# INHIBITION OF MEIS1 ALLOWS EXPANSION OF HEMATOPOIETIC STEM CELLS

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INHIBITION OF MEIS1 ALLOWS EXPANSION OF HEMATOPOIETIC STEM CELLS

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# ABSTRACT


## INHIBITION OF MEIS1 ALLOWS EXPANSION OF HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells (HSCs) are characterized by high capacity of self-renewing and differentiation to all blood lineages. HSCs have been generally used in HSC transplantation (HSCT) to treat the hematological diseases due to their self-renew and differentiation capacities. There are *ex vivo* studies carried out to expand HSC to improve HSCTs. We have been previosly shown that deletion of HSC quiescence related gene Meis1 induces the expansion of HSCs. Targeting Meis1 levels in HSCs appears as a plausible therapeutical target on stem cell expansion technologies. We aimed the development of HSC technologies through inhibition of Meis1 in this study. Therefore, we employed *in silico* and *in vitro* strategies. *In silico* screening of over a million small molecules against crystalised Meis1 homeodomain was carried out by Autodock Vina – PaDel Adv platform. *In vitro* activity of hits were analyzed with Meis1 luciferase reporter assays, gene target analysis by RT-PCR and flow cytometric profiling of HSCs *ex vivo* and in vivo. We have shown that inhibition of Meis1 leads to downregulation of Meis1 target genes, downregulation of CDKIs, and expansion of murine and human HSCs. This findings suggest that inhibition of Meis1 could provide important contributions to stem cell expansion technologies needed on stem cell transplantation and decrease dependency to the cytokines used for stem cell expansion.

# ÖZET


## MEIS1 İNHİBİSYONU İLE HEMATOPOİETİK KÖK HÜCRELERİN ÇOĞALTILMASININ SAĞLANMASI

HKH'lerin en belirleyici özellikleri kendini yenileme ve birden çok hücre tipine farklılaşabilme yetenekleridir. Bu yetenekleri sayesinde HKH'ler tüm kan hücresi tiplerine dönüşebilirken, aynı zamanda vücudun gelecekteki hematopoetik aktivitesi için gerekli olan rejenerasyon kapasitesini de korumuş olurlar. HKH'ler, kendini yenileme yetenekleri sayesinde hematolojik rahatsızlıkların tedavisi için HKH nakillerinde sıklıkla kullanılmaktadır. Bu nedenle bu gibi tedavi edici uygulamalar için *ex vivo* HKH çoğaltılmasına yönelik çalışmalara ihtiyaç duyulmaktadır. Daha önceki çalışmalarımızda Meis1 HKH suskunluk geninin farelerdeki delesyonunun HKH çoğalmasına yol açtığı gösterilmiştir. Meis1 geninin inhibisyonu kök hücre çoğaltılması teknolojilerinde terapötik bir hedef olarak karşımıza çıkmaktadır. Biz bu çalışmada Meis1 inihibisyonu ile birlikte HKH ekspansiyon teknolojileri geliştirmeyi hedefledik. Bu sebeple *in siliko* ve *in vitro* yaklaşımlar geliştirilmiştis. *İn siliko* çalışmalarda, Autodock Vina – PaDel ADV platformu ile kristalize Meis 1 homodomaine karşı bir milyondan fazla küçük molekül incelenmiştir. Bunu takiben, Meis1 lusiferaz raportör deneyleri, gerçek zamanlı PZR ile hedef gen analizleri , akış sitometrisi ile *ex vivo* ve *in vivo* HKH karakterizasyonu gerçekleştirilmiştir. Meis1 inhibisyonunun Meis1 hedef genlerin ve sikline bağımlı kinaz inhibitörlerinin ekspresyonlarını azalttığını ve hem fare hem de insan HKH'lerinin expansiyonunu tetiklediğini belirledik. Bu bulgular, Meis1 inhibisyonunun *ex vivo* HKH ekspansiyon teknolojilerinin geliştirilmesine önemli katkıları olacağını ve sitokinlere bağımlılığın azaltılabileceğini göstermektedir.

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# LIST OF SYMBOLS/ABBREVIATIONS

| | |
|---|---|
| 2D | Two-Dimensional |
| 3D | Three-Dimensional |
| °C | Celcius degree |
| ΔΔC | The Difference Between The Two CT Values |
| Å | Angstrom |
| | |
| µM | Micro Molar |
| Min | Minutes |
| ml | Mililiter |
| rpm | Revolutions per minutes |
| pdbqt | Protein Data Bank, Partial Charge (Q), & Atom Type (T)) format |
| v/v | Volume per Volume |
| | |
| AA | Amino Acids |
| AD-MSC | Adipose Derived Mesenchymal Stem Cell |
| ALL | Acute Lymphoid Leukemia |
| AML | Acute Myeloid Leukemia |
| APbb1 | Amyloid Beta Precursor Protein Binding Family B Member 1 |
| Blastp | Protein–Protein BLAST |
| BM | Bone Marrow |
| CAKs | CDK Activating Kinases |
| CCND2 | Cycline D2 |
| CDKIs | CDK Inhibitors |
| CDKs | Cyclin-Dependent Kinases |
| CHEK1 | Checkpoint Kinase 1 |
| $CO_2$ | Carbon di oxide |
| COBALT | Constraint Based Multiple Alignment Tool |
| CPU | Center Processing Unit |
| CT | Crossing Threshold |

| | |
|---|---|
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| DPBS | Dulbecco’s Phosphate Buffer Saline |
| FBS | Fetal Bovine Serum |
| Glut1 | Glucose Transporter 1 |
| Gpr132 | G Protein- Copuled Receptor 132 |
| HEK293 | Human Embryonic Kidney Cell line 293 |
| HIFs | Hypoxia Inducible Factor |
| Hif-1α | Hypoxia Inducible Factor 1 Alpha Subunit |
| Hif-1β | Hypoxia Inducible Factor 1 Beta Subunit |
| HLA | Human Leukocyte Antigen |
| Hoxa9 | Homeobox A9 |
| HSCs | Hematopoietc Stem Cells |
| HSPCs | Hematopoietic Stem And Progenitor Cells |
| HUVECs | Human Umbilical Vein Endothelial Cells |
| Ink4 | Inhibitors Of CDK4 |
| IL3 | Interleukin 3 |
| IL6 | Interleukin 6 |
| IL11 | Interleukin 11 |
| IRX1 | Iroquois Homeobox 1 |
| KO | Knock Out |
| K562 | Chronic Myelogenous Leukemia |
| L929 | Mouse Fibroblast Cells |
| LDHA | Lactate Dehydrogenase A |
| Lin- | Lineage negative cells |
| LSC | Leukemia Stem Cell |
| LSK | Lin-Sca1+Ckit+ cell population |
| LSKCD34low | Native HSCs |
| LT-HSC | Long-Term Hematopoietic Stem Cell |
| MCM3 | Minichromosome Maintenance Complex Component 3 |
| Meis1 | Myeloid Ecotrophic Insertion Site 1 |
| Meis1-/- | Meis1 Knockout |
| Meis2 | Myeloid Ecotrophic Insertion Site 2 |
| Meis3 | Myeloid Ecotrophic Insertion Site 3 |

| | |
|---|---|
| MEIS3P1 | Meis Homeobox 3 Pseudogene 1 |
| MEIS3P2 | Meis homeobox 3 pseudogene 2 |
| MSC | Mesechymal Stem Cells |
| MP | Mitochondrial Potential |
| NAC | N-acetyl-L-cysteine |
| OD | The Optical Density |
| P1 | Postnatal Day 1 |
| P7 | Postnatal Day 7 |
| PBX1 | Pre-B-Cell Leukemia Transcription Factor 1 |
| PBX2 | Pre-B-Cell Leukemia Homeobox 2 |
| PBX3 | PBX homeobox 3 |
| PBX4 | PBX homeobox 4 |
| PGK | Phosphoglycerate Kinase 1 |
| PMBCs | Peripheral Mononuclear Blood Cells |
| PKNOX1 | PBX/knotted 1 homeobox |
| PKNOX2 | PBX/knotted 1 homeobox 2 |
| PI | Propidium Iodide |
| PSA | Penicillin Streptomycin Amphotericin |
| Rb | Retinoblastoma Family Proteins |
| ROS | Reactive Oxygen Species |
| RPMI | RPMI Roswell Park Memorial Institute |
| SCF | Stem Cell Factor |
| SDF | The Appropriate Formats |
| TAD | The Transcriptional Activation Domain |
| TALE | The 3-Amino-Acid Loop Extension Class |
| TGIF1 | Transforming Growth Factor-Beta-Induced Factor 1 |
| TGIF2 | TGFB induced factor homeobox 2 |
| TGIF2LX | TGFB induced factor homeobox 2 like, X-linked |
| TGIF2LY | TGFB induced factor homeobox 2 like, Y-linked |
| TPI | Turkish Patent Institue |
| TP53 | Tumor Protein P53 |
| TPO | Thrombopoietin |
| U937 | Human Lymphocyte Cells |

| | |
|---|---|
| UCB | Umbilical Cord Blood |
| VEGF | Vascular Endothelial Growth Factor |
| WBM | Whole Bone Marrow |
| WST-1 | Water Soluble Tetrazolium Salts |

# 1. INTRODUCTION

## 1.1. MYELOID ECOTROPHIC INSERTION SITE 1 (MEIS1)

Meis1, myeloid ecotrophic insertion site 1, was discovered in a BXH-2 mouse leukemia model as a common viral integration site [1]. Meis1 is a member of the 3-amino-acid loop extension (TALE) class of transcription factors that activate their target genes by associating with Hox transcription factors [2]. Through its binding domains in the N terminus, Meis1 interacts with its cofactors such as the transcription factors Pbx1 and Hoxa9 [3, 4]. Meis2 and Meis3 are the two other proteins that belong to the same family (Figure 1.1). All of the Meis family members were identified by sequence similarity [5] Meis2 and Meis3 protein sequences demonstrate a high degree of similarity with Meis1 (eighty three percent and sixty six percent, respectively) [6].

