium and hypoxic, provides protection of the quiescent HSC reserve from external factors, and is a relatively immune privileged area, due to the presence of regulatory T lymphocytes (Mercier et al., 2011). Only a fraction of the HSCs enters the cell cycle, but the quiescent cells rest in the endosteal niche and become only active when the need for self-renewal is increased, during stress or when cells are recruited from the niche to the sinusoidal vascular space in response to environmental clues (Kiel et al., 2008).

The vascular niche is rich in blood vessels and cells, such as endothelial cells, pericytes and smooth muscle cells. HSCs, endothelial progenitor cells and MSCs are recruited on the vessel walls. MSCs regulate proliferation and differentiation of HSCs via secretion of stem cell factor (SCF) and SDF1/CXCL12 (Shahrabi et al., 2016). The blood vessels are able to regulate hematopoiesis, stem cell mobilization and homing by creating a microenvironment themselves. The vascular niche interacts with HSPCs by signaling through chemokines and cytokines. SDF-1 and FGF-4 enhance expression of vascular cell adhesion molecule-1 (VCAM-1)- and VLA-4, which promote survival, maturation and platelet release (Kopp et al., 2005). Consequently, different microenvironments take part in different stages of hematopoiesis, and they direct the critical regulation of stem cell trafficking, stem cell migration and mobilization, adherence to the niche, proliferation, maintenance of quiescence or differentiation (Wang and Wagers, 2011; De Lucas et al., 2018).

1.6. Mesenchymal Stem Cells (MSCs)

MSCs are multipotent stem cells that can be easily isolated from BM. Both *in vitro* and *in vivo* MSCs have been shown to support maintenance of HSCs by direct cell-cell interactions, but also through secretion of a wide range of hematopoietic growth factors (Aerts and Wagemaker, 2005). MSCs are also found in peripheral blood, fatty tissue and placenta, albeit in different concentrations (Pittenger et al., 1999). MSCs have the capacity for self-renewal and differentiation into osteoblast, adipocyte and chondrocytes. They also produce and secrete many hematopoietic growth factors and cytokines, and are an important source of enzymes and extracellular matrix proteins within the niche. MSCs express the surface markers CD73, CD90, CD105 and are negative for CD34, CD45, CD14 and HLA-DR (Dominici et al., 2006). However, since none of these surface markers are specific for MSCs, characterization of MSCs should involve the confirmation of absence or expression of a combination of these antigens. MSCs have been shown to interact directly with immune cells during damaged tissue repair and through immunomodulatory effects and they can inhibit cytotoxic T-cells, reduce the activity of B cells and NK cells, and promote proliferation of regulatory T-cells through IL-10 (Nauta and Fibbe, 2007; Selmani et al., 2007).

1.7. Neuropeptide Y (NPY)

The sympathetic nervous system (SNS) plays a role in many physiological activities. There is an important interaction between sympathetic nerve fibers and the stromal cell compartment of the BM. Sympathetic nerves synapse with perivascular cells and regulate BM homeostasis and BM nerve damage/denervation that severely affects the survival of HSCs (Aerts-Kaya et al., 2019). The SNS also influences HSCs mobilization (Kiel et al., 2008). The central nervous system (CNS) plays a direct role in BM homeostasis by expressing neurotransmitters (Lucas et al., 2013).

One of the most abundantly expressed neurotransmitters, is Neuropeptide Y (NPY). NPY is a 36-amino acid peptide of which the gene is located on chromosome 7. NPY

is synthesized as a precursor protein containing a hydrophobic signal peptide, the mature peptide NPY, the amidation-proteolytic site, and as the carboxyterminal extension a 30 aa residue peptide referred to as the CPON. Pro-NPY is next processed by enzymatic cleavage to generate a 39 amino acid peptide that contains NPY and CPON, the role of which is currently unknown (Dumont Y. and Quirion, 2013). NPY is secreted from both central and peripheral sympathetic nerves (Park et al., 2015) and is involved in the regulation of many physiological systems, such as food intake, energy expenditure, cell growth and regulation of pain perception. NPY is a basic neurohormone that controls glucose homeostasis by triggering insulin release from the pancreas and OBs (Lee et al., 2015). Five different G-protein bound receptors of NPY (Y1-Y5) are known (Singer et al., 2013) and found to be expressed not only in the brain, but all throughout the body, including in adipose tissue and liver (Yi et al., 2018). The NPY receptors Y1, Y2 and Y5 most likely developed through duplication of the gene on chromosome 4 and have different affinity for the peptides NPY and Peptide YY (PYY). The Y3 receptor was proposed based on pharmacological experiments but its affinity for NPY is relatively low, and instead is currently known as CXCR4 or the 'homing' receptor (Larhammar et al. 1998, Xu et al., 2015). The known functions of NPY receptors, their chromosomal location, their affinity for different peptides and their tissue distribution are summarized in Table 1.1.

Most studies have focused on the effects of NPY on the hypothalamic arcuate nucleus region (ARC), which plays a role in the regulation of metabolic balance. When the NPY-Y2 receptor is stimulated in the ARC, OB activity is suppressed, whereas proliferation and bone resorption remains unaffected (Khor et al., 2016). The effects of NPY on bone homeostasis are controlled mainly through stimulation of Y1 and Y2 receptors. Y1 signaling in OBs suppresses OB activity and proliferation of mesenchymal progenitors. In the BM, NPY receptors have been found to be expressed by macrophages, OBs and Nestin+ MSCs (Lo Celso et al., 2011; Park et al., 2015; Park et al., 2016). It has been shown that by modifying the levels of NPY, bone and energy metabolism is coordinated (Zhang et al., 2014, Lee et al., 2015) and local

secretion of NPY in the BM by macrophages, OBs and endothelial cells has been shown to regulate homeostasis of immune cells.

Table 1. 1. Function and distribution of NPY receptors. (Adapted from Brothers and Wahlestedt, 2010, Peng et al., 2017).

The NPY receptors	Alternative name	Chromo-some	Ligand	Tissues	Functions
Y1		4q32.2	PYY> NPY	Spleen, adipose tissue, adrenal, kidneys, lymphnodes, brain, heart, placenta, bone marrow	Anxiety, depression and pain modulation, circadian rhythm and cardiovascular sympathetic regulation, bone homeostasis, neuronal stem cell and smooth muscle cell proliferation, angiogenesis, nutrient absorption
Y2		4q32.1	NPY = PYY	Brain, testis, gastrointestinal tract, adipose tissue	Anxiety, depression, pain and dependence modulation, rhythm and circulatory rhythm and cardiovascular sympathetic regulation, bone homeostasis, neuronal stem cell and smooth muscle cell proliferation, angiogenesis
Y3	CXCR4, CD184	2q22.1	CXCL12 NPY>> PYY	Bone marrow, lymphnodes, spleen, placenta	The function / effects of NPY are unknown
Y4	PYY-R1, PPR-1	10q11.22	PP> PYY> NPY	Gastrointestinal tract, skin, prostate, lung, pancreas	Nutrient absorption, regulation of acid secretion
Y5		4q32.2	NPY> PYY	Spleen, adipose tissue, testes, kidneys, placenta, lymphnodes, brain, adrenal	Cardiac hypertrophy, food regeneration and obesity, dependence modulation, MSCs proliferation

NPY triggers the proliferation of MSCs and increases the expression of BMP-2 and VEGF, and NPY signaling through overexpression of Y5 can overcome senescence in MSCs (Igura et al., 2011). In mice with an NPY deficiency a decrease in MSC numbers was detected, as well as increased apoptosis of CNS fibrils and CD31+ endothelial cells (Igura et al., 2011; Liu et al., 2016). Furthermore, NPY deficiency also caused the numbers of HSCs to decrease and resulted in impaired BM regeneration. However, since the differentiation capacity and maturation of HSCs were not affected (Park et al., 2015), this decrease in the number of HSCs was thought to be secondary to the disruption of the hematopoietic microenvironment (Park et al., 2015). Treatment of NPY deficient mice with NPY or a Y1 agonist resulted in an increase in the number of HSCs and a decrease of apoptosis in CNS fibrils and CD31+ endothelial cells (Park et al., 2015).

Both NPY and NPY-Y1 are expressed by macrophages. Macrophages that lack the Y1 receptor have been shown to secrete less IL-12 and tumor necrosis factor-alpha (TNF-α) (Singer et al., 2013; Blank et al., 2015). Binding of NPY to NPY-Y1 results in activation of the PI3K/Akt/mTOR pathway, TGF-β secretion by macrophages and increased neural protection and HSC survival (Park et al., 2015; Lucas et al., 2013). TGF-β is also secreted from myelinated Schwann cells or ECM in the BM (Yamazaki et al., 2011). TGF-β/Smad signaling is important for HSC quiescence and *in vitro* TGF-β inhibits HSC proliferation. Conversely, after inhibition of TGF-β, HSCs were found to reenter the cell cycle (Jun et al., 2010).

1.8. G protein-coupled receptors (GPCRs)

GPCRs belong to the largest group of transmembrane cell surface receptors. They respond to and transfer signals from a variety of external stimuli, emanating from hormones, neurotransmitters, or sensory input. GPCR mediated signaling plays a role in the direct regulation of diverse biological processes, such as maintenance of blood pressure, neurotransmission, sensory systems, but also migration, proliferation and

stem cell differentiation. As such, GPCR signaling is important for maintaining homeostasis of the organism's metabolism (Rosenbaum et al. 2009).

GPCRs have seven α-helical transmembrane domain consisting of an internal and an external loop with an extracellular N-terminal and an intracellular C-terminal sequence. GPCRs are divided into several subfamilies, Classes A, B, C and Frizzled. GPCRs exist both as homomeric and heteromeric dimers. In humans, GPCRs are encoded by over 800 different genes (Cotton and Claing, 2009; Vischer et al., 2011). Distinct GPCRs are expressed in many distinct cell types and trigger diverse reactions in response to the same signal. In addition, different GPCRs can be activated by the same molecule and for example acetylcholine has been shown to activate 5 distinct GPCRs. G_s, $G_{i/o}$, $G_{q/11}$, and G_{12} are major families of G proteins based on the α isoform (Alberts et al., 2012).

The transduction of signals by GPCRs are transferred from the extracellular to the intracellular domains, and occurs through the binding of a ligand to its receptor and consequent activation of intracellular guanine nucleotide-binding (G) proteins, that lead to the activation or inhibition of several different molecular pathways. The activation of GPCR signaling involves several proteins, such as kinases, GTPases and β-arrestins through the classical effectors, such as adenylyl cyclase, phospholipase C, or PDE phosphodiesterase or the non-canonical signaling, mediated by interactions with the cellular cytoskeleton and organelles (Gurevich et al., 2009).

Upon binding of the ligand to its receptor, a signal is transmitted and induces conformational changes of the GPCR resulting in the activation of a G protein heterotrimer that consist of three different subunits, i.e. the α, β and γ subunit (Figure 1.4.). In basal conditions, these three subunits are associated with α binding guanosine diphosphate (GDP). Binding to the ligands and transmission of the signal causes a conformational change of the G protein and downstream signals are propagated by the α, β and γ subunits (Figure 1.4.). The β and γ subunits are interconnected together and called the $G_{\beta/\gamma}$ subunit. There are 27 G_α, 5 G_β, and 13 G_γ subunits are encoded by the

human genome and all $G_{\beta/\gamma}$ subunits function similarly (Rosenbaum et al., 2009; Alberts et al., 2012; Cotton and Claing, 2009; Wettschureck and Offermanns, 2005; Kristiansen, 2004).

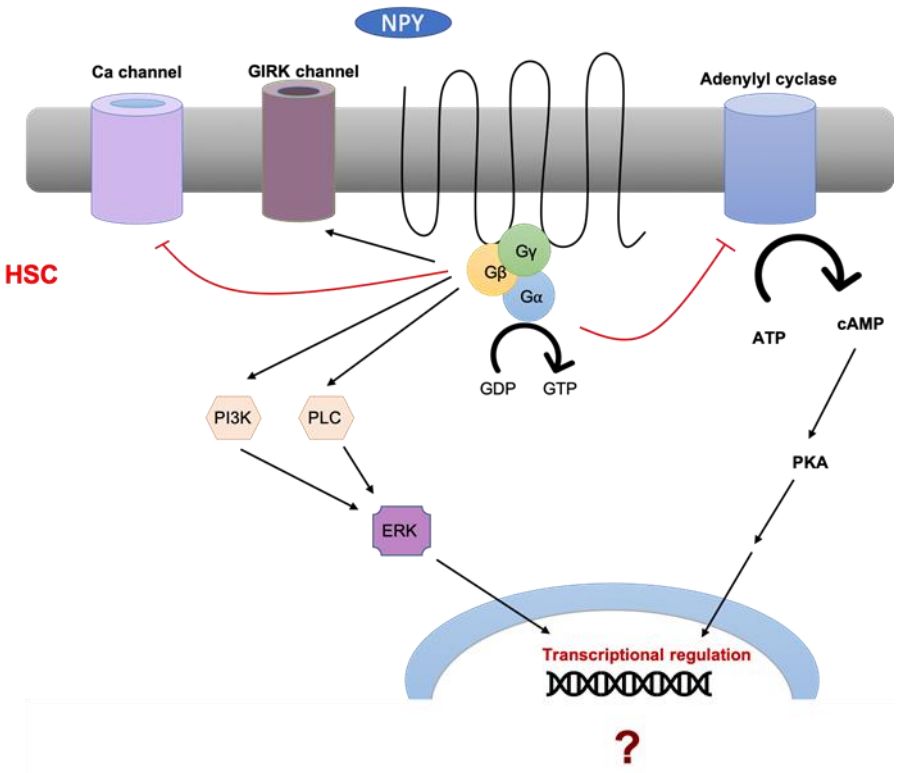

Figure 1. 4. Known intracellular signaling pathways of the NPY receptors. All NPY receptors couple to the Gi signaling cascade, where the Gα (alpha subunit) inactivates adenylyl cyclase. The Gβ/Gγ (beta/gamma subunit) can activate different cascades. Activation of the G protein complex can also lead to suppressed Ca2+ channel activity and enhanced G protein coupled inwardly rectifying potassium (GIRK) currents. Currently, the expression of NPY receptors on HSCs and the effect of NPY binding to its receptors is unknown and the topic of this thesis. GTP; Guanosine triphosphate, GDP; Guanosine diphosphate, PI3K; phosphoinositide 3-kinase, PKA; protein kinase, GIRK; G protein-coupled inwardly-rectifying potassium channels, ERK; extracellular-signal-regulated kinase, PLC; Phospholipase C. (Adapted from Brothers and Wahlestedt, 2010).

Binding of a ligand to the receptor changes the conformation of the GPCR and the α subunit can bind one of two guanine subunits, guanosine triphosphate (GTP) or guanosine diphosphate (GDP). GDP binds to the α subunit, which remains bound to the β/γ dimer subunit to form an inactive trimeric protein. When nucleotides exchange, the G_{α}/GTP is separated from the $G_{\beta/\gamma}$ subunit. This activation leads to second messenger release and to activation of various downstream intracellular signaling pathways, such as phosphoinositide 3-kinase (PI3K) and adenylyl cyclase activation. All signaling cascades result in regulation of diverse biological processes (Kristiansen, 2004; Wettschureck and Offermanns, 2005; Alberts et al., 2012).