Meis1 function has been studied in various types of tissues. In retinal progenitor cells [7] and olfactory epithelial cells [8], Meis1 was shown to sustain the cells in an undifferentiated state. Meis1 is highly expressed in thymic epithelial cells with immature progenitor phenotype [9]. Meis1 plays a critical role in homeostasis by maintaining the epidermal stem cells in the bulge region of the epidermis. Meis1 is highly expressed in the bulge region of the hair follicle, which is one of the stem cell niches. The number of these adult stem cells is decreased by the disruption of Meis1 in the mouse epidermal tissue [10]. Interestingly, epidermal disruption of Meis1 is involved in tumor development and malignant conversion, suggesting a multifunctional model for Meis1 in the epidermis [10].

In addition, Meis1 has been shown to be involved in tumorigenesis of neuroblastomas, nephroblastomas, ovarian carcinomas, prostate cancer, nonsmall-cell lung adenocarcinoma, and leukemia [11-16]. Although correlations have been made regarding Meis1 and tumorigenesis, the molecular mechanism behind it remains undetermined.

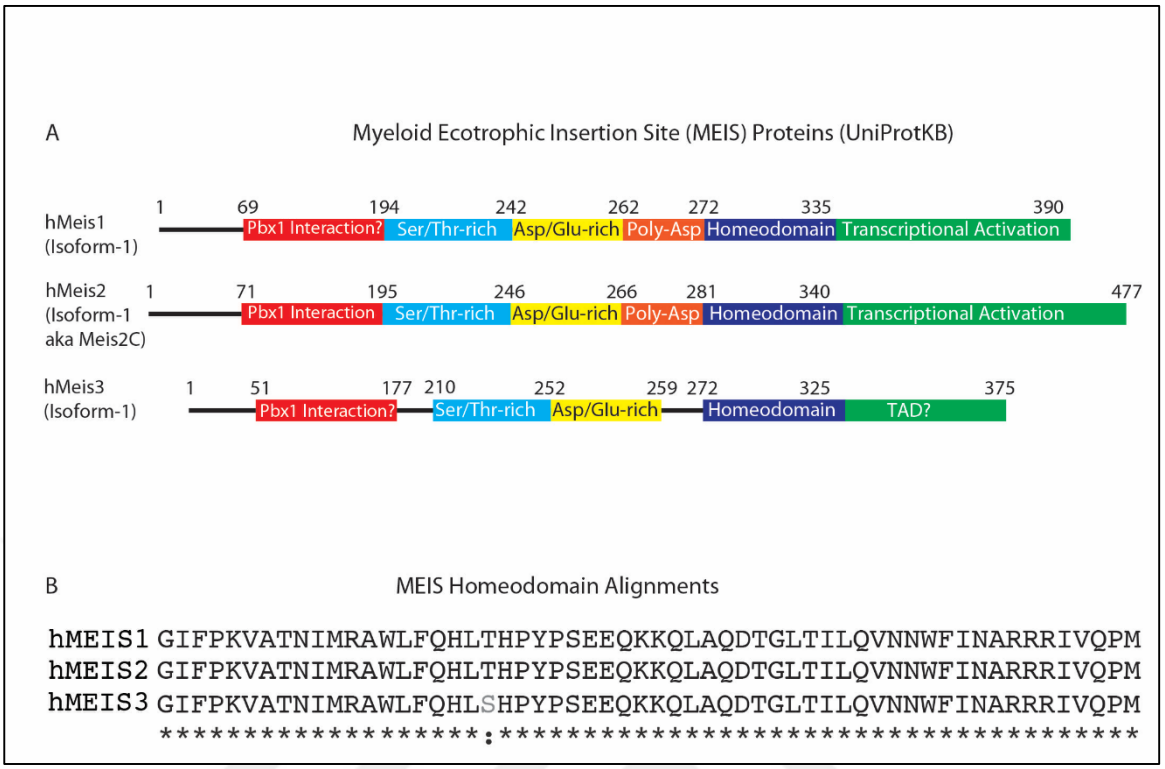


Figure 1.1. Domain structure of MEIS proteins: Meis1, Meis2, and Meis3 are members of the TALE homeodomain transcription factors. They distinctly pose cofactor interaction domains, Ser/Thr- and Asp/Glu-rich domains. Meis1 and Meis2 contain a poly-Asp region followed by a highly conserved Homeobox region. MEIS proteins largely differ at their C terminus where the transcriptional activation domain (TAD) is located

Meis1 is greatly expressed in primitive hematopoietic populations while its expression decreases throughout differentiation [3, 17]. Meis1 knockout ($Meis1^{-/-}$) mice die around embryonic day 11.5–14.5 by virtue of defects in hematopoiesis and vascularization [18-20]. In the liver of $Meis1^{-/-}$ mice embryos, hematopoietc stem cell niches are severely underdeveloped and the number of colony-forming cells is significantly low, highlighting a critical function of Meis1 in hematopoietic stem cells (HSCs) regulation [18-20].

Recent studies showed that inducible Meis1 deletion in HSCs leads to the loss of hematopoietc stem cell (HSC) quiescence, followed by robust HSC expansion and exhaustion *in vivo* [21]. Serial transplantation of Meis1 knockout HSCs consequently led to bone marrow (BM) failure, demonstrating the requirement of Meis1 in HSC function [4, 21]. Meis1 is also an important regulator of the redox state of HSCs. Loss of Meis1 accompanies downregulation of hypoxia inducible factor 1 alpha subunit (Hif-1α) and hypoxia inducible factor 2 alpha subunit (Hif-2α), two major transcriptional regulators of hypoxic response in HSCs, which leads to elevated reactive oxygen species (ROS) generation and apoptosis of HSCs. Manipulation of ROS levels in Meis1 knockout HSCs by use of antioxidants recovers ROS-related BM failure and loss of HSC function [21].

Apart from the role of Meis1 in the regulation of HSC quiescence, it was shown as a transcriptional regulator of neonatal heart regeneration. In mice, Meis1 expression is gradually upregulated from postnatal day 1 (P1) to P7. In contrast to this upregulation, the regenerative capacity of the heart is diminished. We have demonstrated that Meis1 regulates cell cycle arrest in cardiomyocytes. Moreover, conditional deletion of Meis1 in the adult heart results in reactivation of cardiomyocyte proliferation. Recent studies showed that Meis1 is an integral part of the transcriptional network that regulates the cardiomyocyte cell cycle, HSC maintenance, and cellular metabolism. These findings suggest that Meis1 could be a potential therapeutic target for various conditions including alteration of cancer metabolism, targeting of cancer stem cells, HSC expansion, and cardiac regeneration. [22].

## 1.2. REGULATION OF HEMATOPOIETIC STEM CELL METABOLISM BY MEIS1

HSCs are recognized with their high differentiation and self-renewing capacity, as well as their responsibility for generating blood cells throughout life. Considering the hypoxic environment, HSCs generally reside in endosteal regions of the BM which is the zone responsible for vascular organization with partitioning of capillaries that constitute the arterioles [23-25]. The endosteal niches attest to higher glycolytic flux and their partial pressure of oxygen is very low, which results in limited BM perfusion where the blood attains access into sinusoids through the capillaries [26]. In addition, due to the involvement of osteoblasts as a supportive cell type, they can provide persistence of hematopoiesis [23, 24]. Considering all these properties, especially low oxygen tension, the endosteal region is thought to represent the hypoxic niche of HSCs.

Stem cells generally reside in a quiescent state because of their metabolic adaptation to the hypoxic niche [27]. Even though the importance of hypoxic conditions was known for several stem cells, there was not enough knowledge about the metabolic phenotype and its regulatory pathways or how metabolism relates to the cell cycle of stem cells [28, 29]. Recent studies indicate that the cell cycle of HSCs can be regulated transcriptionally and metabolically by involvement of Meis1 [21, 30]. Moreover HSCs prefer anaerobic glycolysis instead of mitochondrial respiration in order to generate energy with a lower rate of metabolism in a quiescent state (reviewed by Uckac et al., 2014). Thus, their unique glycolytic metabolic phenotype allows tolerance to low oxygen tension and minimizes oxidative damage. In addition, murine HSCs and human hematopoietic progenitor and stem cells (HSPCs) are characterized by low mitochondrial potential (MP) [21]. Two major transcriptional regulators, Hypoxia Inducible Factor 1 Alpha Subunit (Hif-1α) and Meis1, are involved in the low MP profile, hypoxic response, and glycolytic metabolism of HSCs [21, 31].

Hif-1 is an essential regulatory transcription factor for cellular and systemic responses in hypoxic conditions. Hif-1, when stabilized, causes a shift from mitochondrial oxidative phosphorylation to glycolysis [32-34]. It is a heterodimeric protein that is composed of two subunits, Hif-1α and Hypoxia Inducible Factor 1 Beta Subunit (Hif-1β ). Hif-1α is known as an oxygen-regulated subunit that can control the main Hif-1 function and forms a complex with constitutively expressed Hif-1β [31]. Hif-1α has a key role in maintaining quiescence of HSCs and stress resistance [26], as well as in stabilization of protein [32-34] or transcriptional activation [35-38].

Meis1 is a transcription factor involved in the regulation of HSC metabolism through the optimal expression of Hif-1α [30]. Meis1 and its cofactors Pre-B-Cell Leukemia Transcription Factor (Pbx1) and HoxA9 cooperatively play a role in Hif-1α activation through conserved and corresponding Meis1, Pbx1, and HoxA9 binding sites in the Hif-1α gene [39]. Recent studies prooved that Meis1 plays a major role in anaerobic glycolysis in the hypoxic niche of HSCs by positively regulating expression of not only Hif-1α but also Hif-2α [21, 31]. Meis1 deletion in HSCs downregulates expression of both Hif-1α and Hif-2α. The role of Hif-1α and Meis1 in anaerobic metabolism was shown by conditional and tissue-specific deletion of Hif-1α and Meis1 in HSCs *in vivo.* Takubo et al [40]. showed that conditional deletion of Hif-1α in BM initiates loss of HSC quiescence, increases HSC cycling, and induces loss of long-term repopulation. Recently demonstrated that the shifting from cytoplasmic glycolysis to mitochondrial oxidative phosphorylation can be triggered by deletion of Hif-1α or Meis1 [21, 31]. As a result, a lower rate of glycolysis and increased mitochondrial activity occur, which leads to an increase in reactive oxygen species (ROS) and loss of HSC quiescence.

As discussed above, murine HSCs utilize glycolysis rather than mitochondrial oxidative phosphorylation as their primary energy source, but what human hematopoietic stem and progenitor cells (HSPCs) use remained unknown until recently. Recently reported that human HSPCs have similar metabolic characteristics as shown by high levels of glycolysis and low levels of oxygen consumption [39]. In addition, repopulation ability of human low MP cells or HSPCs is markedly better than that of high MP cells [39]. Moreover, Hif-1α expression is markedly high in human HSPCs. Similar to murine counterparts, Meis1 has a key role in the regulation of human HSPCs through regulation of Hif-1α together with its cofactors Pbx1 and Hoxa9 [39].

The role of Meis1 is not only limited to the regulation of HSC metabolism; it is also responsible for the regulation of oxidative stress through transcriptional regulation of Hif-2α [21, 31]. Hif-1α and Hif-2α generally have discrete targets even though they are extremely homologous and may also target similar genes such as Glut1 (glucose transporter 1) and VEGF (vascular endothelial growth factor). A well-defined example of having different targets is that while glycolytic enzymes such PGK (phosphoglycerate kinase 1) and LDHA (lactate dehydrogenase A) are exclusively stimulated by Hif-1α, expressions of antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxide are regulated by Hif-2α [41, 42].

Increased ROS, multiorgan dysfunction, cardiac hypertrophy, hepatic steatosis, and defects in spermatogenesis and hematopoiesis are the main challenges that Hif-2α knockout mice face [41, 42]. During mitochondrial oxidative phosphorylation, generation of ROS occurs. High levels of ROS cause senescence or apoptosis in HSCs. Thus, increase in ROS generation in Meis1 knockout HSCs is associated with downregulation of Hif-2α in HSCs (Figure 1.2) [21]. Moreover, treatment with N-acetyl-L-cysteine (NAC) a ROS scavenger, was efficiently used to compensate oxidative damage in Meis1 knockout HSCs [21].

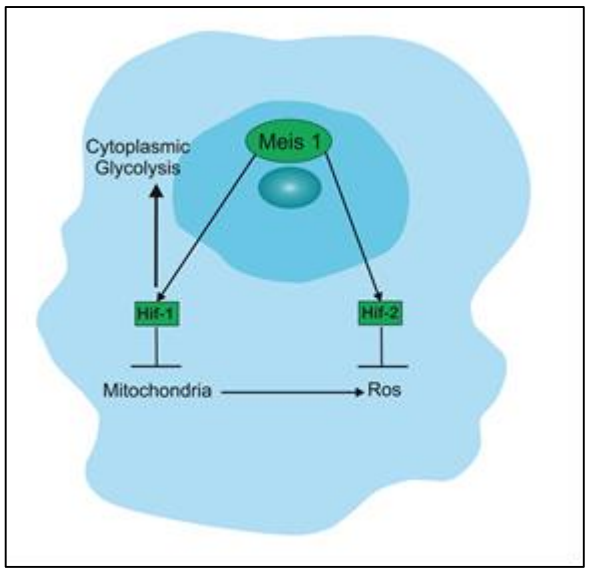


Figure 1.2. Regulation of cellular metabolism and oxidative stress through transcriptional regulation of Hif-1α and Hif-2α in HSCs.

## 1.3. INVOLVEMENT OF MEIS1 IN CARCINOGENESIS, CANCER STEM CELL ESTABLISHMENT AND METABOLISM

Cell cycle regulation is one of the most significant areas of research with implications for regenerative biology and treatment of cancer [43-45]. Dysregulation of cell cycle checkpoints results in genomic and chromosomal instability that contributes to carcinogenesis [46, 47]. Cancer cells metabolize glucose at an elevated level compared to normal cells in a lack of oxygen, which is known as the Warburg effect [48]. Anaerobic glycolysis allows survival of cancer cells in hypoxic microenvironments of tumors [49]. Even though there is a relationship between metabolic adaptation and upregulation of Meis1 in various malignant cells, functions of Meis1 and its cofactors remain to be discovered in the process of carcinogenesis, cancer stem cell establishment, and involvement in cancer metabolism.

Involvement of Meis1 in cancer was first shown by a study of myeloid leukemia cells of acute myeloid leukemia (AML) patients [11, 50-52]. Increased expression of Meis1 was detected in the BM of acute lymphoid leukemia (ALL) and AML patients (Table 1.1) [18, 53]. Overexpression of Meis1 accelerates the onset of the disease in murine AML models [54, 55]. Meis1 associates with Hox proteins in leukemic transformation [3, 11]. Hoxa9 along with Meis1 remarkably accelerate leukemogenesis [11]. In addition, Meis1 is upregulated in neuroblastomas [12-14] nephroblastomas [15], and ovarian carcinomas [16]. Furthermore, a recent study on epidermis showed that Meis1 is involved in the development of papilloma and formation of skin tumors [10].

Wong et al.showed that Meis1 is involved in cancer stem cell function as shown in leukemia stem cell (LSC) establishment, LSC frequency through regulating cancer stem cell self-renewal, induction of differentiation arrest, and establishment of *in vivo* LSC generation from hematopoietic progenitors [52]. Intriguingly, Meis1 expression is reduced in prostate cancer, which suggests the involvement of Meis1 in prostate cancer development [56]. The reason why Meis1 is differentially expressed in different types of tumors is not known yet.

However, it suggests that Meis1 is involved with tumorigenesis of different types of tumors through different mechanisms. Moreover, Meis1 represses nonsmall-cell lung adenocarcinoma cancer cell proliferation [57]. This situation is similar to the results of our study on hematopoietic stem cells, in which the expression of Meis1 promoted HSCs to stay in a quiescence state [21], and overexpression of Meis1 also reduces neonatal and adult cardiomyocyte proliferation [22]. All these findings suggest that Meis1 has distinct functions in the proliferation of a cell. This could be dependent on the presence of cofactors and on the type of metabolism needed. For instance, we believe that glycolytic cancer cell metabolism might be triggered by increased expression of Meis1 in cancer, which in turn upregulates cytoplasmic glycolysis through Hif-1α and downregulates oxidative damage through Hif-2α.

Environmental and intrinsic stem cell factors influence stem cell fate, function, and metabolism [58]. Meis1 and Hif-1α are two regulators that play critical roles in HSC metabolism and function [21, 26]. In addition, Meis1 controls the oxidative stress response through Hif-2α regulation. Increased expressions of Meis1 and its cofactors are observed in HSCs and a wide variety of leukemias [1, 3, 11, 18, 51-54] and in other cancer cell types [12-16]. Even though it is known that Meis1 plays a role in cancer and HSCs, further studies are required to understand its role in the mechanism of transformation and regulation of cancer cell metabolism and cancer stem cell function.

Table 1.1. Expression of the Meis1 gene and its cofactors in various cancer types.

| **Cancer type** | **Upregulated TALE family members** | **Reference** |
|---|---|---|
| Leukemia | Meis1 and HoxA9 | [3, 11, 51-54] |
| Neuroblastoma | Meis1 | [12] |
| Nephroblastoma | Meis1 | [15] |
| Ovarian carcinoma | Meis1 and Pbx | [16] |
| Skin tumor | Meis1 | [10] |

## 1.4. TARGETING MEIS1 IN HEMATOPOIETIC STEM CELL TECHNOLOGIES

HSCs underlie BM transplantation and demonstrate great importance in targeted gene therapies. Transplantation of HSCs is applied in the treatments of leukemia, lymphoma, and autoimmune disorders [59]. An human leukocyte antigen (HLA) matched donor is required to overcome immunologic responses by the host. In addition, access to a large enough quantity of HSCs from the donor is necessary for successful engraftment [60]. Therefore, it is required to develop *ex vivo* methods to provide efficient numbers of HSCs.