1.9. Interactions between NPY receptors and their Ligands

Most of the currently known NPY receptor ligands are peptides and their use in clinical applications is limited. Development of agonists and antagonists of NPY receptors have been mostly tested for their function and efficacy in the treatment of obesity and anxiety disorders (Heilih, 2004; Stephens et al.,1995). PYY, which is mostly expressed in the gastrointestinal system, has the highest affinity for NPY-Y1 (approximately 10-fold) higher than for the other receptors (Vona-Davis and McFadden, 2007). In addition shorter versions of NPY have been shown to bind more potent to the NPY receptors (Park et al., 2018). Of these, NPY (13- 36) and PYY (3-36) are selective peptide agonists of the Y2 receptor. Affinity of NPY for the NPY-Y3 receptor is low, and the primary ligand of this receptor has now been found to be SDF-1 (Oberlin et al., 1996; Bleul et al., 1996). Obinepitide (TM30338) is a Y2 and Y4 receptor agonist and has been used for the treatment of obesity (Sato et al., 2009). 1229U91 is potent agonist of Y4 receptor but also has been shown to possess antagonistic activity on the Y1 receptor (Hegde et al, 1995, Schober et al., 1998). BWX-46 is selective agonist of Y5 (Kothandan and Cho, 2012). BIBP3226 is a selective and potent non-peptide receptor antagonists of the Y1 receptor (Mollereau et al, 2001). BIIE0246 is non-peptide Y2R antagonist as well as Y1 receptor agonists

(Doods et al, 1999). The Y5 receptor antagonists MK-0557 and S-2367 have been used in clinical trials of obesity (Sato et al, 2009). MK-0557 is Y5 receptor antagonist that was tolerated by patients but failure in obesity clinical trials (Small & Bloom, 2005).

Table 1. 2. Some of the NPY receptor agonist and antagonist (Kothandan and Cho, 2012; De Clercq, 2010).

NPY receptor	Agonist	Antagonists
Y1	BIIE0246	PD 160170, 1229U91, BIBP3226, BVD-10, GR-231, GR-118, Peptide YY, GR-231,118
Y2	NPY (13- 36), PYY (3-36), Obinepitide	BIIE0246, JNJ 5207787, SF 11
Y3	SDF-1	AMD3100, Mozobil, KRH-1636
Y4	Obinepitide, 1229U91, Peptide YY, GR-231,118	UR-AK49
Y5	BWX-46, PYY	MK-0557, S-2367, CGP-71683, FMS-586, L152, L804, Lu AA-33810

1.10. Role of NPY in hematopoiesis

The SNS regulates the BM environment, but its mechanisms and neurotransmitters are still largely unknown. Sympathetic nerve fibers and the BM hematopoietic cells produce and secrete NPY, which has a regulatory role in the BM microenvironment. In addition, BM cells, especially OBs, endothelial cells (ECs) and macrophages express Y receptors that regulate bone and immune cell homeostasis (Park et al. 2015). NPY deficiency causes a decrease in HSC survival, mobilization, BM regeneration, the number of ECs and destruction of the sympathetic nerve fibers. Inhibition of NPY-Y1 receptor signaling impairs survival of HSCs and BM cells. NPY or Y1 agonist administration has been shown to protect BM cells and SNS fibers in $NPY^{-/-}$ mice.

NPY also mediates the survival of HSCs by protecting BM SNS fibers ECs and Nestin+ MSCs (Park et al. 2015).

One of the major side effects of cancer treatments is the chemotherapy or radiotherapy-induced hematopoietic dysfunction and impaired BM regeneration. Chemotherapy causes chronic and irreversible BM failure (Park et al. 2018). Cisplatin is a widely used chemotherapeutic drug that causes nerve injury and damages hematopoietic regeneration in the BM. In mice, treatment with NPY restored HSC impairment and chemotherapy-induced cell damage in the BM microenvironment via neuroprotection and resulted in a recovery of the blood cell count (Park et al., 2015; Park et al., 2018).

Quiescence provides a balanced system that protects against depletion and overproduction of HSCs. Loss of balance within this system may cause BM failure or leukemia (Blank et al., 2015). Leukemia is a malignancy of the blood forming apparatus and occurs as the result of clonal proliferation of immature blood cells. In children with acute leukemia, plasma NPY levels were found to be high and it was suggested that NPY might be used as a biomarker in patients with acute lymphocytic leukemia (ALL). NPY mRNA was detected in CD10+ lymphoblasts in leukemic BM (Kogner et al. 1994). Myelodysplastic syndrome (MDS) is a clonal stem cell disorder, characterized by ineffective hematopoiesis and a decreased peripheral blood count. Overactivation of inhibitory pathways, such as TGF-β has been proposed to cause inefficient blood production in MDS (Blank et al., 2015). In MDS, blood cells in the BM cannot mature and blood cells in the BM display abberant development. As a result of a reduction in the production and quality of red blood cells MDS is characterized by anemia. Some subgroups of MDS may transform into leukemia (Fozza et al., 2016; Koeffler et al., 2016).

1.11. Hypothesis

In order to protect the HSC pool, the number of actively dividing progenitor cells is tightly regulated in the BM niche and there is a controlled balance between

quiescence, proliferation, maturation and migration of HSCs. The neurotransmitter NPY controls homeostasis of bone and immune cells. In NPY deficiency, the numbers of HSCs were decreased and BM regeneration was impaired. Therefore, it was hypothesized that NPY directly affects proliferation and quiescence of HSCs through interactions with its receptors.

Here, firstly the presence of NPY receptors on HSCs and mature hematopoietic cells from different sources was assessed. Then the response of HSCs to direct application of NPY agonists and antagonists was investigated using assays to measure differences cell cycle activity, metabolism, cell viability, apoptosis and differences in gene expression pathways in control and NPY-treated HSCs. Differences in gene expression pattern were confirmed with RT-qPCR.

The results of this study may help to establish novel protocols that support expansion of HSC *ex vivo*, while promoting their long-term engraftment potential; to give a better understanding of the role of neurotransmitters in the healthy hematopoietic BM niche and the development of BM impairment syndromes. It may also provide a more general knowledge on the regulation of stem cells quiescence and their cell cycling.

CHAPTER 2

MATERIALS AND METHODS

2.1. Isolation and Expansion of Human BM MSCs

Collection of BM samples from healthy donors was approved by the Hacettepe University Ethical Committee, approval no GO-16/693-13. Small BM samples were obtained from healthy individuals, scheduled to serve as donors for transplantation purposes BM mononuclear cells (MNCs) were collected using density centrifugation and cultured in complete medium (DMF10), consisting of 60% DMEM-LG and 40% MCDB-supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% Penicillin/ Streptomycine), 2 mM L-Glutamine. Cultures were maintained at 37ºC, 5% CO_2 and the medium were replaced every 3-4 days. All experiments were carried out with MSCs at passage 3.

2.2. Umbilical Cord Blood (UCB)

Human umbilical cord blood (UCB) was collected from the umbilical cords of healthy term newborns born by Cesarean section at the Hacettepe University, Department of Obstetrics and Gynecology. For each cord blood, 20 mL phosphate buffered saline (PBS) (Applichem,USA) and 20 uL (50 IU/mL) heparin were added to a sterile autoclaved glass bottle. UCB was collected up to 80 mL per unit. This study was approved by the Hacettepe University Non-Interventional Ethics Committee, GO16/693 and GO18/133.

2.3. CD34+ Hematopoietic Stem Cell Isolation from UCB

UCB was transferred from the sterile bottles to 50 mL polypropylene tubes (Greiner, USA) and total volume was determined. The total number of nucleated cells was counted with Turk's dye. UCB was diluted with PBS at a ratio of 1:1 and layered over

15 mL Ficoll (Cegrogen Biotech, USA) added in the same volume in 50 mL tubes. Samples were centrifuged at 2000 rpm for 15 minutes room temperature without brake (Eppendorf Centrifuge 5810, USA). After centrifugation, the mononuclear cells (MNCs) were collected from the buffy coat and washed with PBS at least 2 times at 1500 rpm for 5 minutes at 4°C. Cells were counted with Turk's dye and cell recovery was calculated (Figure 2.1.).

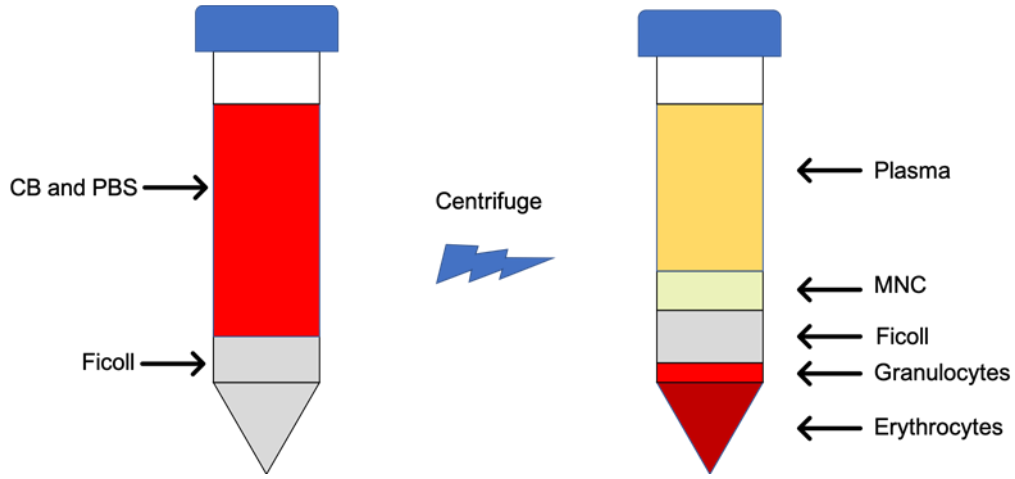

Figure 2. 1. Isolation of MNC by Ficoll density gradient. CB: Cord Blood, MNC: Mononuclear cells.

For every 10^8 MNCs; 300 uL of MACS buffer, 100 uL of FcR blocking solution and 100 uL of anti-CD34 microbeads (Miltenyibiotec, USA) were added and incubated for 30 minutes at 4°C on the MACS rotator (Miltenyibiotec, USA). After incubation, the sample was washed twice with cold MACS buffer and centrifuged at 1500 rpm for 5 minutes at 4°C. The cell pellet was suspended in cold MACS buffer and passed through a 35 µm cell strainer (Corning, USA) to obtain a single cell suspension. For isolation, the LS column (Miltenyibiotec, USA) was placed in the magnetic field of the MidiMACS separator and prepared by washing 2 times with 6 mL ice cold MACS

buffer. After rinsing, 3 mL of cell suspension was loaded onto the column and washed 3 times with 3 mL cold MACS buffer. Whereas the magnetically labeled CD34+ cells remained in the column, the CD34 negative (CD34-) fraction was washed away with MACS buffer. After collection of the CD34(-) fraction, the column was removed from the magnetic field and placed onto a fresh sterile 15 mL tube (Greiner, USA) and the CD34+ cells were eluted from the column by gentle force using a piston and washing twice with 6 mL cold MACS buffer. The CD34- and CD34+ fractions were centrifuged at 1500 rpm for 5 minutes at 4°C and counted with Turk's dye. 10^5 cells were used for FACS analysis.

2.4. Real-Time Cell Proliferation Assessment (xCELLigence)

Real time proliferation was assessed using measurements of impedance. Passage 3 MSCs were seeded into 100 µL of DMF10 at 2000 cells per well in triplicate onto special 96-well xCELLigence E-Plates (Roche, cat no 05232368001). Attachment, spreading and proliferation of the cells was monitored every 60 minutes using the xCELLigence RTCA DP instrument (Roche, Germany). NPY was tested at concentrations of 0.01 nM, 0.1 nM and 1 nM. The NPY-Y1 antagonist PD 160170 was tested at doses of 0.5 µM, 1 µM and 2 µM. Cells were maintained in DMF10 medium and real-time proliferation was monitored for 10 consecutive days. Medium was changed after 5 days (peak at 100h).

2.5. Co-Cultures of MSCs and HSCs

The following conditions were tested in co-culture experiments. All experiments were carried out in 6-well plates. Plates that contained MSCs were grown to confluence before the beginning of the experiment. CD34+ cells were added as 100.000 cells/well. Concentrations of NPY and NPY-Y1 antagonist (PD 160170, TOCRIS, cat no 2200) were 0.1 nM and 1.0 µM, respectively. Cells were co-cultured in serum-free StemMACS Hematopoietic Expansion Medium (Miltenyi, cat no 130-100-463), supplemented with 1% STF (SCF, TPO, Flt3-ligand) StemMACS hematopoietic growth factor cocktail 100X (Miltenyi, cat no 130-100-843).

Table 2. 1. MSCs and HSCs Co-culture conditions.

Group	Condition
1	MSC
2	MSC + HSC
3	MSC + HSC + NPY
4	MSC + HSC + PD 160170
5	MSC + HSC + PD 160170 + NPY
6	HSC
7	HSC + NPY
8	HSC + PD 160170
9	HSC + PD 160170 + NPY

After 7 days of co-cultures, cells were collected and used for the following experiments. To separate MSCs from HSCs, cells from co-cultures were fractionated into two cell populations, based on cell size and immunophenotype. Cells were collected using cell scraping, run through cell sieves and CD34+ cells were isolated using a MiniMACS column (Miltenyi).

2.6. CD34+ HSC Culture

The effect of NPY on HSCs was tested in a range of cell culture experiments. All experiments were carried out in 12 mL round bottom polypropylene (Fisherscientific, USA) culture tubes with 100.000 CD34+ cells/tube. Tested NPY concentrations were 0.01, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 nM. CD34+ cells were cultured for 2, 4 or 7 days in serum free StemMACS Hematopoietic Expansion Medium (Miltenyi, cat no. 130-100-463) with 30 ng/mL Thrombopoietin (TPO, R&D systems) or

SCF/TPO/Flt3-ligand HSC expansion cocktail (1X STF, Miltenyi, cat no 130-100-843).

2.7. Immunophenotypic Analysis of CD34+ HSCs

For FACS analysis, BM and UCB total nucleated cells (TNCs), UCB-MNCs, CD34 negative and the CD34 positive cell fractions were analyzed. Where needed, cell populations were treated with 1X Lysis buffer ($NH_4Cl/KHCO_3$/EDTA buffer, pH 7.4) for 10 minutes to lyse remaining red cells. Cells were then centrifuged at 1500 rpm for 5 minutes and washed with PBS/BSA/NaN_3 (PBN). Single cell suspensions were stained with 5 µL/tube primary antibodies against CD34 (BioLegend, 343510), CD38 (BD Biosciences, 555459), CD45 (BD Biosciences, 555485; eBioscience, pp. 17-9459-42), CD14 (BD Biosciences, 555399; BioLegend, 367104), CD16/56 (BD Biosciences, 342403), CD3 (BioLegend, 344812) and CD19 (BD Biosciences, 555413). For detection of NPY-receptors: 1 µL NPY-Y1 (1: 1000, Abcam, ab55730), 4 µL NPY-Y 2 (1/25, ThermofisherScientific / Invitrogen, PA5-72223), 5 µL NPY-Y3 (CXCR4 / CD184-PE-Cy7, BioLegend, 306506, clone 12G5), 10 µL NPY-Y4 (1: 100, Abcam, ab188915) and 1 µL NPY-Y5 (1: 1000, Abcam, ab 133757) were used. At least 100,000 cells were seeded into each tube and incubated with 100 %L of PBN (PBS, Calf Serum Albumin, Sodium Azide) containing 2% human serum AB in the dark at room temperature for 15 min with the above-mentioned primary antibodies. Subsequently, the cells were washed twice with PBN and goat-anti-rabbit IgG-Alexa Fluor 568 (GAR-AF568, Abcam, fold no ab150077) or rat-anti-mouse IgG-FITC (BD Biosciences, fold no 553443) with secondary antibodies and incubated in 100 µL PBN with 2% normal mouse serum for 15 minutes at room temperature, in the dark. After incubation, cells were washed twice with PBN and centrifuged at 1500 rpm for 5 min. PBN was added to the pellets (150-300 µL). At least 10.000 list mode events were measured for each sample using a BD Accuri (Becton Dickinson) and analyzed using BD CSampler Analysis software for Mac (Becton Dickinson).