*Ex vivo* HSC expansion approaches mainly rely on growth factors and cytokines [59]. The balance between proliferation and quiescence of HSCs is strictly controlled to ensure lifelong homeostasis and maintenance of HSCs [61]. However, small molecules that target quiescence factors involved in HSC quiescence have not been widely used for expansion of HSCs. It is anticipated that hematopoietic small molecules will bring new approaches to the expansion of HSCs in cell culture.

*Ex vivo* expansion of HSCs may involve many difficulties, such as decreased self-renewal ability, senescence, apoptosis, and differentiation. Apart from this, knowledge about the constituents of the HSC microenvironment and regulators of HSC function in *ex vivo* expansion procedures is limited. Many studies concentrated on cytokines and growth factors when defining how to expand HSCs in *ex vivo* cultures. In order to be proved to expansional functions of HSC, thrombopoietin (TPO), FL3, IL3 (interleukin 3), IL6 (interleukin 6), IL11 ( interleukin 11) cytokines and stem cell factor (SCF) have been used [59].

Cytokines stimulate HSCs that are arrested in the G0 phase to enter the cell cycle by upregulating factors responsible in self-renewal and by downregulating inhibitors of the cell cycle. Interestingly, p38 and some other factors that inhibit the cell cycle are upregulated during the *ex vivo* expansion procedure [62-64]. Use of p38 inhibitor was shown to increase *ex vivo* expansion of mouse HSCs [62-64].Earlier studies showed the applicability of *ex vivo* HSC expansion using small molecules [21, 65, 66]. These studies demonstrated the expansion of human and mouse HSCs utilizing Garcinol (nonspecific HAT inhibitor), StemRegenin (AhR antagonist), and nicotinamide (SIRT1 inhibitor) [61, 66-69].

These studies focused on the targeting of cell cycle inhibitors, HSC quiescence regulators, and inhibitory factors of *ex vivo* HSC expansion by applying hematopoietic small molecules [67, 70-73]. One of the recently discovered targets for *ex vivo* expansion of HSCs is Meis1 (Figure 1.3). We previously reported that HSCs could be expanded *in vivo* by specific deletion of Meis1 or Hif-1α genes (Table 1.2). This expansion of HSCs was evident in increase in HSC frequency, reduction of the number of HSCs in the quiescent state (G0 phase of the cell cycle), and induction of percentage of HSCs in G1 and S-G2-M phases of the cell cycle [21]. This is accompanied by increased cell cycle activity as measured by increased percentage of G1 and S-G2-M phase cells in Meis1 (eighteen percent , three percent, respectively) and increased G1 phase in Hif-1α KO (twenty percent) HSCs. Thus, the Meis1-/- or Hif-1α -/- long-term hematopoietic stem cell (LT-HSC) phenotype indicates an altered quiescence in HSCs as well as a tendency to proliferate [21].

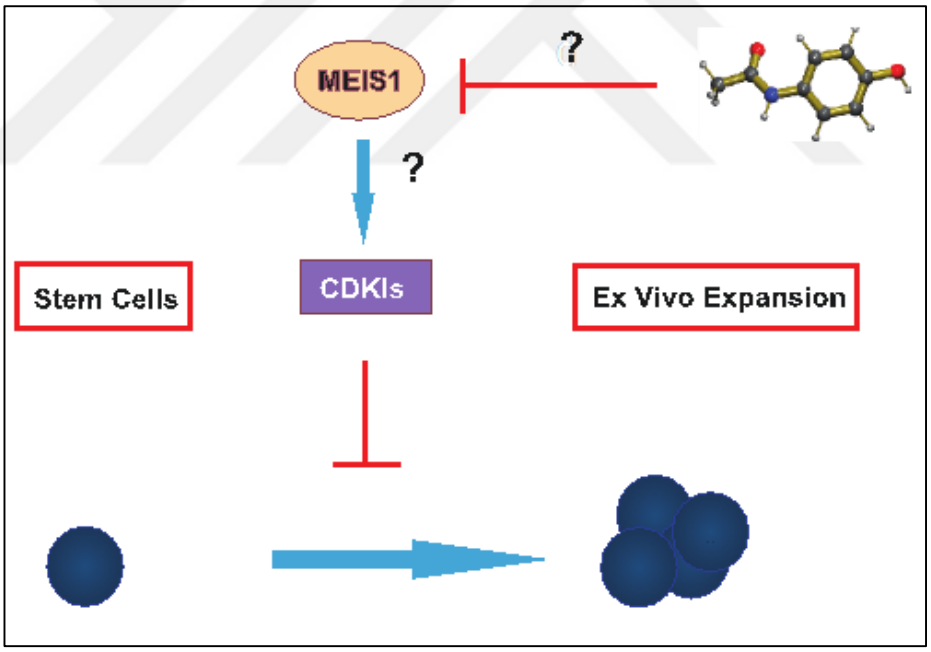


Figure 1.3. Targeting Meis1 to expand HSCs: Meis1 has been shown to target CDKIs and control cellular proliferation. Targeting of Meis1 may induce significant increase in the number of adult HSCs through decreasing inhibitory effects of CDKIs on cell cycle progression.

Table 1.2. *In vivo* expansion of HSC pool and increased cell cycle activity in Meis1 and Hif-1α knockout HSCs. HSC-specific deletion of Meis1 or Hif-1α results in 4.5- and 1.75-fold increase in their HSC pools *in vivo*, respectively.

| **Genotype** | **Percent of LT-HSC** | **Percetn of cells in $G_0$ , $G_1$, S-$G_2$-M phases of HSC cell cycle** |
|---|---|---|
| WT | 0.0020 | 82.9 ± 0.2, 11.4 ± 0.2, and 2.0 ± 0.1 |
| Meis1-/- | 0.0092 | 71.2 ± 1.1, 18.4 ± 1.0, and 3.2 ± 0.4 |
| Hif1α-/- | 0.0035 | 77.5 ± 0.8, 20.9 ± 0.7, and 0.9 ± 0.1 |

## 2. AIM OF THE STUDY

In our previous study, we demonstrated that Meis1 expression is detected in the postnatal heart associated with postnatal cardiomyocyte cell cycle arrest. On the other hand, deletion of Meis1 in adult cardiomyocytes was related to induction of cell cycle reentry [22]. This is similar to the findings from the HSC cell cycle that a lack of Meis1 in LT-HSCs results in loss of quiescence and increased HSC expansion [21]. These studies clearly showed that Meis1 have a critical role in HSC pool expansion. Moreover, Meis1 is involved in the regulation of cellular metabolism through transcriptional regulation of Hif-1α and Hif-2α. Meis1 therapeutics could potentially be useful in various fields of research and treatment of disorders.

# 3. MATERIALS

## 3.1. CELL CULTURE MEDIA

Cell culture media and their supplements are delivered from Invitrogen, Gibco, UK Company, in order expansion of primarily isolated diverse cells and cell lines. The requisite list with ordered number are listed on the Table 3.1and Table 3.2.

Table 3.1. Types of cell culture medium and the supplement varieties of GIBCO, UK Company and STEM CELL TECH, Canada Company

| **Requisite** | **Catalog Number #** |
|---|---|
| Dulbecco's Modified Eagle's Medium (DMEM ) low glucose | 31885-023 |
| Dulbecco's Modified Eagle's Medium (DMEM) high glucose | 41966-052 |
| RPMI Roswell Park Memorial Institute (RPMI) 1640 Medium | 12001-534 |
| Fetal Bovine Serum, qualified , heat inactivated, Canada origin | 12483020 |
| Antibiotic-Antimycotic (100X) | 15240062 |
| StemSpan SFEM (STEM CELL TECH) | 9650 |
| Phosphate Buffer Saline (DPBS ) | 14190250 |

Table 3.2. The growth factors used in sfem media and their related companies/catalog numbers which ordered from StemCell Technologies.

| **Sfem Supplementary** | **Catalog Number #** | **Experimental Use** |
|---|---|---|
| TPO (StemCell Technologies) | 78072 | Megakaryocyte colony-stimulating factor, MGDF |
| Flt3/Flk-2 ligand (StemCell Technologies) | 78011.1 | Fms-like tyrosine kinase 3 ligand |
| SCF (StemCell Technologies) | 78064.1 | Mast cell growth factor (Stem Cell Factor) |
| StemSpan™ CC100 (Stemcell Technologies) | 02690 | Human cytokine cocktail |

## 3.2. CELL LINEAGES

Each single cell lines beside all types of hematopoetic and mesenchymal stem cells were sent from ATCC, USA Biotechnology Company. Cell lines are listed below with ATCC catalog numbers on the Table 3.3.

Table 3.3. Cell lineages that were used and their related companies/catalog numbers which mostly ordered from ATCC, USA.

| **Cell Lines** | **Catalog Number#** |
|---|---|
| HDF (Human Dermal Fibroblast) | PCS-021-012 |
| HEK 293 (Human Embryonic Kidney) | CRL-11268 |
| HUVEC (Human Umbilical Vascular Cells ) | CRL-1730 |
| L929 (mouse subcutaneous connective tissue) | CCL-1 |
| K562 (chronic myelogenous leukemia ) | CCL-243 |
| U937 human lymphocyte) | CRL- 1593.2 |

Balb/c mice had been used for isolate whole bone marrow and mesenchymal stem cell primarily and also Scid mice had been used *in vivo* analysis in this projet. All animal studies were approved by the Institutional Animal Care and Use Committee of Yeditepe University (YUDHEK, Decision Number:468). In addition to Human umbilcal cord blood, humane bone marrow and adipogenic tissue had been used primer cell culture and stem cell isolation. All human were approved by the Institutional Clinical Studies Ethical Committee of Yeditepe University (Decision Numbers: 547 and 548). Each Primarily isolated cells that were taken from different sources and isolated in Yeditepe University Regenerative Biology Research Lab that was seen individually in Table 3.4.

Table 3.4. Primarily isolated cells that were taken from different sources and isolated in Yeditepe University Regenerative Biology Research Lab.

| **Cell Types** | **Origin** |
|---|---|
| Mouse Bone Marrow derived Hematopoetic Stem Cells | mBM obtained from YUDETAM and Cells Isolated by Yeditepe University Biotechnology Laboratories |
| Umbilical Cord Blood derived Hematopoetic Stem Cells | Umbilical Cord Blood Obtained from ONKİM and Cells Isolated by Yeditepe University Biotechnology Laboratories |
| Human Bone Marrow derived Mesenchymal Stem Cells | hBM obtained from Anatolian Health Center and Cells Isolated by Yeditepe University Biotechnology Laboratories |
| Adipogenic Mesenchymal Stem Cells | Adipogenic Tissue obtained from Serdar Bora Bayraktaroğlu Beauty Clinics and Cells Isolated by Yeditepe University Biotechnology Laboratories |
| Human Bone Marrow derived Mesenchymal Stem Cells | Isolated by Yeditepe University Biotechnology Laboratories |

## 3.3. PLASMIDS AND LUCIFERASE ASSAY

Plasmids that were utilized for the transfections and all of them were prepared and used previous study Kocabas and collegues, and that was shown in Table 3.5

Table 3.5. Isolated vectors that were reproduced in Yeditepe University Regenerative Biology Research Lab.

| **Plasmids** | **References** |
|---|---|
| p21-pGL2 | [21] |
| pEGFP-N1 | |
| pCMVSPORT6-Meis1 | |
| pGL2-elb | |

Meis1 expressions of the cells either induced or inhibited via plasmids and their results analyzed via Luciferase Assay. Transfection reagent and Luciferase assay kit were taken commercially and their catalog number listed on the Table 3.6.

Table 3.6. Related companies /catalog numbers of specific kits and other chemicals that were used for luciferase reporter assay and their comparative result analysis.

| **A Specific Kits** | **Catalog Number#** | **Clinical Use** |
|---|---|---|
| Dual-Glo Luciferase Assay System (Promega) | E2920 | Luciferase Assay |
| Poliethyleneimine Transfection Reagent (SantaCruz) | Sc-360988A | Transfection Reagent |
| Hoechst 33342 (SigmaAldrich ) | 14533 | Cell dye used for Transfection Control |

## 3.4. LINEAGE NEGATIVE CELLS ISOLATION

Lineage negative cells (Lin-) isolation process will be performed via magnetic separation from primarily isolated whole bone marrow cells. All materials provided from BD Bioscience that applied for the process are listed on Table 3.7

Table 3.7. Related companies /catalog numbers of specific kits, specific materials and other chemicals that were used for Lin Isolation and expansion materials. which ordered from BD Biotechnologies.

| **Specific Kits and Equipments** | **Catalog Number** |
|---|---|
| BD I-Magnet | 552311 |
| Biotinylated antibody plus Streptavidin | 51-9000810 |
| Cell Strainer, 70 µM, white | 352350 |
| Biotin Mouse Lineage Depletion Cocktail | 558451 |
| Purified anti-mouse CD16/CD32 (Fc gamma III/II Receptor) Mouse Fc Block™ (BD Biotechnologies) | 553141 |
| BD Stemflow™ Mouse Hematopoietic Stem Cell Isolation Kit (CD34, c-Kit, Sca-1, Lineage Cocktail) | 560492 |

## 3.5. UMBILICAL CORD BLOOD DERIVED HEMATOPOETIC STEM AND PROGENITOR CELLS ISOLATION

Umbilical cord blood mononuclear cells (PMBCs) were obtained from whole blood by using ficoll gradient centrifuge technique. Table 3.8 show that where specific chemicals provided with catalog numbers.

Table 3.8. Related companies /catalog numbers of specific materials and specific chemicals that were used for PMBCs isolation

| Specific Chemicals | Catalog Number | Clinical Use |
|---|---|---|
| Ficoll-Paque Histopaque™ (Sigma-Aldrich ) | 1083 | *In vitro* isolation of Blood |
| Plastic Blood Bag T&T PED CPD/1 | 20000006204 | Collection bag of UCB |

## 3.6. FLOW ANALYSIS

Lin-cells expansion analysed with flow cytometry by labelling surface markers.Table 3.9 and Table 3.10 illustrated flow markers which specialized for surface antigens and slam markers of mHSPCs with ordered company and their catalog numbers respectively.

Table 3.9. Surface markers with their conjugated florochromes which were ordered from BD Biotechnologies.

| **Surface Antigens** | **Florochromes** | **Catalog Number** |
|---|---|---|
| Anti-mouse Sca-1 (BD Biotechnologies) | PE-Cy7 | 51-9006308 |
| Anti-mouse CD117 / c-Kit (BD Biotechnologies) | PE | 51-9006307 |
| Anti-mouse CD34 (BD Biotechnologies) | FITC | 51-9006306 |
| Anti-mouse Lineage Cocktail (BD Biotechnologies) | APC | 51-9006309 |

Table 3.10. Flow markers with their conjugated florochromes which were ordered from BD Biotechnologies and eBioscience Companies

| **Antigens** | **Conjugated Florochrome** | **Catalog Number** |
|---|---|---|
| Anti-Mouse CD150 (eBioscience) | FITC | 11-1501-80 |
| Anti-Mouse CD48 (eBioscience) | APC | 17-0481-80 |
| Anti-mouse Sca-1 (BD Biotechnologies) | PE-Cy7 | 51-9006308 |
| Anti-mouse CD117 / c-Kit (BD Biotechnologies) | PE | 51-9006307 |
| Anti-mouse CD34 (BD Biotechnologies) | FITC | 51-9006306 |
| Anti-mouse Lineage Cocktail (BD Biotechnologies) | APC | 51-9006309 |

hHSPCs isolated from UCB. In order to analyse expansion of hHSPCs cells labelled various combination of flow markes which shown ordered company and their catalog numbers in Table 3.11.

Table 3.11. Flow markers that specialized for surface antigens and their catalog number which were ordered from eBioscience Companies.

| **Antigens** | **Florochromes** | **Catalog Number** |
|---|---|---|
| Anti-human CD34 (eBioscience ) | PE | 12-0349-42 |
| Anti-human CD133 (eBioscience ) | APC | 17-1338-42 |
| Anti-human CD38 (eBioscience ) | PE-Cyanine7 | 25-0389-42 |
| Anti-human CD90 (eBioscience ) | FITC | 11-0909-41 |
| Anti-human CD73 (eBioscience ) | APC | 344006 |
| Anti-human CD90 (eBioscience ) | FITC | 328108 |
| Anti-human CD105 (eBioscience ) | PerCP/CY5.5 | 323216 |
| Anti-human CD45 (eBioscience ) | PE | 304008 |

## 3.7. CELL CYCLE ANALYSIS AND ANNEXIN V-FITC APOPTOSIS DETECTION ASSAYS

LSK cells used for cell cycle and apoptosis analysis. Each assay requirement will be ordered from BD Biotechnology. List of the kits and chemicals are specified below in Table 3.12.

Table 3.12. Different Kits for specialized functions and their catalog numbers/companies.

| **Specific Kits and Chemicals** | **Catalog Number#** | **Clinical Use** |
|---|---|---|
| Annexin V-FITC Apoptosis Detection Kit (BD Biotechnology) | BD 556547 | Apoptosis Assay |
| Propidium Iodide Staining Solution (BD Biotechnology) | 556463 | Cell Cycle Analysis |

## 3.8. CLONOGENIC ASSAY

Methylcellulose-based medium with recombinant cytokines (including EPO) for mouse and human cells used for clonogenic assay. Methocult media is kind og viscous type media by the virtue of need specific equipments to seed cells these are listed below with Stemcell Technologies catalog numbers on the Table 3.13.

Table 3.13. Specific Chemicals/Equipments for Clonogenic Assay which were provided by Stemcell Technologies.

| **Specific Chemicals/Equipments** | **Catalog Number#** |
|---|---|
| MethoCult™ GF (Stemcell Technologies) | M3434 |
| MethoCult™ Classic (Stemcell Technologies) | H4434 |
| 16 gauge blunt-end needles (Stemcell Technologies) | 28110 |
| 3cc syringes (Stemcell Technologies) | 28240 |

## 3.9. QUANTITATIVE REAL TIME PCR

Genes expression patterns examined in three basic ways. First of all, Meis expression was analyzed in CDKI pool via the quantitative real time PCR. Table 3.14 show that specific kits for quantitative PCR analysis that were used listed below. Predesigned primers from NIH mouse primer depot [74] were ordered from Sentegen and identified in Table 3.15.