Table 2. 2. Antibodies used in immunophenotyping.

Antibody	**Company**	**Cat no**
CD34-PE	eBioscience	343510
CD38-APC	BD Biosciences	555459
CD45 APC	BD Biosciences	555485
CD14 PE	BioLegend	367104
CD16 FITC	BD Biosciences	342403
CD3 APC	BioLegend	344812
CD19 PE	BD Biosciences	555413
NPY-Y1	Abcam	ab55730
NPY-Y2	Invitrogen	PA5-72223
NPY-Y3	BioLegend	306506
NPY-Y4	Abcam	ab188915
NPY-Y5	Abcam	ab133757
Goat-anti-rabbit IgG-Alexa Fluor 568	Abcam	ab150077
Rat-anti-mouse IgG-FITC	BD Biosciences	553443

2.8. Cell Cycle Analysis by Flow Cytometry

The determination of the percentage of cells in S-phase is dependent upon the detection of a thymidine analogue, bromodeoxyuridine (BrdU), which when added to culture medium is incorporated into DNA during DNA replication. The cultured CD34+ HSCs were incubated with 10 mg/mL BrdU (BioLegend, USA) for 60 min at 37^{o}C and then collected and centrifuged for 5 minutes at 1500 rpm. Cells were washed once with Cell Staining Buffer (BioLegend, USA). Cell suspensions were stained with antibodies against CD34, and incubated in 100 µL PBN with 2% human AB serum for 15 minutes at room temperature in the dark. After incubation, cells were washed twice with PBN and centrifuged at 1500 rpm for 5 min. Cells were fixed with 100 µL of Buffer A (BioLegend, USA) to each tube and incubated at 4°C for 20 min. After incubation, cells were washed with Buffer B, and centrifuged at 1500 rpm for 5 min. The cells were then permeabilized by adding 100 µL of Buffer C for 10 minutes at room temperature. Cells were washed once and treated with 20 µg of DNAse (Cat.

No. D4513, Sigma-Aldrich) diluted in Ca^{2+}/Mg^{2+} DPBS (BioLegend, USA) to unwind the DNA for BrdU staining and incubated at 37°C for 1 hour. Cells were washed once and 5 µL anti-BrdU antibody was added to each tube. Cells were incubated for 20 minutes at room temperature in the dark. Cells were washed once and then incubated with 1 µg 7-AAD (BioLegend, USA) for 5 minutes prior to acquisition.

2.9. Immunofluorescent Staining

Cytospins were used to immobilize cells onto glass slides. Briefly, 300 µL of CD34+ HSC suspensions were pipetted into each of the sample chambers of the cytospin and centrifuged at 300g for 3 min (Thermo Shandon, USA). CD34+ HSCs were fixed for 10 min in 70% Ethanol and stored at -80ºC before staining. The slides were washed with PBS and blocked with 3% goat serum, 10% FBS (Life Technology, USA) and 0,1% Tween-20 (Merck, Germany) in PBS and incubated for 1 hour at room temperature. The NPY-receptors and dilutions used were as follows: 1 µL NPY-Y1 (1/1000, Abcam, cat no ab55730); 0,1 µL NPY-Y2 (1/2000, ThermofisherScientific / Invitrogen, Cat. No. PA5-72223); 1 µL NPY-Y3 (1/100, CXCR 4-Alexa Fluor 555, Abcam, cat no. 216926); 4 µL of NPY-Y4 (1/2500, Abcam, cat. no. Ab188915) and 5 µL of NPY-Y5 (1/200, Abcam, cat no. Ab133757). Primary antibodies for NPY-Y receptors were prepared in 0.1% Tween-20 / PBS. One hundred µL of antibody was added to the cells and incubated overnight at 4°C. The next day, samples were washed 3 times with PBS. Goat-anti-rabbit IgG-Alexa Fluor 568 (1/1000, GAR-AF568, Abcam, cat no ab150077) or rat-anti-mouse IgG-FITC (1/1000, BD Biosciences, cat no 553443) secondary antibodies were used in 0.1% Tween-20/PBS solution. Samples were incubated for 40 minutes at room temperature with 100 µL of secondary antibody added and then washed 3 times with PBS. DAPI (1/4000 ratio) was prepared as cell core dye. The cells were incubated with DAPI at room temperature for 1 min. Prolong Antifade (Invitrogen) coating solution was added to the slides and permanently closed with a coverslip. Leica Application Suit 3.1 was used to capture fluorescent photographs.

2.10. Analysis of Cell Proliferation

CD34+ HSCs were cultured at a density of 10.000 cells per well in a 96-well plate, in presence of 0.01, 0.1, 0.3, 1, 3, 10, 30, 100 and 300 nM NPY in triplicate. To assess cell proliferation and viability in cell populations, Cell Proliferation Reagent WST-1 (Roche, Germany) was used and color changes were assessed spectrophotometrically. Cultures were maintained for 4 or 7 days. For measurements, 20 µL/well (1:10) WST-1 was added directly into the 96-well plates and cells were incubated at 37ºC. Results were measured 4 hours later at 450 nm using 620 nm as the reference wave length on the Tecan ELISA microplate reader.

2.11. Total RNA Isolation from HSCs

For the determination of changes in gene expression patterns of HSCs, total RNA was isolated using the miRNeasy minikit (Qiagen, USA). The RNeasy Mini Kit allows efficient purification of total RNA and ensures high-quality RNA preparations from small amounts of cells. Cells were treated with Qiazol Lysis Reagent (Qiagen, cat. No. 79306) and incubated at room temperature for 5 minutes. Qiazol facilitates lysis of cells and homogenizes and inhibits RNases. Qiazol treated samples were stored at -80°C until use. For further processing, samples were thawed and homogenized at room temperature. 140 µL of chloroform (Applichem, cat. No. A3633) was added to each tube and the tubes were shaken up and down 20 times and allowed to stand at room temperature for 5 minutes. The samples were then incubated for 3 minutes at room temperature and centrifuged at 12.000xg for 15 minutes at 4oC. At the end of the centrifugation, three layers were observed. The lower layer contained proteins; the interphase DNA and the upper aqueous phase contains total RNA. In order to isolate the total RNA fraction, free from protein and DNA contamination, the upper aqueous phase was collected and transferred to a new microcentrifuge tube. Ethanol (100%) was added 1.5 volumes and the samples were vortexed for 30 seconds. The samples were loaded onto the RNeasy mini spin column and precipitated for 30 seconds at 10.000xg. RWT buffer (350 µL) was added and samples were precipitated for 15

seconds at 10.000xg. Then 80 µL DNase was added and samples were incubated at room temperature for 30 minutes. 350 µL RWT buffer was then added and samples were precipitated for 15 seconds at 10.000xg. Finally, 500 µL RPE Buffer was used and samples were spun down for 2 minutes at 10.000xg. RNA was eluted from the column and stored at -80°C until used. RNA concentrations of the samples and RNA purity were measured using a NanoDrop 2000 (Thermo Scientific, US). 1 µL of the samples were loaded onto the NanoDrop sensor, and the concentrations were measured at A260/A280 to check for protein or phenol contamination and at A260/A230 for nucleic acid contamination.

2.12. Transcriptome Array

Differentially expressed transcripts were determined with The GeneChip™ Human Gene 2.0 ST Array (Affymetrix, USA) analysis. At first, control RNA and total RNA/Poly-A RNA control mixture were prepared. After, first-strand cDNA, 3' Adaptor cDNA, double-stranded cDNA and cRNA by *in vitro* transcription were synthesized respectively. Then, cRNA was purified by using purification beads and cRNA yield was assessed. Following the last step, 2nd-cycle single-stranded cDNA was synthesized and RNA was hydrolyzed by using RNase H. After, 2nd-cycle single-stranded cDNA was purified and single-stranded cDNA yield was assessed. Then, single-stranded cDNA was fragmented and labelled. At the end, cartridge arrays were hybridized and scanned. The signals obtained after hybridization were analyzed by the scanner. After analysis, signal intensities from the microarray chips were compared. Each chip received the same concentration and all were within the evaluation limits.

2.13. RNA Sequencing Analysis

Transcriptome sequencing using BGISEQ was performed for identification of differentially expressed RNAs in NPY treated and untreated samples. Required the total amount of human RNA samples was > 5 µg and average concentration of mRNAs collected from HSCs were sufficient for further analysis (65 ng/µL). The purity of the obtained mRNAs was OD260 / 280 ≥1.8, OD260 / 230 ≥1.8 and RNA 28S: 18S ≥ 1.0,

RIN ≥ 7.0. mRNA and poly A enrichment was performed on total RNA samples to remove ribosomal RNAs. The HSC transcriptome in presence and absence of NPY was analyzed using the Agilent Bioanalyser 6000 Nano Kit (Agilent Technologies,USA) to determine the quality and amount of mRNAs obtained in presence and absence of NPY. In order to perform next generation sequencing, mRNA samples were split into smaller samples of an appropriate size using RNAse III. After treatment with RNAse III, samples were purified for further sequencing and cDNA was obtained by reverse transcription. Fragments obtained from the prepared library were calculated as 1 fragment to 1 ion capture particle and the beads and fragments were bound to each other. The particles were then emulsified in an oil-containing water mixture. For each particle the Emulsion Polymerase Chain Reaction (emPZR) was encapsulated in a microreactor where amplification takes place. The micro-reactors were then broken and the amplified particles were used for sequencing. Basically, the stages of the RNA sequencing process were performed. At first, complete primers were connected and primers were added during selection of target regions to fragments. After the sequencing solution was loaded into the flow cell. Then, the first base after the primer was connected and non-bonded bases were removed in the environment. Flow cell was pictured. Each base realizes fluorescent glow of different colors. For this reason, the bound base was determined by taking the photo of the flow cell behind each connected base. Transcriptome sequencing was carried out using the BGISEQ sequence system by BGI.

After removal of low-quality, adaptor-polluted and high content of unknown base (N) reads, the clean reads were mapped according to a reference genome using HISAT2 (Kim D., et al., 2015). After genome mapping, StringTie was used to reconstruct transcripts (Pertea M. et al., 2015), and novel transcripts were identified using Cuffcompare (Trapnell C. et al., 2012; Kong L. et al., 2007.). After genome mapping, rMATS was used to detect differentially spliced genes (DSG) between samples (Shen S. et al., 2014). Novel coding transcripts were merged with reference transcripts to get a complete reference, and mapped to clean reads using Bowtie2 (Langmead B. et al.,

2012). Then the gene expression levels were calculated for each sample with RSEM (Li B. and Dewey C.N., 2011). To assess the correlation of gene expression between samples, Pearson correlation coefficients were calculated. Then further gene expression cluster analysis was performed using cluster software and Euclidean distance matrix for hierarchical clustering analysis. Genes with similar expression patterns usually have same functional correlation. Gene ontology classifications were done for differentially expressed genes.

2.14. Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

First, cDNA preparation was performed according to the manufacturer's instructions using the High-Capacity cDNA Reverse Transcription Kit (ThermoScientific, Germany). After the determination of the RNA concentration, the concentration of the RNA to be used in cDNA preparation was adjusted accordingly. Quantitation was performed based on measurements of fluorescence. The reactions were carried out using a LightCycler® 480-II system (Roche, Germany) with the selected appropriate primers. Changes in expression due to variables in the experiments were calculated using the relative ($2^{-\Delta\Delta Ct}$) quantitation. *ACTB* reference gene expression was used as a normalizer. All the RNA steps were confirmed based on MIQE guidelines (Dorak, 2006).

2.15. Statistical Analysis

Statistical significance between groups were calculated using the Excel spreadsheet program using the two-tailed Student's T-test. The significance value (p) was <0.05. Data are expressed as mean ± standard deviation. RT-qPCR calculations and graphs were made using the free Graphpad software (version 6) using the two-tailed Student's T-test. For microarray studies, the limma ebayes test and Benjamini Hochberg multi-test corrections were performed. Limma ebayes test is a slightly different version of the T-test to find statistically significantly different genes between the two groups.

CHAPTER 3

RESULTS

3.1. Determination of effective doses of NPY and NPY-Y1 antagonists using measurements of real time proliferation of MSCs

The effect of NPY on proliferation of MSCs was tested at concentrations of 0.01, 0.1 and 1 nM. None of the tested doses showed negative effects on proliferation of MSCs (Figure 3.1.). The dose of NPY was found to be optimal at 0.1 nM and the dose of the NPY-Y1 antagonist PD 160170 was selected to be 1 μM in the subsequent experiments.

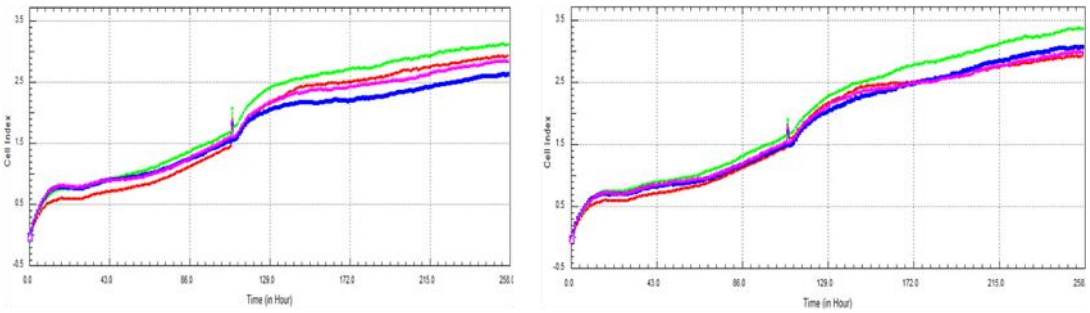

Figure 3. 1. MSC proliferation with NPY and NPY-Y1 inhibitor (real time). Different colors indicate different groups and concentrations of NPY and NPY-Y1. Left: effects of NPY on MSC proliferation with red: control; green: NPY 1 nM; blue: NPY 0.1 nM and pink: NPY 0.01 nM. Right: effects of NPY-Y1 inhibitor PD160170 on proliferation of MSCs with red: control; green: NPY 1 nM; blue: NPY 0.1 nM and pink: NPY 0.01 nM. Cells were monitored after treatment and impedance was measured and recorded as Cell Index (CI) values, which reflect the proliferation of the adherent cells. None of the tested doses affected proliferation in a negative fashion.

3.2. The Effect of NPY and NPY-Y1 Antagonist on Cell Cycling of HSCs

To assess the effect of NPY and NPY-Y1 antagonist treatment on the hematopoietic niche and indirectly on cell cycling of HSCs, three experimental groups of HSCs were evaluated:

Table 3. 1. Three conditions of experimental groups.