Table 3.14. Related companies /catalog numbers of specific kits that were used for quantitative PCR analysis.

| **Special Kits** | **Catalog Number#** |
|---|---|
| Quick RNA MiniPrep Isolation Kit (Zymo Research) | R1054 |
| GoTaq qPCR Master Mix (Promega) | A6002 |
| ProtoScript® First Strand cDNA Synthesis Kit (NEB) | E6300S |

Table 3.15. Primers 5 prime to 3 prime that utilized for the real time PCR of CDKIs genes that ordered from SENTEGEN, TURKEY

| GENE | FORWARD PRIMER | REVERSE PRIMER |
|---|---|---|
| GAPDH | TTGATGGCAACAATCTCCAC | CGTCCCGTAGACAAAATGGT |
| Meis1 | GTTGTCCAAGCCATCACCTT | ATCCACTCGTTCAGGAGGAA |
| P15 (CDK2NB) | CAGTTGGGTTCTGCTCCGT | AGATCCCAACGCCCTGAAC |
| P16-v1 (CDKN2A) | GCAGAAGAGCTGCTACGTGA | CGTGAACATGTTGTTGAGGC |
| P16-v2 (CDKN2A) | GGGTTTCGCCCAACGCCCCGA | TGCAGCACCACCAGCGTGTCC |
| P18 (CDK2NC) | CTCCGGATTTCCAAGTTTCA | GGGGGACCTAGAGCAACTTAC |
| P19 (CDKN2D) | TCAGGAGCTCCAAAGCAACT | TTCTTCATCGGGAGCTGGT |
| P19-ARF | GTTTTCTTGGTGAAGTTCGTGC | TCATCACCTGGTCCAGGATTC |
| P21 (CDKN1A) | ATCACCAGGATTGGACATGG | CGGTGTCAGAGTCTAGGGGA |
| P27 (CDKN1B) | GGGGAACCGTCTGAAACATT | AGTGTCCAGGGATGAGGAAG |
| P57 (CDKN1C) | TTCTCCTGCGCAGTTCTCTT | CTGAAGGACCAGCCTCTCTC |
| Hif-1α | CGGCGAGAACGAGAAGAA | AAACTTCAGACTCTTTGCTTCG |
| Hif-2a (EPAS1) | ATCACGGGATTTCTCCTTCC | GGTTAAGGAACCCAGGTGCT |
| Hif-3a | TGTGAACTTCATGTCCAGGC | GCAATGCCTGGTGCTTATCT |

## 3.10. EXPERIMETAL ANALYSIS EQUIPMENTS

Many specific devices have been used in this experiment for research purposes. Areas of utilization and their companies were identified individually inTable 3.16.

Table 3.16. Specific devices that were used during research.

| **Equipments** | **Vendor** | **Catalog Number** | **Experimental Use** |
|---|---|---|---|
| Luminoskan Ascent | Thermo Scientific | 92-480L | Luciferase Reporter assay |
| Light Cycler 96 | Roche Health Care e | 12953 | qPCR Analysis |
| MyCycler Thermal Cycler | BIORAD | 580BR1719 | Thermal Cycler |
| Steri- cycler $CO_2$ Incubator | Thermo Scientific | 3141 | Cell culture |
| FACS Calibur | BD Bioscience | 342975 | Flow Analysis |
| FACS Aria III | BD Bioscience | 23-11539-00 | Sorting Cells |
| Carl Zeiss Inverted Microscopy | ZEISS | 3849000464 | Visualizing cells |
| Thermo Labsystem Multiskan Spektrum | Thermo Scientific | 1500-176 | Colorymetric Measurement |

# 4. METHODS

## 4.1. TALE FAMILY PROTEIN ALIGNMENTS

TALE family involve these genes; Meis1, Myeloid Ecotropic Viral Integration Site 1 Homolog (MEIS1), MEIS2, MEIS3, Meis homeobox 3 pseudogene 1 (MEIS3P1), Meis homeobox 3 pseudogene 2 (MEIS3P2), PBX/knotted 1 homeobox (PKNOX1), PBX/knotted 1 homeobox 2 (PKNOX2), Transforming Growth Factor-Beta-Induced Factor 1 (TGIF1), TGFB induced factor homeobox 2 (TGIF2), TGFB induced factor homeobox 2 like, X-linked (TGIF2LX), TGFB induced factor homeobox 2 like, Y-linked (TGIF2LY), Pre-B-Cell Leukemia Transcription Factor 1 (PBX1), Pre-B-Cell Leukemia Homeobox 2 (PBX2), PBX homeobox 3 (PBX3), PBX homeobox 4 (PBX4), Iroquois Homeobox 1 (IRX1), IRX2, IRX3, IRX4, IRX5, IRX6, Mohawk genes. Protein sequences aligned to identify TALE family-protected amino acids (aa), including the Meis1 protein. The aa sequences of each TALE family proteins collected from NCBI using a blastp (protein–protein BLAST) algorithm. FASTA sequence of the related protein hits was collected to perform an amino acid alignment and to determine the conserved amino acid residues in TALE family. Multiple alignments were performed using the "constraint based multiple alignment tool" (COBALT) (NCBI). The accession numbers of the TALE family-related protein sequences are provided in Figure 4.1.

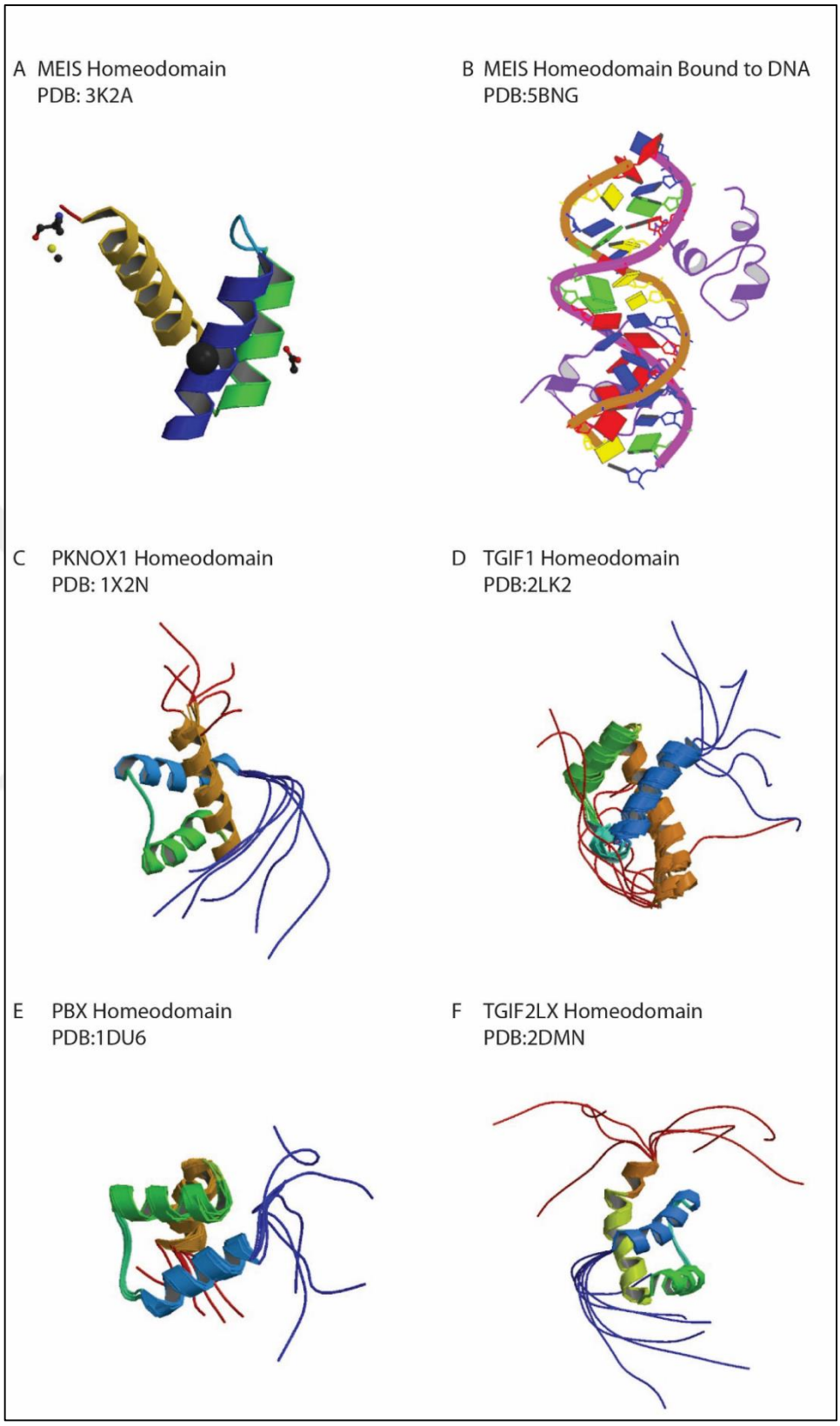


Figure 4.1. Crystal Structure of TALE-types Homeodomain.