Groups	Conditions
1	MSC + HSC
2	MSC + HSC+ NPY
3	MSC + HSC+ NPY + PD 160170

During cell cycle progression, proliferating cells sequentially undergo a transition of $G_1 \rightarrow S \rightarrow G_2 \rightarrow M$ phases for synthesis of DNA (S-phase), preparation of cell division (G_2-phase) and subsequent mitosis (M-phase). Quiescent cells (G_0 phase) are characterized by having minimally active cell cycle machinery and maintaining specialized cellular functions rather than by proceeding to cell proliferation. FACS analyses showed that although the purity of the CD34+ HSC populations isolated was sufficient (<2.0% contamination with MSCs), the fraction of recovered cells was not sufficient to perform the RNA experiments. In contrast, MSC fractions still contained a considerable number of contaminating HSCs. The cycling of HSCs was not affected by treatment with either NPY or NPY-Y1 antagonists at the tested doses. Cells in G_0 phase were similar in all three conditions, ranging from 57.8-59.9% and cells in S/G2/M phases were between 24-25% in all groups.

3.3. Expression of NPY Receptors by BM and UCB Cell Fractions

The expressions of NPY-Y1 on BM (n=1) and UCB (n=10) hematopoietic fractions were assessed and results are presented in Table 3.2. The NPY-Y1 expression was detected at varying levels in all hematopoietic subgroups, including CD34+ HSCs.

However, considerable differences in the expression of NPY-Y1 was observed between donors. Many genes are differentially expressed through populations and even individuals. Mostly the genes that show the highest expression variability among human individuals are significantly associated with diseases. This also effect the susceptibility to diseases (Li et al., 2010). The main reason can be differences in epigenetic signature. The other reason is the differences binding sites of transcription factors person to person. These differences are also can be heritable and effect the level of gene expression (Paul, 2004, Kasowski et al., 2010).

Table 3. 2. Surface expression of NPY-Y1 by BM and UCB cells (%)

Source	CD3	CD14	CD16	CD19	CD34	CD38	CD45
UCB (n=10)	41.0 ± 33.8	9.1 ± 11.4	30.6 ± 39.2	29.4 ± 28.7	46.2 ± 46.5	21.4*	73.9 ± 38.7
BM (n=1)	12.2	17.5	2.9	2.8	43.7	88.0	

* Single sample

In Figure 3.2., the expression of NPY receptors Y1 throughY5 by UCB-CD34+ cells were assessed. In order to minimize the differences between the experiments, UCB cells from 3 donors were combined in each experiment (n=5). Despite combining of cells from 3 donors, there were still considerable differences in the NPY-R expression between experiments. To assess the effect of culture on the NPY receptors expression, NPY receptors Y1 to Y5 were also assessed after 4 and 7 days of CD34+ cell culture in presence or absence of NPY. NPY receptor Y1-Y5 expression was evaluated before and after cell culture (Figure 3.2.).

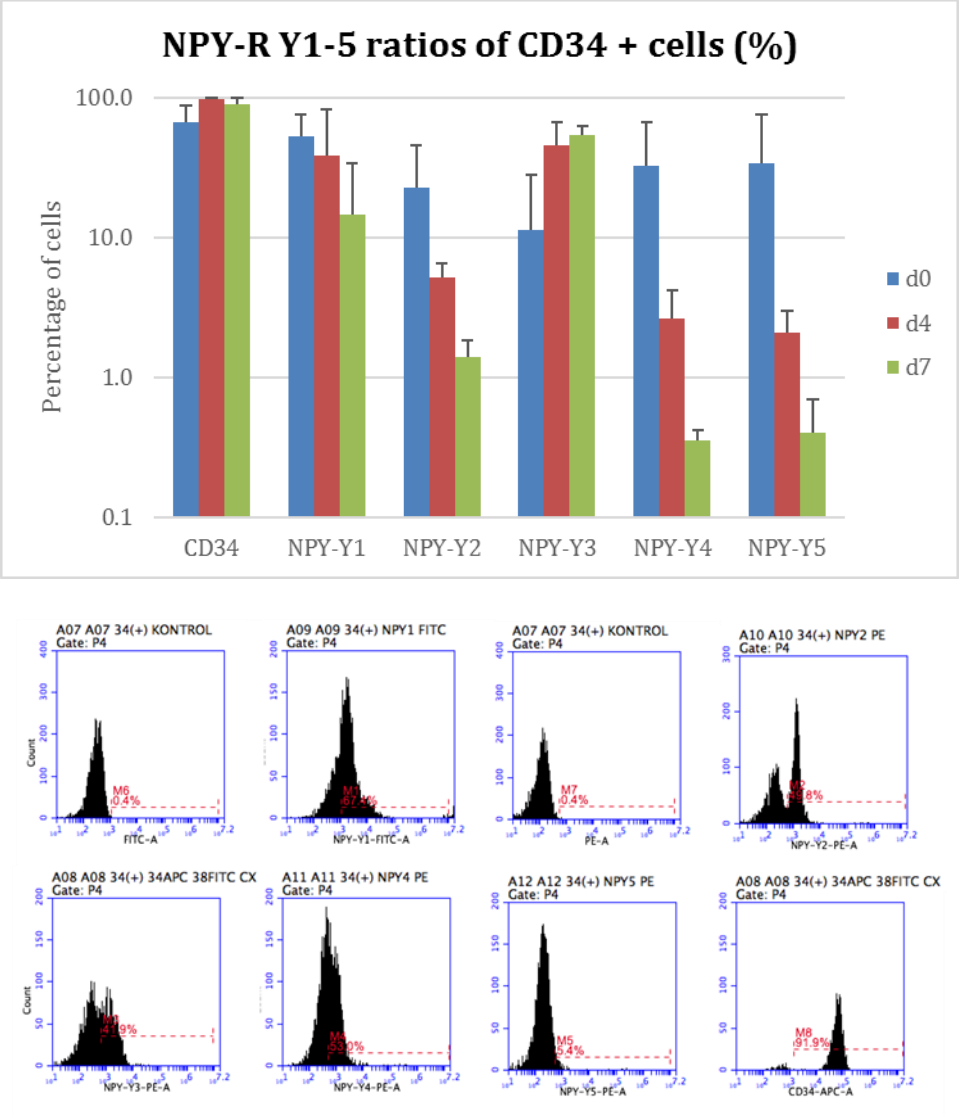

Figure 3. 2. Percentage of cell surface expression of NPY receptors. Y1 to Y5 by uncultured and cultured CD34+ HSCs (%).Upper panel: NPY receptor expression of CD34+ UCB cells were evaluated by flow cytometry before culture (blue) and 4 days (red) and 7 days (green) of culture in presence in serum-free HSC expansion medium. Values are given as the mean of five individual experiments ± SD. Lower panel: NPY receptor expression of CD34+ UCB cells as day 0 before culture. Representative histogram.

NPY-Y1 expression was high before culture and decreased rapidly on the 4th and 7th days of the cell culture. Whereas expression of NPY-Y2, NPY-Y4 and NPY-Y5 decreased in time, expression of NPY-Y3 (or CXCR4), the most important receptor for the homing of HSCs, increased over time in culture. In order to investigate the effect of NPY on NPY receptor expression, CD34+ HSCs were cultured for 4 and 7 days in serum-free HSC expansion medium containing 1X STF and supplemented with 0-100 nM NPY. NPY receptor expression was evaluated on day 4 (Figure 3.3. upper panel) and day 7 (Figure 3.3. lower panel) of the culture. NPY did not have any negative effect on HSCs and NPY receptor Y1 to Y5 expression was not markedly affected. As a positive control SH-SY5Y, Hela and HEK cells were used (Table C.1.).

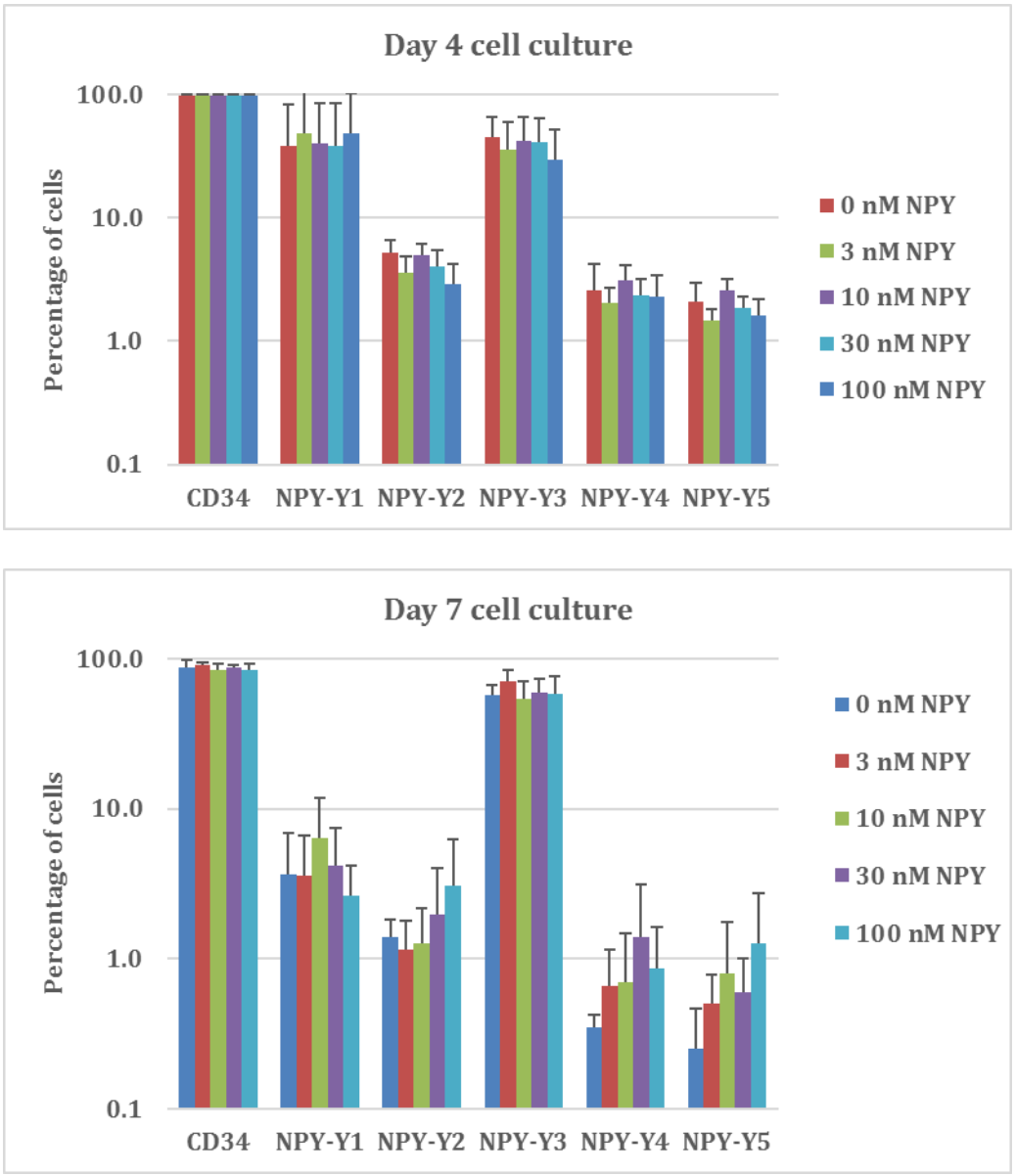

Figure 3. 3. Effect of NPY on HSC NPY receptor expression after 4 and 7 days. The effect of NPY on CD34 + HSC NPY receptor expression was assessed by flow cytometry after culturing in serum-free HSC expansion medium containing 0-100 nM NPY after 4 days (upper) and 7 days (lower panel), (n=3).

3.4. Direct Effect of NPY on HSC Proliferation

NPY doses of 0-300 nM were applied to the CD34+ cell cultures for 4 or 7 days to evaluate the effect of NPY on HSCs proliferation (Figure 3.4.) in CD34+ cell cultures with or without NPY.

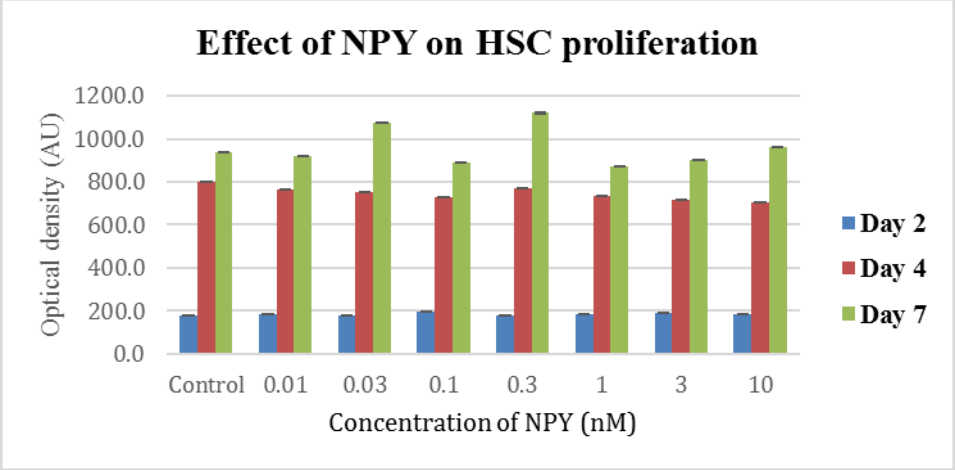

Figure 3. 4. The effect of 0-10 nM NPY on HSC proliferation. CD34+ HSCs were cultured for 2, 4 or 7 days with serum-free HSC expansion medium and 1X STF. Cell proliferation was assessed with WST-1. Data are given as average + standard deviation. Results of a single experiment, all experimental doses were tested in triplicate.

Since proliferation was minimal at day 2 of the culture, for further assessments only day 4 and day 7 cultures were used. Doses of 0-10 nM NPY did not affect proliferation of HSCs, therefore, higher concentrations were used in following tests. That 100 and 300 nM were used on days 4 and 7 and the effects on HSCs were investigated in serum-free medium containing either STF or TPO alone (Figure 3.5.). Cell numbers in TPO-containing cell cultures were generally lower, therefore, only STF-containing cell culture media were used for the subsequent experiments. In the 7-day cultures, doses exceeding concentrations of 30 nM NPY were found to slightly suppress

proliferation of HSCs. To verify these data, the same experiment was repeated in cultures containing 300 nM NPY and only STF (Figure 3.6.).

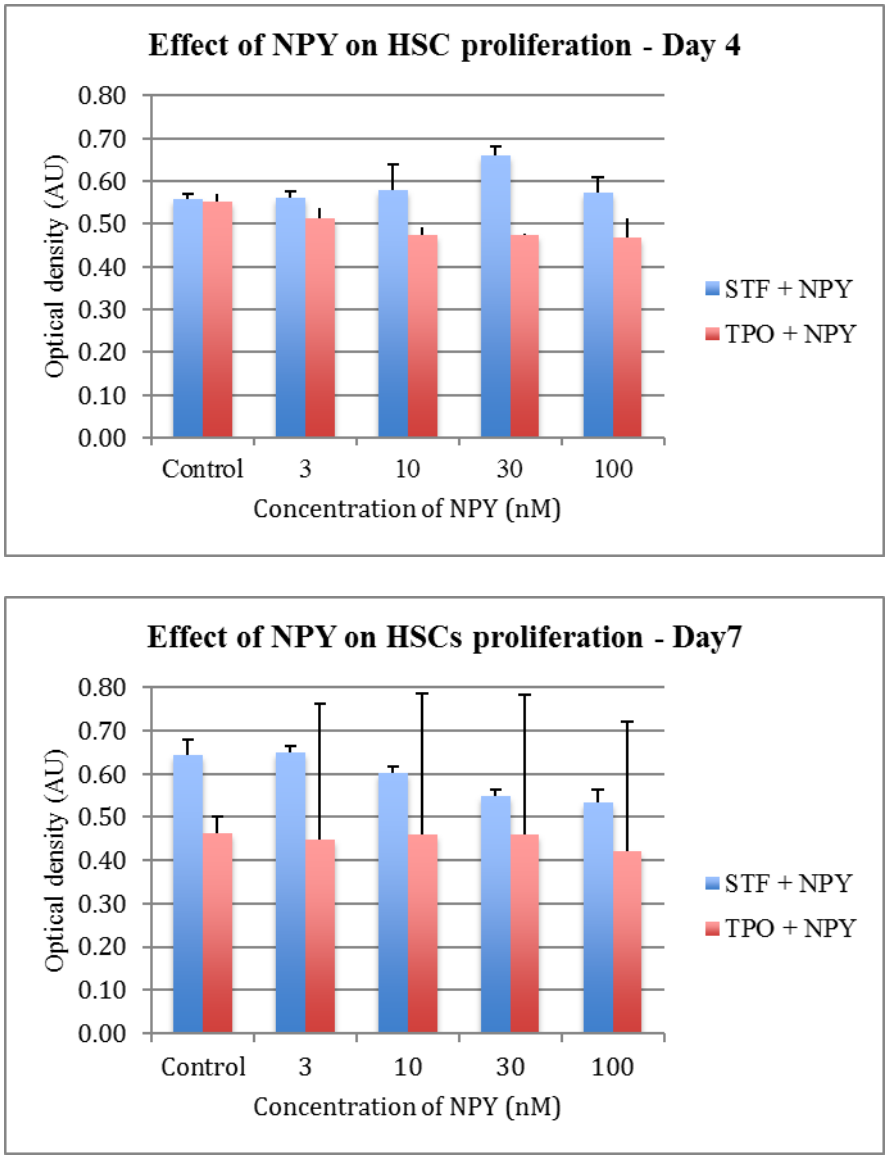

Figure 3. 5. Assessment of the effects of NPY on HSC proliferation in the presence of different growth factors. CD34+ HSCs were cultured for 4 (upper panel) or 7 (lower panel) days with serum-free HSC expansion medium and 1X STF (blue) or TPO alone (orange). NPY (0-100 nM) was added to the cultures and cell proliferation was evaluated with WST-1. Data are given as the average ± SD. All doses were tested in triplicate (n=3).

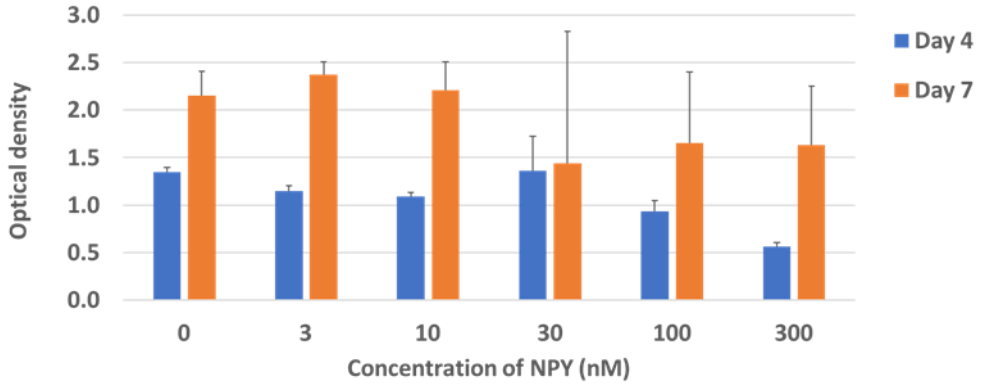

Figure 3. 6. The effect of different doses of NPY on HSC proliferation. CD34+ HSCs were cultured in serum-free StemMACS medium and STF medium for 4 (blue) or 7 (orange) days. NPY (0-300 nM) was added to the cultures and cell the proliferation was evaluated with WST-1 (n=3). Tests were performed in triplicate. Data are provided as mean ± SD.

Doses of NPY >30 nM appeared to have a slightly suppressive effect on proliferation of HSCs, especially in the 7-day cell cultures. In order to study whether the observed inhibition of proliferation was due to cell death (toxicity) or inhibition of the cell cycle, Ann-V/PI apoptosis assays and BrdU incorporation tests were performed after 4 and 7 days of the culture. Figure 3.7. shows the results of the cell cycle analyses using a BD Accuri (n=3). At the day 4, a dose of 300 nM NPY resulted in an increase of HSCs in the G_0 phase of the cell cycle. The number of the cells in S and G_2/M phases decreased in the 300 nM NPY treated cells compared to control sample and other doses. The same pattern was observed at the day 7.

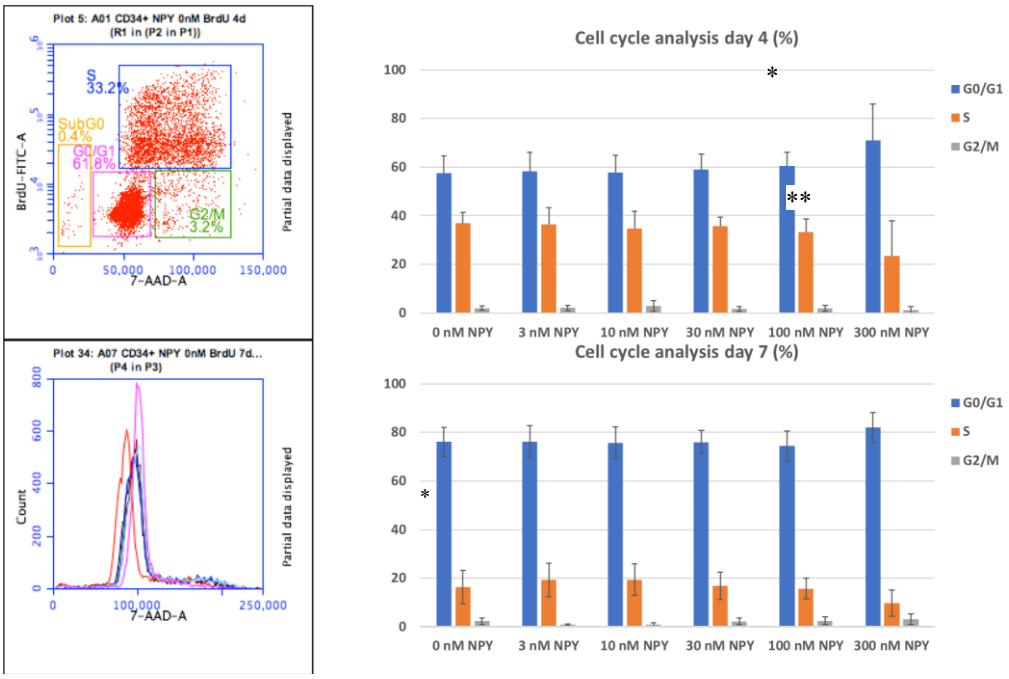

Figure 3. 7. The effect of different doses of NPY on cell cycle of HSCs. CD34+ HSCs were cultured in serum-free HSC expansion medium with 1X STF for 4 or 7 days. NPY (0-300 nM) was added to the cultures and cell cycle was evaluated by BrdU/7-AAD staining. Top left: Day 4; Bottom left: Day 7 (n=5). Top right: a representative example of a dot plot; Bottom right: a representative sample of a histogram plot. A dose of 300 nM NPY resulted in an increase of HSCs in the G0 phase of the cell cycle. * p: 0.12; ** p: 0.09.

WST proliferation tests showed that proliferation of HSCs was particularly suppressed under serum-free culture conditions containing 1X STF at a concentration of 300 nM NPY. These results were also confirmed in cell cycle tests and a significant increase was found in the fraction of cells in the G_0/G_1 phase of the cell cycle at a dose of 300 nM NPY, whereas a relative decrease was found in the number of HSCs in the S and G_2/M phases of the cell cycle (Table 3.3.).

Table 3. 3. Cell cycle analysis of CD34+ HSCs.

Average Day 4	G0/G1	S	G2/M	SubG0	S+G2/M
0 nM NPY	57.6	37.0	2.0	1.4	39.0
3 nM NPY	58.4	36.3	2.1	0.9	38.4
10 nM NPY	57.6	34.8	2.9	1.9	37.8
30 nM NPY	59.0	35.7	1.6	1.4	37.3
100 nM NPY	60.5	33.1	1.9	1.0	35.1
300 nM NPY	71.0	23.4	1.2	1.0	24.6

Average Day 7	G0/G1	S	G2/M	SubG0	S+G2/M
0 nM NPY	76,1	16,4	2,4	2,5	18,7
3 nM NPY	76,1	19,3	0,8	1,6	20,2
10 nM NPY	75,7	19,4	1,1	1,6	20,5
30 nM NPY	76,0	16,9	2,3	2,3	19,2
100 nM NPY	74,5	15,8	2,4	2,7	18,2
300 nM NPY	82,0	9,8	3,2	2,0	13,1

With increasing day of culture and the dose of NPY, cell numbers of HSCs in G_0 increased, whereas cell proliferation decreased (S+G2/M) (n=5). Average of 3 separate experiments. With increasing doses of NPY the number of cells in S+G2/M phase decreased, whereas the number of cells in G0 increased. Representative flow image sample shown at the right.

3.5. Microarray Analysis

After 2 days of cultures of HSCs, total RNA samples of HSCs were isolated using the miRNeasy minikit. After the analysis, the signal intensities from the array chips were compared. All samples were within the evaluation limits. Graph of the results of the limma ebayes test performed as a classic in microarray studies is shown in Figure 3.8.

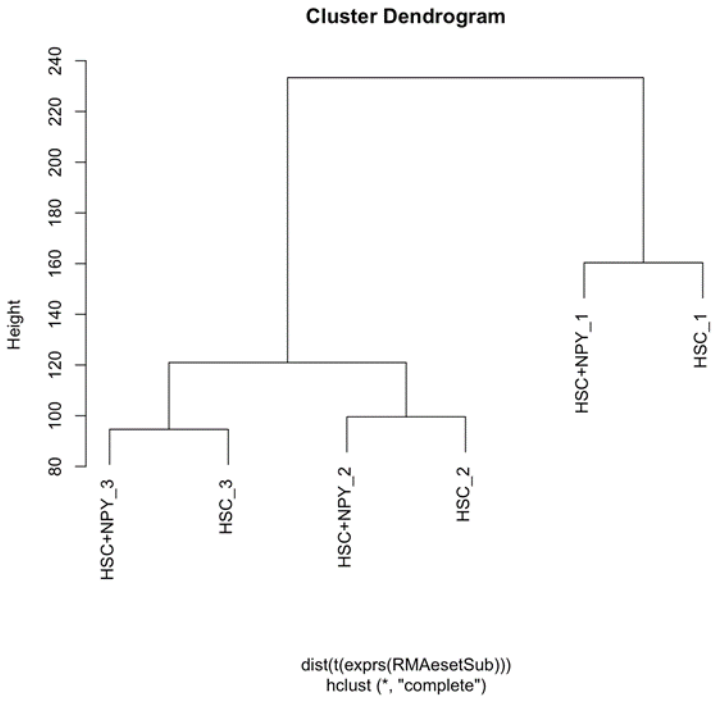

Figure 3. 8. Clustering of arrays after normalization to housekeeping genes. Group 1; HSC_1 and HSC+NPY_1, Group 2; HSC_2 and HSC+NPY_2, Group 3; HSC_3 and HSC+NPY_3

Using the e-bayes test, a volcano graph was obtained (Figure 3.9.). On the y-axis, the logarithm of each gene (log10) is converted to the positively converted p-value, the x-axis (log2) represents the logarithmic value of the change-value (number of changes between two conditions). However, statistically significant differences between the HSCs cultured in presence or absence of NPY could not be detected.

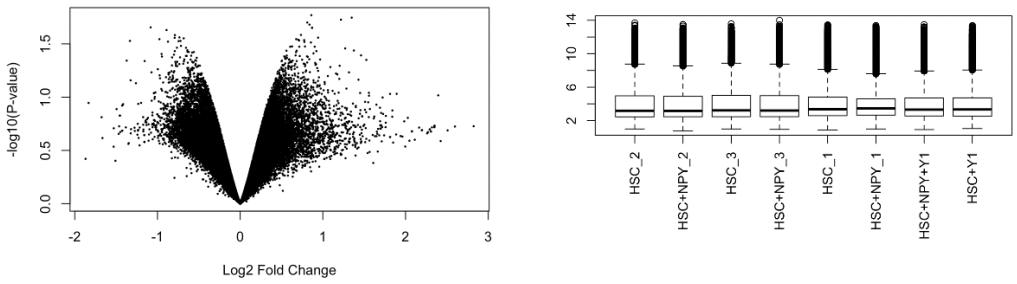

Figure 3. 9. Volcano graph and box plots of arrays after normalization. No significant genes were found.

Considering that the microarray is categorized according to general similarities, it was decided that further pursuing this type of analysis would not be meaningful. Therefore, we analyzed each group with another (Figure 3.10.). When the NPY treated and control groups were compared, the genes that were changed more than two folds (corresponding to 1fold in log2 scale) were listed. However, at the 0,1 nM NPY dose used for this study we couldn't detect any significant differentially expressed candidate genes between the groups. Therefore, it was decided to perform a broader transcriptome analysis using RNAseq and increased doses of NPY to find new transcripts.

Common Genes
1 and 2:
33 IDs not annotated
as Genes

Gene Symbol
A2M-AS1
FFAR3
GTF2IRD1P1
LOC105372906
LOC105376997
LOC199882 /// LOC101929805
LOC283177
MIR3147
MIR3665
MIR486-2
NDUFAF4
POU5F1P3
PRSS21
RASA4B
RNASE3
RNU1-13P
RNU1-13P
SNORD114-4
SNORD115-24
SNORD90
TARP /// TRGJP1
TRAJ17
TRDJ2
WDR5B
ZNF675

Common Genes
1 and 3:
59 IDs not annotated
as Genes

Gene Symbol
BEND3P3
DGCR6L
FMO4
HNRNPA1P33
KRTAP6-3
LINC00202-1
LOC100508046 /// POTEH-AS1
LOC100652901
LOC102724587
LOC105369830
LOC105379854 /// LOC105379521
MIR3198-1
MIR514A1
MIR548D1
MIR548H2
RORA-AS1
RPL23 /// SNORA21
SNORD113-8
SNORD124
TRAJ41 /// OR6C4
TRAJ49 /// TRAV20
TRBV7-7
USP17L5

Common Genes
2 and 3:
15 IDs not annotated
as Genes

Gene Symbol
IGKV1-27 /// IGKC
MIR345
MIR451B
ZNF582

Figure 3. 10. Microarray data of the genes affected by NPY treatment of HSCs. Common genes within each group were compared. The first column shows common genes in Group 1 and 2. The second column shows common genes in Group 1 and 3. The third column showed common genes in Group 2 and 3.

3.6. RNASeq Analysis

The quality of the RNA obtained for RNAseq analysis was sufficient for further analysis (Ek E). After removal of low-quality, adaptor-polluted and high content of

unknown base (N) reads, the clean reads were mapped according to a reference genome using HISAT2 (Kim D., et al., 2015 HISAT: a fast-spliced aligner with low memory requirements). On average 94.39% reads were mapped and the uniformity of the mapping suggested that the samples were comparable. In total 13.457 novel transcripts were identified and the distribution of SNP and INDEL variants were assessed (Figure 3.11.).