## 4.2. CREATION OF *IN SILICO* SMALL MOLECULES LIBRARY

*In silico* small molecule library was created by collecting small molecules from three different sources for *in silico* screening. First of all, the ZINC databases was used [75]. The small molecules in the Drugs-now class are p.mwt <= 500 and p.mwt> = 150 and p.xlogp <= 5 and p.rb <= 7 and p.psa <150 and p.n_h_donors <= 5 and p.n_h_acceptors <= 10 and these are molecules that can be obtained from the market or synthesized. In addition, the Sigma-Aldrich LOPAC1280 library was included in the study. Finally, known and potential homeobox gene family inhibitors have been compiled from PubChem. Throughout compilation process, inhibitor reports were examined at PubChem for 314 genes and small molecule library, which may be inhibitors, was prepared as a SDF format.

The three-dimensional structure of the Meis1 homeodomain was downloaded from PDB.org (PDB code: 3K2A) and then opened in the Autodock Tools package and converted to the pdbqt format required for virtual screening in the Autodock Tools-1.5.6 program (see Figure 4.2). Prior to that, the water molecules were removed and the electrons in the atoms were checked and the crystal structure of the homeodomain was recorded in the form of pdbqt.

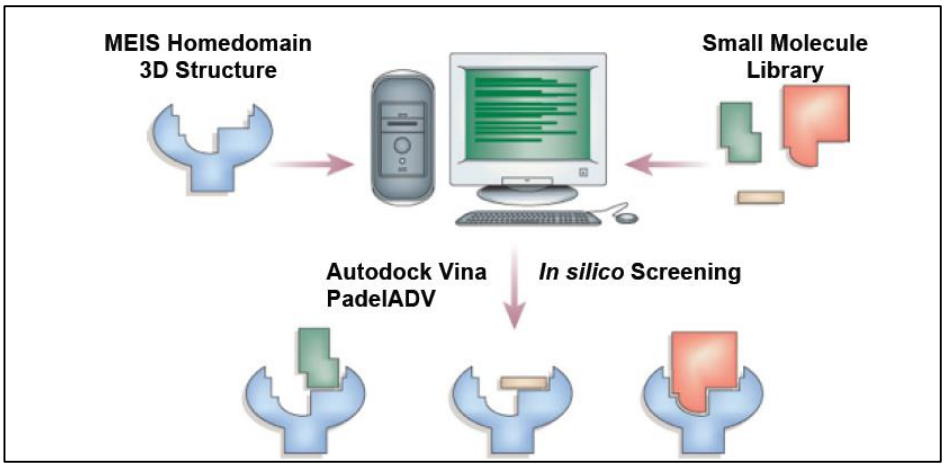


Figure 4.2. General Strategy of *in silico* Secreening.

## 4.3. DETERMINATION OF CARDIOTOXIC SMALL MOLECULES

The collaborative study with Associate Professor Serdar Durdağı revealed those that may have possible cardiotoxicity from the identified hit molecules were eliminated by affinity calculation of the hERG channel. Determination potential cardiotoxicity risk, based on binding affinity of Cisapride [76]. For this reason cisapride have been reported with much cardiovascular problems. Cardiotoxic novel small molecules determined with using "In-silico design of hERG-neutral cisapride analogues with retained pharmacological activity" method by Associate Professor Serdar Durdağı.

## 4.4. SMALL MOLECULES PREPERATION

MEISi-1 and MEISi-2 were determined according to virtual secreening. These small molecules occur as commercially available in PubChem small molecules libray. MEISi-1 and MEISi-2 were ordered from HIT2LEAD with Cat.No 7930037 and 5112931 respectively. MEISi-1 is 4-[2-(benzylamino)-2-oxoethoxy]-(2,3-dimethylphenyl) and its molecular weight is 388,49 g/mol. Following calculation of molarity, lyophilized MEISi-1 small molecules dissolved in Dulbecco's Phosphate Buffered Saline (DPBS, Gibco Cat.No. 14190250). MEISi-2 is 4-hydroxy-N'-[(Z)-(2-oxonaphthalen-1-ylidene)methyl] benzohidrazit and its molecular weight is 306,32 g/mol. Following calculation of molarity, lyophilized MEISi-1 small molecules dissolved in zero point five percent dimethyl sulfoxide (DMSO, Calbiochem, Cat.No.317275). These two molecules have been patent pending to Turkish Patent Institue (TPI No: 2016/16602).

## 4.5. MUTAGENICITY ANALYSIS

Anti-microbial activity was analysed prior to test the mutagenicity of inhibitors. For the anti-microbial activity test, the disk test was used. 200 µg of MEISi-1 and MEISi-2 were added onto the two separate disks, respectively. Salmonella Typhimurium (ATCC Cat.No. 29629) was swabbed uniformly across the Nutrient Agar plate. After inoculation with this bacterium, the disk containing both anti-microbial agent and Meis inhibitors was placed on the center of agar plates. After obtaining negative results from anti-microbial activity test, AMES test was done to check the mutagenicity. Salmonella Typhimurium (ATCC Cat.No.29629) was inoculated by using streak plate method. After incubation at 37℃, one colony was selected. This selected colony was inoculated into Nutrient Broth at 37℃ for overnight. After incubation, the optical density (OD) of this bacterial culture was measured at 545 nm with Thermo Labsystem Multiskan Spektrum (Thermo, Cat.No.1500-176). OD was measured as almost zero point four to zero point six and diluted with nutrient broth. It should be at least zero point fifteen. 500 µl Sodium phosphate buffer was mixed with 13 µl sodium azide(Sigma Aldrich Cat.No.S2002) as a mutagen then 100 µl diluted bacterial culture and 10 mM concentration of the MEISi-1 and MEISi-2 were mixed, respectively. The assays were incubated sequentially with the following reagents, each step being for ten minutes at room temperature. For negative control, mixture includes sodium phosphate buffer and bacteria. Different from negative control, positive control comprise mutagen. Prepared all negative control, positive control and assay mixtures stirred with 2 ml top agar and poured onto bacto-agar (BD Bioscience. 4220779) plate then incubate at 37℃ for two days. After two days observed and counted colonies for analyse mutagenicity.

## 4.6. LUCIFERASE REPORTER ASSAY

p21-pGL2 (2 μg) was co-transfected with Meis1 expression vector pCMVSPORT6-Meis1 (OpenBiosystems, 400 ng ) and pEGFP-N1 (transfection control, 2 μg ) pGL2-elb (internal control, 400 ng) into HEK293 cells in six-well plates using poliethyleneimine transfection reagent (SantaCruz, Cat.No. Sc-360988A) (Table 4.1). After 48 hours of transfection, the cell lysate was processed for luciferase activity using the luciferase reporter system, according to the manufacturer's instructions (Promega Dual-Glo, Cat.No. E2920). Luciferase measurements were calculated as relative light units normalized to transfection control (Renilla-Luci) measured with Luminoskan Ascent Mikroplate (Thermo Lab System Cat.No. 1500-176).

Table 4.1. Luciferase reporter assay set-up ratio of plasmids.

| **Plasmids** | **Control** | **200ng Meis1** | **400ng Meis1** | **GFP Control** |
|---|---|---|---|---|
| p21-PGL2 | 1500ng | 1500ng | 1500ng | - |
| Meis1 Plasmid | - | 200ng | 400ng | - |
| pGL2-Filler | 400ng | 200ng | - | - |
| Transfection Control (Renilla) | 100ng | 100ng | 100ng | - |
| GFP Plasmid | - | - | - | 2000ng |

## 4.7. WHOLE BONE MARROW ISOLATION

Balb-C mice provided by YUDETAM within the context of ethics committee approval. Balb-C mice placed into visible inside the euthanasia chamber and turning on gas from the $CO_2$ tank until death can be ensured. Mouse bone marrow (mBM) was extracted from the femurs and tibias of Balb-C mice by flushing the bone marrow space with insulin syringe and DPBS (Figure 4.3) Collecting bone marrow homogenized with tradeoff by using 5 ml syringe. Homogenized cells filter the samples through 70 µM cell strainer (BD, Cat.No. 352350).

Figure 4.3. Whole bone marrow isolation from Balb-C mice was shown as step by step [77].

## 4.8. LINEAGE NEGATIVE CELL ISOLATION

Balb-C mice provided by YUDETAM within the context of ethics committee approval. Balb-C mice placed into visible inside the euthanasia chamber and turning on gas from the $CO_2$ tank until death can be ensured. Mouse bone marrow (mBM) was extracted from the femurs and tibias of Balb-C mice by flushing the bone marrow space with insulin syringe and DPBS. Collecting bone marrow homogenized with tradeoff by using 5ml syringe.Homogenized cells filter the samples through 70 μM cell strainer (BD, Cat.No.352350). Isolated WBM cells centrifuged and remove the supernatant. Resuspended cells into DPBS suplemented with two percent fetal bovine serum (FBS, v/v) and just after thoroughly dissolved cells treated with FC block and incubate for fifteen minutes (min) on ice. Blocked cells deal by Lin Coctail (BD, Cat. No.560492) and incubate again.