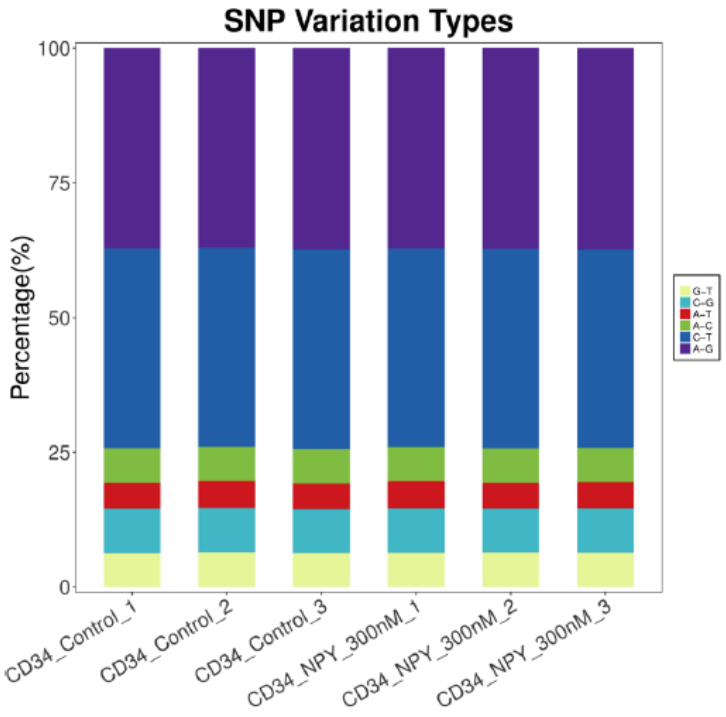

Figure 3. 11. SNP variant type distribution. The X-axis represents the type of SNP, whereas the Y axis represents the number of SNPs.

Alternative splicing causes the production of different isoforms or differentially spliced genes (DSGs) from a single gene. Changes in relative abundance of these isoforms, regardless of the expression change, indicates a splicing-related mechanism. In total five types of AS events were detected, including Skipped Exons (SE),

Alternative 5' Splicing Sites (A5SS), Alternative 3' Splicing Sites (A3SS), Mutually exclusive exons (MXE) and Retained Introns (RI) as shown in Figure 3.12.

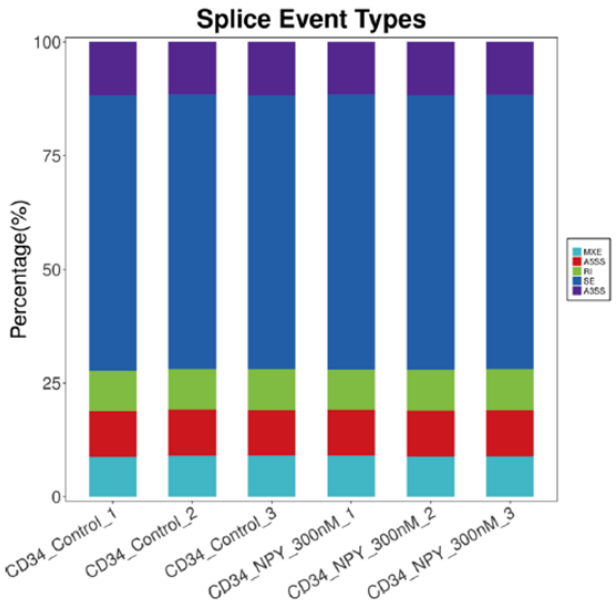

Figure 3. 12. Detected splicing variants in each sample. X-axis represents the type of splicing. The Y-axis means the amount. Different columns represent different splicing events. SE: Skipped Exons; A5SS: Alternative 5' Splicing Sites; A3SS: Alternative 3' Splicing Sites; MXE: Mutually exclusive exons; and RI: Retained Introns.

Gene and novel coding transcripts are shown in Table 3.4. and Table 3.5.

Table 3. 4. Genes and transcripts

Sample	Total GeneNumber	Total TranscriptNumber
CD34_Control_1	15,658	29,970
CD34_Control_2	15,647	29,691
CD34_Control_3	15,732	30,078
CD34_NPY_300nM_1	15,774	30,107
CD34_NPY_300nM_2	15,649	29,707
CD34_NPY_300nM_3	15,789	30,094

Table 3. 5. Overview of expressed genes.

gene_id	transcript_id(s)	length	expected_count	FPKM	SymbolID
1	NM_130786	1,766.00	155.00	4.97	A1BG
10	NM_000015	1,317.00	2.00	0.09	NAT2
100	NM_000022,...,NM_001322051	1,568.38	1,422.00	51.62	ADA
1000	NM_001308176,NM_001792	3,859.00	4.00	0.06	CDH2
10000	NM_001206729,...,NM_181690	6,924.71	757.85	6.02	AKT3

Correlations between samples are shown in Figure 3.13. The closer the samples, the higher the similarity in expression levels. Interestingly, differences between the NPY-treated group and control samples are larger than differences between samples treated with or without NPY, resulting in clustering of separate controls and NPY-treated together. Previous data from microarrays and receptor expression studies already indicated a wide variation between groups, hence the cells were pooled from 3 different donors before use. Nevertheless, despite pooling differences between samples remained larger than the effect of NPY treatment.

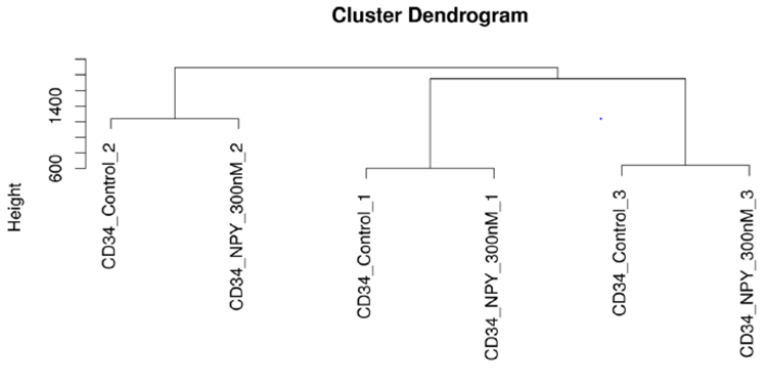

Figure 3. 13. Analysis of hierarchical clustering between samples.

Gene expression cluster analysis was further performed using cluster software and data are shown in Figure 3.14.

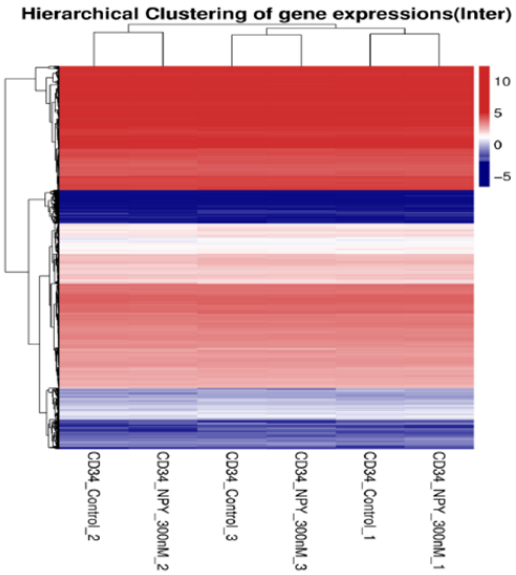

Figure 3. 14. Hierarchical clustering of gene expression.

The gradient legend at the top right of the graph represents the FPKM (Fragments per Kb of transcript per Million mapped reads) value that has been logarithmically converted. Each column represents a sample, each row represents a gene, different colors represents different expression levels, red for highest expression, and blue for lowest expression.

Based on the gene expression level differentially expressed genes (DEG) were assessed. Using the methods as described above, only two DEGs were detected, both of which were unknown genes (Gene iD 8341, log2 fold change 1.15 and Gene iD 8293, log2 fold change 7.85) and both were downregulated (Figure 3.15.).

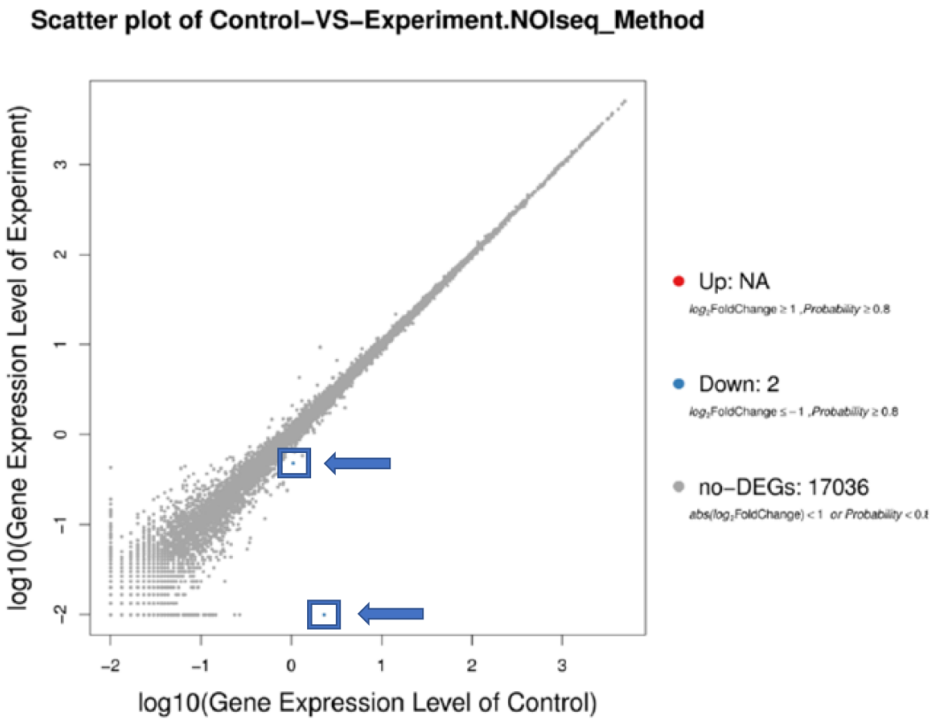

Figure 3. 15. Scatter plot of differentially expressed genes (DEGs). Blue arrow show that downregulated genes.

X and Y-axes represent log10 transformed gene expression level, red color represents up-regulated genes, blue color represents the down-regulated genes, gray color represents the non-DEGs.

With the two found DEGs, Gene Ontology (GO) classification and functional enrichment was performed. GO has three ontologies: molecular biological function, a cellular component and biological processes (Figure 3.16).

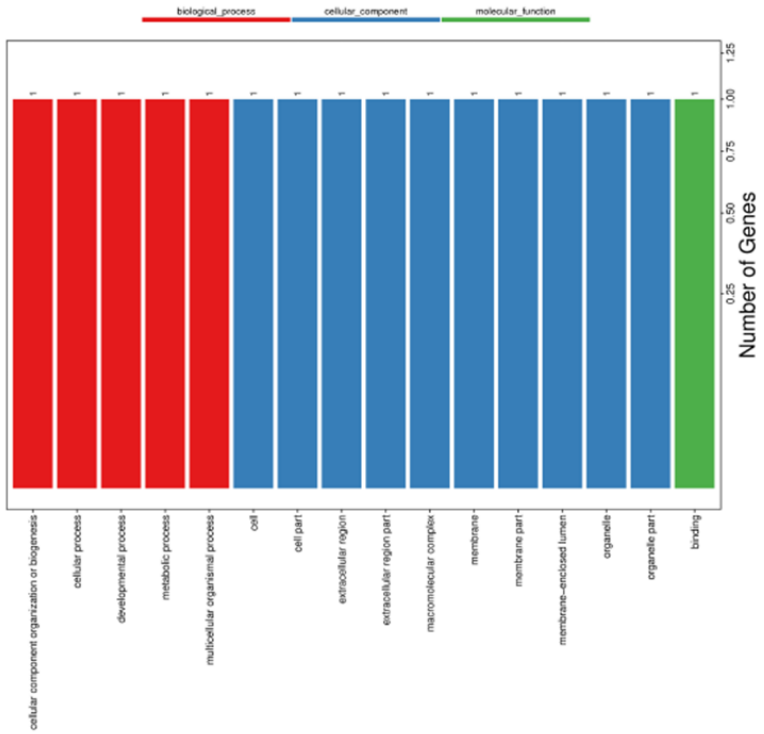

Figure 3. 16. Gene ontology classification of differentially expressed genes.

3.7. RT-qPCR Results

HSCs were cultured for 7 days in serum-free HSC expansion medium supplemented with 1X STF with or without 300 nM NPY. Total RNA was collected and used for RT-qPCR analysis of the *FOXO3, DICER1, PCNA, SMARCA2* and *PDK1* genes. *ACTB* gene expression was used as a normalizer. Figure 3.17. showed that all genes tested showed a significant increase in expression after NPY treatment.

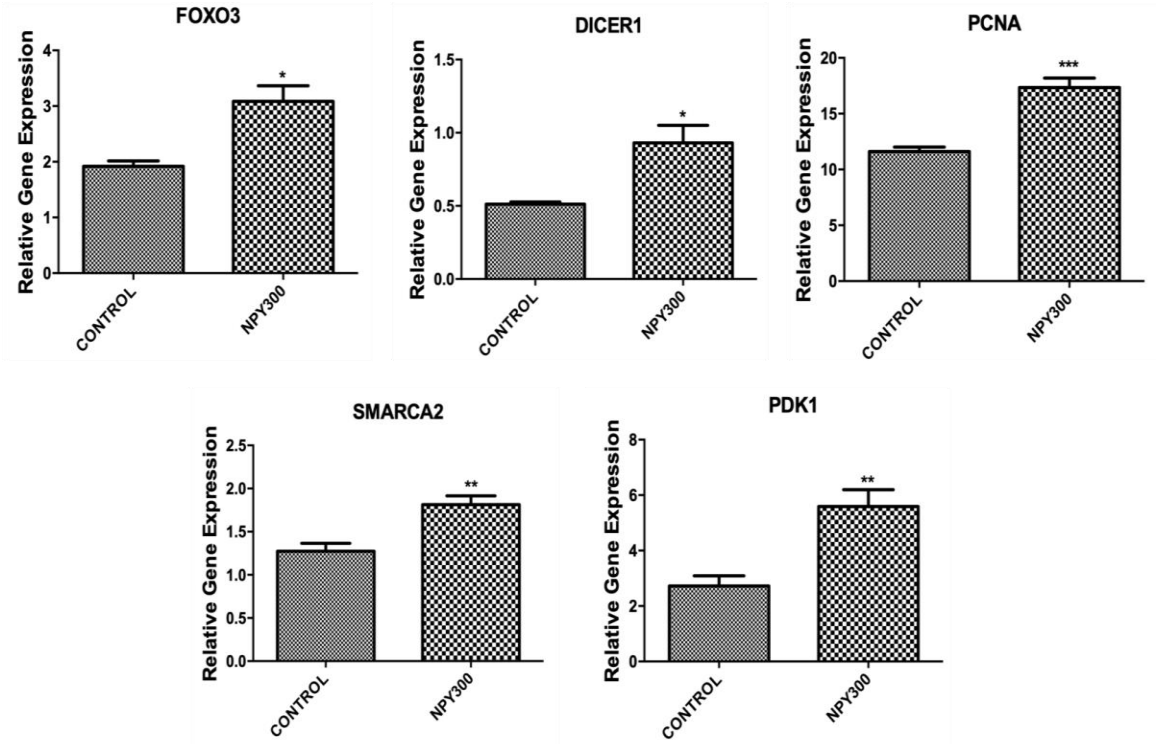

Figure 3. 17. Relative gene expression levels of FOXO3, DICER1, PCNA, SMARCA2 and PDK1 genes were measured using RT-qPCR. Gene expressions were normalized according to ACTB. *p<0.05; **p<0.01; ***p<0.001.

CHAPTER 4

DISCUSSION

Although the neurotransmitter NPY is classically known to be expressed in the CNS and PNS, it has now been shown to also play a considerable regulatory role in several peripheral tissues, including bone, BM, adipose tissue and the gastrointestinal system. Currently, six NPY receptors have been identified, and named Y1, Y2, Y3 (CXCR4), Y4, Y5, and Y6. These receptors belong to G protein-coupled receptor family and differences in their pattern of expression between tissues dictates the different effects of NPY on distinct tissues and organs.

NPY has been shown to play a regulatory role on the differentiation, proliferation, and survival of different types of neural stem cells. For example, NPY has proliferative and differentiative effect on hippocampal precursor cells, Subcallosal Zone (SCZ) Precursor Cells, Olfactory Neuronal Precursor Cells and Subventricular Zone (SVZ) Neural Precursor Cells. These effects however are largely mediated by interactions with the Y1 and Y2 receptors followed by ERK1/2 activation (Howell et al., 2005, Hansel et al., 2001, Agasse et al., 2008). In human ESCs, which were indicate to express both NPY itself, and two of its receptors (Y1 and Y5), NPY supported self-renewal and proliferation by activation of AKT and ERK1/2 signaling (Son et al.,2010).

The role of NPY in the regulation of hematopoiesis and the BM niche has thus far been investigated indirectly. It is well known that NPY modulates bone cells and bone homeostasis by interactions with the Y1, Y2 and Y5 receptors (Lee et al., 2010). Evidence for the protective role of NPY in the BM microenvironment largely came from studies assessing the effects of NPY in NPY knockout mice, which revealed that 1) NPY deficiency causes a significant BM dysfunction, and 2) transplantation of

healthy HSCs into NPY knockout mice display impaired engraftment (Park et al., 2015). However, it was thought that the effect of NPY on hematopoiesis was circumstantial and caused by a defective hematopoietic microenvironment, rather than a direct effect on the HSCs itself. Indeed, both NPY and its receptors have been presented to be expressed by a wide range of BM cells, including OBs, endothelial cells, macrophages and MSCs. Furthermore, NPY was shown to have proliferative, differentiation supportive and protective effects on the latter through activation of the canonical Wnt pathway (Lee et al., 2010).

Based on the literature, it is assumed that the NPY receptor was not expressed on HSCs. In this study, to assess the effects of NPY on HSCs and hematopoiesis, a series of co-culture experiments were carried out, in which HSCs and MSCs were co-cultured in medium supportive of HSC expansion in the presence or absence of NPY agonists and/or antagonists. Then, it was hypothesized that in order to investigate the effects of NPY, the hematopoietic environment was mimicked by co-culturing NPY receptive cells, i.e. NPY receptor expressing MSCs together with CD34+ HSCs. Any observed effects of NPY on HSCs were thought to be the result of changes in the gene expression and protein expression profile of MSCs, which would then affect nearby HSCs through cell-cell interactions or by changing their secretory profile.

In the initial co-culture experiments, we did not find significant differences after NPY treatment on quiescence or proliferation of HSCs. The most likely reasons for the absence of any effects may have been 1) insufficient concentrations of NPY tested (0,1 nM based on effects on MSCs, instead of the later determined dose of 300 nM in HSC-based WST tests); 2) duration of incubation in presence of NPY and its antagonist (2 vs 4 vs 7 days); and 3) the half-life of NPY in culture (NPY was only added once during the initiation of the culture, however, some studies have shown that the NPY half-life may be a short as 30 minutes *in vivo*) (Strand F. L., 1999).

In healthy individuals, plasma concentrations of NPY have been found to be in the range of 50 pM, whereas in pediatric leukemia patients levels of NPY were increased

and ranged from 50 to 385 pM (Kogner et al. 1992). When used in culture, recommended doses of NPY for maintenance of human embryonic stem cells (hESCs) ranged from 100-500 nM (Son et al. 2011). At these doses, NPY was able to support stemness of hESCs and keep the cells in an undifferentiated state, whereas hESCs at lower doses of NPY (<100 nM) showed spontaneous differentiation (Son et al. 2011). The effects of NPY on human umbilical vein endothelial cells (HUVEC) were shown to be biphasic and occur at doses between 0.1 – 10 pM and again at doses of 100 nM and higher (Zukowska-Grojec et al. 1998). At these doses NPY was shown to increase HUVEC attachment, proliferation and migration. Several groups have shown that NPY promotes proliferation, viability and osteogenic differentiation of MSCs at doses between 1 pM – 10 nM NPY (Liu et al. 2016; Lee et al. 2010; Wu et al. 2017; Igura et al. 2011). Thus, it appears that the effect of NPY is strongly dependent on the type of stem cell, the type of medium used and expression of NPY receptors by the target cells and in view of these data, the doses used in this study to assess the effects on HSCs appear to be in line with the literature.

The half-life of NPY has been shown to be as short as 20 minutes *in vitro* up to 5 days *in vivo*, depending on the presence and concentration of aminopeptidase P or the serine protease dipeptidyl-peptidase IV (DP-IV, CD26), which may through cleavage affect the function of NPY (Michel et al. 1998). In addition, the effects of neuropeptides may extent longer than their biological half-life due to binding to cell-membranes, which may protect them from proteolysis and allows extended activation of GPCR signaling (Strand 1999).

In addition, the HSC numbers obtained for microarray analysis after co-culture were fairly low, and despite different treatment regimens, the intergroup variability remained high, thus obscuring significant differences between groups. Therefore, to obtain significant results, these experiments need to be repeated with higher cell numbers. This can be done by upscaling the cultures (more cells, larger plates, more medium) and by increasing cell purity by using FACS sorting, rather than MACS separation.

The NPY-Y1 expression was detected at varying levels in all hematopoietic subgroups, including CD34+ HSCs. Previous studies showed that some of the NPY receptors (Y1, Y2 and Y5) were expressed by several mature hematopoietic and immune cell subsets, such as macrophages, T cells, natural killer (NK) cells, B cells, dendritic cells, monocytes and mast cells (Pettito et al., 1994; Kawamura et al., 1998; Farzi et al. 2015). Our findings are in agreement with these publications. NPY was shown to display immunological effects, such as NK cell activation, modification of the functions of macrophages and differentiation/adhesion of T-cells (Straub et al., 2000, Bedoui et al., 2003). NPY-Y1 receptor activation causes an anti-inflammatory effect on macrophages by stimulating the release of TGFβ through activation of the PI3K pathway (Zhou et al., 2008). NPY was also shown to interact directly with T-cells through binding to NPY-Y2 and activate β1 integrins that cause T-cell adhesion to the extracellular matrix (Levite, 2000).

However, after detection of high levels of NPY-Y1 expression on particularly HSCs, direct effects of NPY on HSCs were assessed without the confounding presence of MSCs. NPY was applied directly to HSCs and we assessed the presence of the other NPY receptors. Expression of the other NPY receptors Y2-Y5 by UCB-CD34+ cells was evaluated using flow cytometry, before cell culture and on days 4 and 7 of cell culture. Interestingly, NPY-Y1 expression was very high before culture and decreased rapidly on the 4th and 7th day of cell culture. Similarly, expression of NPY-Y2, NPY-Y4 and NPY-Y5, which was detected in different levels also decreased in time proportionally to the time of the cell culture. In contrast, expression of NPY-Y3 (or CXCR4), the most important receptor for the homing of HSCs, was shown to increase significantly over time in culture.

Whereas HSCs express the chemokine receptor CXCR4 (NPY-Y3), its ligand CXCL12 (SDF-1) is expressed on the cell surface of and secreted by BM stromal cells. CXCR4 has been shown to regulate quiescence, proliferation and most importantly migration and chemotaxis of HSCs (Wright et al., 2002, Kahn et al., 2004). CXCR4 signaling supports homing and quiescence of HSCs into BM. High doses of CXCL12

were shown to inhibit cell cycling of primitive hematopoietic cells through upregulation of the cell cycle inhibitor p57 (Nie et al., 2008). Since CXCR4 is not only the receptor for SDF-1, but also for NPY, we hypothesized that administration of NPY might affect quiescence of HSCs, since Park and colleagues showed an increase of 20% in the numbers of quiescent primitive hematopoietic cells in NPY deficient mice, without affecting motility, homing and differentiation capacity of these cells (Park et al., 2015).

NPY doses of 0-300 nM were applied.to the CD34+ cell cultures for 4 or 7 days to evaluate the effect of NPY on HSC proliferation. Whereas doses of 0-10 nM NPY did not affect proliferation of HSCs, higher concentrations (30, 100 and 300 nM) appeared to slightly suppress HSC proliferation, especially in the 7-day cell cultures. Suppression of proliferation of HSCs by 300 nM NPY was not caused by an increase in cellular apoptosis or loss of cell viability, but was directly translated into a significant increase in the fraction of cells in the G_0/G_1 phase of the cell cycle, and a relative decrease in the number of HSCs in the S and G2/M phases of the cell cycle. Therefore, this dose was chosen in further culture, RT-qPCR and RNA Seq experiments.

Using the DNBseq platform data from the RNAseq analysis were analysed. Interestingly, differences between the NPY-treated group and control samples were larger than differences between samples treated with or without NPY, resulting in clustering of separate controls and NPY-treated together. Previous data from microarrays and receptor expression studies already indicated a wide variation between groups, hence the cells were pooled from 3 different donors before use. Nevertheless, despite pooling differences between samples remained larger than the effect of NPY treatment. However, as a result of the wide interdonor variation, only two previously unknown genes were found to be differentially expressed between the NPY treated and control groups. Gene ontology classification suggested these genes to play a role in cellular metabolism, cellular and developmental processes, which is compatible with the hypothesis that NPY might play a role in control of cell cycling

and would confirm the data obtained from the separately performed RT-PCR, using the exact same samples. Nevertheless, the identification and function of these two genes should be pursued to gain a better understanding of their role in hematopoiesis. Also, different analysis programs and biostatistics will be used to reanalyze the RNAseq data.

Using RT-qPCR the gene expression levels of *FOXO3, DICER1, PCNA, SMARCA2*, and *PDK1* genes were assessed in HSCs cultured in the presence or absence of NPY. These genes were previously found to play a significant role in the regulation of stem cell proliferation and quiescence. PDK1 phosphorylates the catalytic domain of AGC family kinases, including PI3K-controlled serine kinases and plays a role in cell cycle progression and checkpoint control (Nakamura et al., 2008). PCNA plays a role in DNA replication and chromosome segregation and is usually downregulated in quiescent stem cells (DeMorrow et al., 2013). DICER1 is a miRNA processing factor that when upregulated triggers spontaneous activation of quiescence (Cheung and Rando, 2013). SMARCA2 encodes the probable global transcription activator SNF2L2, which plays a role in chromatin and nucleosome assembly and is upregulated during quiescence (Guerrero-Martínez and Reyes, 2018). FOXO3 is a transcription factor that functions as a trigger for apoptosis through upregulation of genes involved in the regulation of cell death, including *Bim* and *PUMA* or downregulation of anti-apoptotic proteins, such as FLIP (Zhang et al., 2011). All together these genes have all been shown to control entry and exit from the cell cycle through activation of different pathways. All genes were significantly up-regulated after NPY treatment, indicating that at high doses NPY may suppress proliferation and determine HSCs fate by promoting exit from the cell cycle. These effects appear to be dependent on dose and time. Interestingly, of all these genes also *PCNA* gene expression was found to be increased, whereas other studies have specifically found that during quiescence these genes were downregulated. In fact, increased expression of PCNA was previously found to be associated with S phase entry. In addition, *in vivo* use of NPY into rats was shown to decrease PCNA expression and induce cell cycle arrest in

cholangiocytes (DeMorrow et al., 2013). In contrast, *PCNA* expression levels in NPY-treated epithelial cells were increased, suggesting that NPY promotes cellular survival and proliferation of this type of cells (Jeppsson and Chandrasekharan, 2014). Thus, the effects of NPY on PCNA expression appear to be dose and tissue related, and thus may depend on the expression and distribution of the NPY receptors. Altogether these data support the idea that NPY regulates quiescence and fate of HSCs.

CHAPTER 5

CONCLUSION

NPY has been shown to play an important role in the CNS and PNS, but its role in the regulation of Hematopoietic Stem Cells has never been explored, even though NPY deficient mouse models indicated that the absence of NPY causes a significant BM impairment. Here, we aimed to explore the effect of NPY on the regulation of HSCs by performing a detailed analysis of the effects of NPY on HSC cell cycling and gene expression. It was found that expression of the NPY receptors Y1-Y5 was variable in HSCs and mature hematopoietic cell subsets, changed widely between donors and was not affected by the NPY treatment. However, culture of HSCs under serum-free conditions but in the presence of the hematopoietic growth factors SCF, Fl3-ligand and TPO resulted in a significant decrease in the expression of Y1, Y2, Y4 and Y5 in time, but a signficant increase in the cell surface expression of NPY-Y3 (CXCR4). The latter has important implications for the homing potential of HSCs. The presence of these receptors were found in HSCs for the first time. When the effect of NPY on HSC proliferation was evaluated, we observed that high concentrations of NPY (30, 100 and 300 nM) suppressed HSC proliferation in cell cultures, especially after 7 days. This suppression of proliferation was not associated with any cell toxicity or induction of apoptosis and in particular 300 nM NPY caused an important increase in the number of CD34+ HSCs in G_0/G_1 phase.

We then used this dose of NPY in 7 days HSC cultures to assess the effect of NPY on gene expression levels of *FOXO3, DICER1, PCNA, SMARCA2* and *PDK1,* which all have been shown to play an important role in the regulation of cell proliferation and quiescence. In line with data obtained from the cell cycle and proliferation studies, all genes were shown to be significantly upregulated after NPY treatment. In this study, data provide an indication that NPY plays a direct effect on the regulation of HSCs fate, by modulating their cell proliferation and quiescence.

Future Perspectives

- The effects of NPY on HSCs migration should be evaluated in transwell assays.
- RNASeq analyses should be re-performed and the results obtained should be confirmed by RT-PCR.
- Confirmation of activation of NPY receptors on HSCs by assessment of the signaling pathway
- Assessment of NPY levels in healthy and patients with impaired BM function

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APPENDICES

A. Buffers And Solutions

Turk's dye; 50 mg Cristal Violet, 495 mL distilled water, 5 mL Acetic Acid

PBN (PBS + 0,5% BSA, %0,05 NaAzide)

MACS Buffer; PBS, 0,5% BSA, 2 mM EDTA

1X Lysing Buffer ; 8.26 g Ammonium Chloride [Merck 1145], 1.0 g KHCO3 [Merck 4854], 0.037 g EDTA [Sigma ED2SS], 1 L MilliQ water)

B. Tables Of Materials For PCR

Table B. 1. 2X RT Master Mix.

Reagents	**Volume**
10x RT buffer	2,0 µL
25x dNTP (100 mM)	0,8 µL
10X RT Random Primers	2,0 µL
Transcriptase (50 U/µl)	1,0 µL
Nuclease-free H2O	4,2 µL

Table B. 2. Thermal cycling conditions.

Temperature	**Duration**
25ºC	10 min.
37ºC	120 min.
85ºC	5 min.
4ºC	∞

C. Positive Control For NPY Receptors Antibodies

Table C. 1. Positive control for NPY receptors antibodies on the neuroblastoma cell line SH-SY5Y, the cervical cancer line HeLa and the human embryonic kidney cell line HEK-293T.

	NPY-Y1	**NPY-Y2**	**NPY-Y3**	**NPY-Y4**	**NPY-Y5**
SH-SY5Y	3.9	26.8	56.2	11.5	0.1
HeLa	71.1	0.4	34.6	1.3	0.5
HEK-293T	23.0	3.4	67.7	0.1	0.0

D. Immunofluorescent Staining Of Freshly Isolated Cd34+ UCB Cells

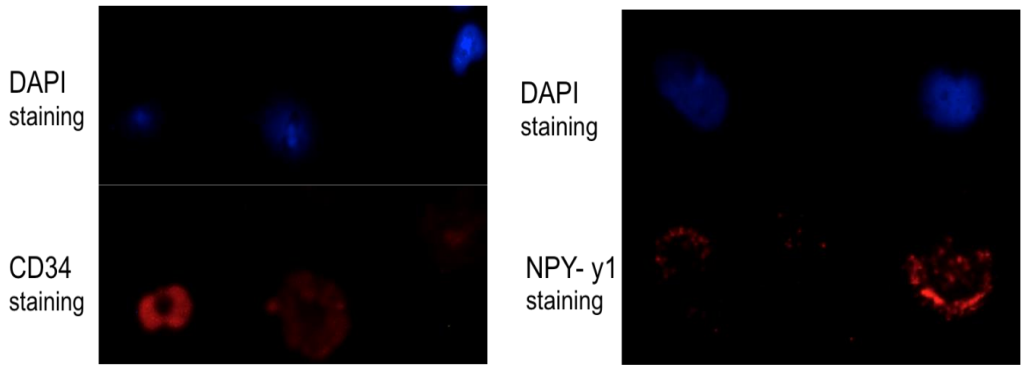


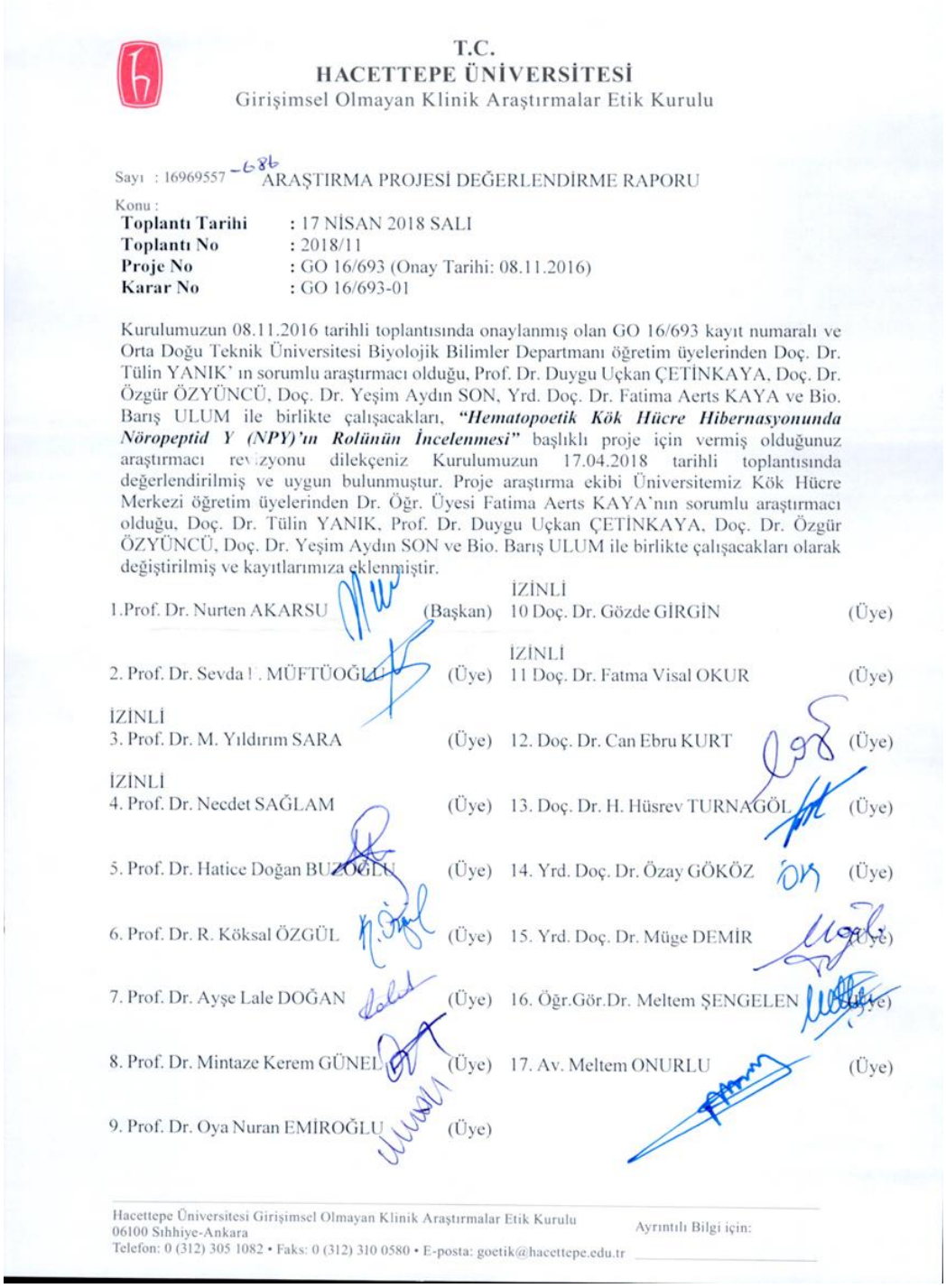
Figure D. 1. Immunofluorescence images of CD34+ HSCs stained with anti-NPY-Y1. CD34+ HSCs from the same sample were spun onto different slides and stained with antibodies against CD34 and NPY-Y1. Unfortunately, since the secondary antibodies used for staining of CD34 and NPY-Y1 were both PE-labeled, overlap of these slides was not possible. Shown are therefore slides stained separately with anti-CD34 antibody (left) and anti-NPY-Y1 antibody (right). Nuclei were counterstained with DAPI.

E. Quality control (QC) of RNA

Table E. 1. Quality control (QC) of RNA

No.	Sample Name	Sample Number	Concen-tration (ng/μL)	Volume (μL)	Total Mass (μg)	RIN	28S/ 18S	Library Type	Test Result
1	1. CD34(+) Control	8521904 -002261	61	12	0.73	10	2.3	BGISEQ-500 Transcriptome	Qualified
2	1. CD34(+) NPY 300 nM	8521904 -002262	68	12	0.82	10	2.4	BGISEQ-500 Transcriptome	Qualified
3	2. CD34(+) Control	8521904 -002263	67	12	0.8	10	2.2	BGISEQ-500 Transcriptome	Qualified
4	2. CD34(+) NPY 300 nM	8521904 -002264	73	12	0.88	10	2.2	BGISEQ-500 Transcriptome	Qualified
5	3. CD34(+) Control	8521904 -002265	74	12	0.89	10	2.1	BGISEQ-500 Transcriptome	Qualified
6	3. CD34(+) NPY 300 nM	8521904 -002266	44	12	0.53	9.9	2.2	BGISEQ-500 Transcriptome	Qualified

F. Ethical Approval



T.C.
HACETTEPE ÜNİVERSİTESİ
Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16969557-686 ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Konu :
Toplantı Tarihi : 17 NİSAN 2018 SALI
Toplantı No : 2018/11
Proje No : GO 16/693 (Onay Tarihi: 08.11.2016)
Karar No : GO 16/693-01

Kurulumuzun 08.11.2016 tarihli toplantısında onaylanmış olan GO 16/693 kayıt numaralı ve Orta Doğu Teknik Üniversitesi Biyolojik Bilimler Departmanı öğretim üyelerinden Doç. Dr. Tülin YANIK' ın sorumlu araştırmacı olduğu, Prof. Dr. Duygu Uçkan ÇETİNKAYA, Doç. Dr. Özgür ÖZYÜNCÜ, Doç. Dr. Yeşim Aydın SON, Yrd. Doç. Dr. Fatima Aerts KAYA ve Bio. Barış ULUM ile birlikte çalışacakları, ***"Hematopoetik Kök Hücre Hibernasyonunda Nöropeptid Y (NPY)'ın Rolünün İncelenmesi"*** başlıklı proje için vermiş olduğunuz araştırmacı revizyonu dilekçeniz Kurulumuzun 17.04.2018 tarihli toplantısında değerlendirilmiş ve uygun bulunmuştur. Proje araştırma ekibi Üniversitemiz Kök Hücre Merkezi öğretim üyelerinden Dr. Öğr. Üyesi Fatima Aerts KAYA'nın sorumlu araştırmacı olduğu, Doç. Dr. Tülin YANIK, Prof. Dr. Duygu Uçkan ÇETİNKAYA, Doç. Dr. Özgür ÖZYÜNCÜ, Doç. Dr. Yeşim Aydın SON ve Bio. Barış ULUM ile birlikte çalışacakları olarak değiştirilmiş ve kayıtlarımıza eklenmiştir.

1.Prof. Dr. Nurten AKARSU	(Başkan)	İZİNLİ 10 Doç. Dr. Gözde GİRGİN	(Üye)
2. Prof. Dr. Sevda [illegible]. MÜFTÜOĞLU	(Üye)	İZİNLİ 11 Doç. Dr. Fatma Visal OKUR	(Üye)
İZİNLİ 3. Prof. Dr. M. Yıldırım SARA	(Üye)	12. Doç. Dr. Can Ebru KURT	(Üye)
İZİNLİ 4. Prof. Dr. Necdet SAĞLAM	(Üye)	13. Doç. Dr. H. Hüsrev TURNAGÖL	(Üye)
5. Prof. Dr. Hatice Doğan BUZOĞLU	(Üye)	14. Yrd. Doç. Dr. Özay GÖKÖZ	(Üye)
6. Prof. Dr. R. Köksal ÖZGÜL	(Üye)	15. Yrd. Doç. Dr. Müge DEMİR	(Üye)
7. Prof. Dr. Ayşe Lale DOĞAN	(Üye)	16. Öğr.Gör.Dr. Meltem ŞENGELEN	(Üye)
8. Prof. Dr. Mintaze Kerem GÜNEL	(Üye)	17. Av. Meltem ONURLU	(Üye)
9. Prof. Dr. Oya Nuran EMİROĞLU	(Üye)		

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Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16969557 – 521
Konu : ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 06 MART 2018 SALI
Toplantı No : 2018/07
Proje No : GO 18/133 (Onay Tarihi: 13.02.2018)
Karar No : GO 18/133- 52

Kurulumuzun 13.02.2018 tarihli toplantısında GO 18/133 kayıt numarası ile onaylanmış olan Üniversitemiz Kök Hücre Araştırma ve Uygulama Merkezi öğretim üyelerinden Yrd. Doç. Dr. Fatima Aerts KAYA' nın sorumlu araştırmacı olduğu, Prof. Dr. Duygu Uçkan ÇETİNKAYA, Doç. Dr. Özgür ÖZYÜNCÜ, Doç. Dr. Yeşim Aydın SON ve Bio. Barış ULUM ile birlikte çalışacakları, GO 18/133 kayıt numaralı, ***"Hematopoetik Kök Hücre Regülasyonunda Nöropeptid Y (NPY)' nin Rolünün İncelenmesi"*** başlıklı projeye "***Nöropeptid Y (NPY) Reseptörlerinin Varlığının Ve Fonksiyonunun Araştırılması***" iş paketinin eklenmesi araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş olup, etik açıdan uygun bulunmuştur.

1.Prof. Dr. Nurten AKARSU	(Başkan)	10 Doç. Dr. Gözde GİRGİN	(Üye)
2. Prof. Dr. Sevda F. MÜFTÜOĞLU	(Üye)	11 Doç. Dr. Fatma Visal OKUR	(Üye)
3. Prof. Dr. M. Yıldırım SARA	(Üye)	12. Doç. Dr. Can Ebru KURT	(Üye)
4. Prof. Dr. Necdet SAĞLAM	(Üye)	13. Doç. Dr. H. Hüsrev TURNAGÖL	(Üye)
5. Prof. Dr. Hatice Doğan BUZOĞLU	(Üye)	14. Yrd. Doç. Dr. Özay GÖKÖZ	(Üye)
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Sayı : 16969557 – 316
Konu :

ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 13 ŞUBAT 2018 SALI
Toplantı No : 2018/05
Proje No : GO 18/133 (Değerlendirme Tarihi: 06.02.2018)
Karar No : GO 18/133- 05

Üniversitemiz Kök Hücre Araştırma ve Uygulama Merkezi öğretim üyelerinden Yrd. Doç. Dr. Fatima Aerts KAYA' nın sorumlu araştırmacı olduğu, Prof. Dr. Duygu Uçkan ÇETİNKAYA, Doç. Dr. Özgür ÖZYÜNCÜ, Doç. Dr. Yeşim Aydın SON ve Bio. Barış ULUM ile birlikte çalışacakları, GO 18/133 kayıt numaralı, ***"Hematopoetik Kök Hücre Regülasyonunda Nöropeptid Y (NPY)' nin Rolünün İncelenmesi"*** başlıklı proje önerisi araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş olup, etik açıdan uygun bulunmuştur.

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BSs	Hacettepe University	2003
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Year	Place	Enrollment
2008-Present	Hacettepe University	Biologist

FOREIGN LANGUAGES

English

PUBLICATIONS

1. **Baris Ulum**, Hikmet Taner Teker, Aysun Sarikaya, Gunay Balta, Baris Kuskonmaz, Duygu Uckan-Cetinkaya, Fatima Aerts-Kaya. Bone Marrow Mesenchymal Stem Cells from Donors with a High BMI Display ER Stress and Are Functionally Impaired. Journal of Cellular Physiology, 2018:1-8. DOI: https://doi.org/10.1002/jcp.26804

2. Fatima Aerts-Kaya, **Baris Ulum**, Aynura Mammadova, Sevil Köse, Gözde Aydın, Petek Korkusuz, Duygu Uçkan-Çetinkaya. Neurological regulation of

the bone marrow niche. Adv Exp Med Biol. 2019. DOI: https://doi.org/10.1007/5584_2019_398

3. **Baris Ulum**, Aynura Mammadova, Özgür Özyüncü, Duygu Uçkan Çetinkaya, Tülin Yanık, Fatima Aerts-Kaya. Neuropeptide Y is involved in the regulation of quiescence of Hematopoietic Stem Cells. Submitted to Neuropeptides.

Abstracts and posters:

1. **Baris Ulum**, Fatima Aerts-Kaya, Hikmet Taner Teker, Günay Balta, Duygu Uckan-Cetinkaya. Chemical inducers of Endoplasmic Reticulum (ER) Stress suppress proliferation and adipogenic differentiation of human Mesenchymal Stem Cells. Abstract EBMT15-ABS-1164. EBMT 2015. Poster P054. Bone Marrow Transplant 2015(50), Supplement 1, S145.

2. **Baris Ulum**, Fatima Aerts-Kaya, Hikmet Taner Teker, Günay Balta, Duygu Uckan-Cetinkaya. The Effect of Endoplasmic Reticulum (ER) Stress on Human Bone Marrow Derived Mesenchymal Stem Cells. Abstract ISSCR 2015. Poster #1582.

3. Aysun Sarikaya, Taner Teker, **Baris Ulum**, Duygu Cetinkaya, Fatima Aerts-Kaya. Endoplasmic Reticulum Stress During Adipogenic Differentiation of Mesenchymal Stem Cells. Abstract ICSCCT 2015, Antalya, Turkey. Oral Presentation.

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HOBBIES

Tennis, Movies, Motor Sports