In order to achieved negative selection, cells treated with biotinylated antibody plus Streptavidin and incubate cells thirty min on the ice. The labelled cells wash with centrifugation and resuspended in DPBS with two percent FBS. Finally cells transferred into 12x75 mm round bottom test tubes (flow tubes) and placed into BD IMagnet (BD, Cat.No. 552311). After approximately forty five min, labelled cells are attracted to the magnet and positive cells stick and observed as a pinky on the wall of flow tubes (Figure 4.4). Supernatant which were negative fraction, was aspirated without removing inside BD-IMagnet and without disturbing pellet. Aspirated supernatant were transferred new flow tube inside BD IMagnet for achieving pure culture, at the same time pellet which was positive fraction, was resuspend into fresh PBS with two percent FBS inasmuch as retain cells and. Finally collect all supernatant without removing inside BD IMagnet, lineage negative (Lin-) cells count and then centrifuged. Depending on an experiment, cells resuspend into proportionately StemSpan SFEM media which was supplemented with Scf, Tpo, Flt-3land one percent PSA (penicillin streptomycin amphotericin) their ratio shown in Table 4.2.

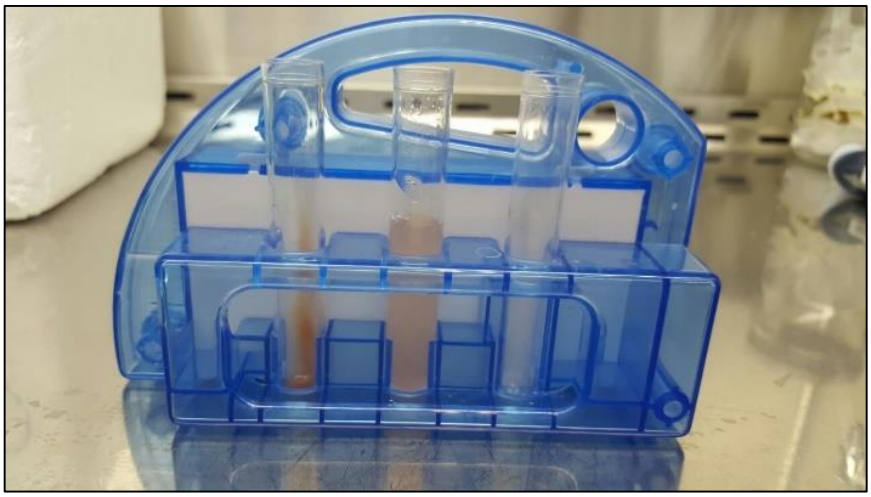

Figure 4.4. Isolated Lin-cells placed in the BD-IMagnet and positive cells stick and observed as a pinky on the wall of flow tubes.

Table 4.2.The growth factors used in sfem media their related companies/catalog numbers which ordered from StemCell Technologies.

| **Sfem Supplementary** | **Amount in 1 ml Sfem media** |
|---|---|
| TPO (StemCell Technologies) | 0,2µl/1ng |
| Flt3/Flk-2 ligand (StemCell Technologies) | 1µl/1ng |
| SCF (StemCell Technologies) | 1µl/1ng |

## 4.9. UMBILICAL CORD BLOOD CELL ISOLATION

Umbilical Cord Blood (UCB) was collected into spesific blood bag (Plastic collection blood bag, T&T PED CPD/1 200ML Cat.No. 20000006204 ) from the newborn at the birth time with parent's consent by Onkim Stem Cell Technologies. UCB mononuclear cells were isolated with density gradient centrifugation method by using Ficoll-Paque (HistopaqueTM, Sigma, Cat.No. 10771). Fist of all, cord blood cells were aliquat as a 25 ml into 50 ml falcon tubes ( Isolab , CatNo. 078.02.004) and cord blood was diluted with 1:1 proportion of (v/v) DPBS 1:1 proportion (v/v) of Ficoll-Paque was added drop by drop onto diluted cord blood.These mixtures centrifuged ( without brake). After centrifugation three different phases were obtained and cloudy interphases were collect which are mononuclear cells. Isolated mononuclear cells were washed with DPBS and centrifugation (with breake). Finally isolated cells seeded on 96 well-plate (Corning-Costar, Cat.No.CLS3599) in expansion medium at 10,000 cells / well. The expansion medium consists of Serum-Free Expansion Medium (StemSpan™ SFEM, Stemcell Technologies, Cat.No. 09650) supplemented with one percent PSA (10,000 units/ml penicillin and 10,000 ug/ml streptomycin and 25 µg/mL of Amphotericin B, Gibco, Cat.No.15240062) and human cytokine cocktail (StemSpan™ CC100, Stemcell Technologies, Cat.No. 02690) and these cells growth in normal cell culture condition that is humidified incubator at 37ºC with five percent 5% $CO_2$ .

## 4.10. MESENCHYMAL STEM CELL ISOLATION FROM MOUSE BONE MARROW

Mouse bone marrow mesenchymal stem cells were isolated according to optimized refitted protocol from Soleimani and Nadri [78]. Balb-C mice provided by YUDETAM within the context of ethics committee approval. Balb-C mice placed into visible inside the euthanasia chamber and turning on gas from the $CO_2$ tank until death can be ensured. Mouse bone marrow (mBM) was extracted from the femurs and tibias of Balb-C mice by flushing the bone marrow space with insulin syringe and PBS. Collecting bone marrow homogenized with tradeoff by using 5 ml syringe.Homogenized cells filter the samples through 70 µM cell strainer (BD, Cat.No.352350).

Isolated WBM cells centrifuged and remove the supernatant. WBM cells harvested as described and seeded at a density of 30 x 106 cells and pellet resuspended into Dulbecco's Modified Eagle's Medium (DMEM Gibco, Cat.No.31885-023) supplemented with fifteen percent (v/v) FBS (Sigma Aldrich, USA, Cat.No. 12103C) and one percent (v/v) PSA (10,000 units/ml penicillin and 10,000 ug/ml streptomycin and 25 µg/mL of amphotericin B, Gibco, Cat.No.15240062) and cells seed on T-75cm$^2$ flasks (Corning- Costar, Cat.No. CLS3290). Mesechymal stem cells (MSC) used for up to passage number three (P3).

## 4.11. ADIPOGENIC MESENCHYMAL STEM CELL ISOLATION

Adipose tissue was collected from liposuction operation by Clinic SBB with patients's consent. Collected adipose tissue is processed within three to four hours. 150 ml adipose tissue was washed with equal volume of DPBS twice then tissue was placed into a 500 ml bottle which comprise of equal volume of collagenase solution (zero point two, v/v). The tissue was digested at 37 ºC for one to four hour by continuous shaking on 250 rpm. After the digestion, ten percent FBS was added for inhibition of the collagenase activity. The digested tissue was centrifuged at 2000 rpm for ten min at room temperature. The supernatant that contains the collagenase solution and adipocytes was discarded and then, the pellet was resuspended with Ammonium Chloride, (STEMCELL Technologies CatNo:07850) for red blood cell lysis. The cell suspension was incubated at 37 ºC for ten min by continuous shaking on 250 rpm. The cell suspension was washed centrifugation by using DPBS. Isolated Adipogenic Mesenchymal Stem Cell (AD-MSC) were resuspended in DMEM which were supplemented with ten percent FBS and then, filtered through 100 µM cell strainer. The cells seeded onto T-75cm$^2$ flasks (Corning- Costar, Cat.No. CLS3290).

## 4.12. HSPCS AND MSCS EXPANSION WITH FLOW CYTOMETRY ANALYSIS

Primarily isolated hematopoietic stem and progenitor stem cells (HSPCs) were seed on 96 well plate (Corning-Costar, Cat.No. CLS 3599 ) as 30.000 cells /wells. MEISi-1 and MEISi-2 small molecules was applied on cells at 0,1 μM, 1 μM and 10 μM concentrations just after seeding. The cells used as positive control were treated with zero point five percent dimethyl sulfoxide (Calbiochem, Cat.No.317275). HSPCs were incubated in normal cell culture conditions that is humidified incubator at 37ºC with 5% $CO_2$ throughout seven days. After seven days, HSPCs was labelled depeding on the source of cells and analysed with flow cytometer via FACS Calibur (BD Bioscience, Cat.No.342975).

Primarily isolated MSCs both human adipose derived and mouse bone marrow, seed on T-75 cell culture flask (Corning-Costar, Cat.No. CLS3290-100EA ). When passage number became two ( P2 ), cells trypsinized and seed on 96 well plates as 10.000 cells/wells. MSCs differ from HSPCs has adherent cell type. For this reason primarily isolated MSCs (does not matter of their origins) treated with different concentrations of MEISi-1 and MEISi-2 (final concentrations; 10 μM, 1 μM, 0.1 μM) after 24 hours cultured.

These cells used as positive control were treated with zero point five percent DMSO (Calbiochem, Cat.No.317275). MSC staining was applied according to the manufacturer's protocol at the end of the seven days of incubation. Then flow cytometry analysis was performed with FACS Calibur (BD Bioscience, Cat.No.342975).

The expansional effect of MEISi-1 and MEISi-2 on HSPCs and MSCs consist in flow analysis. Hematopoietic stem and progenitor cells were characterized by Lineage-, Sca-1+, c-Kit-+ for LSK and LSKCD34low population. This strategy comprise of Lin-and LSK cell population which is inside of the live gate and CD34FITC gating respectively that was clearly shown in Figure 4.5.

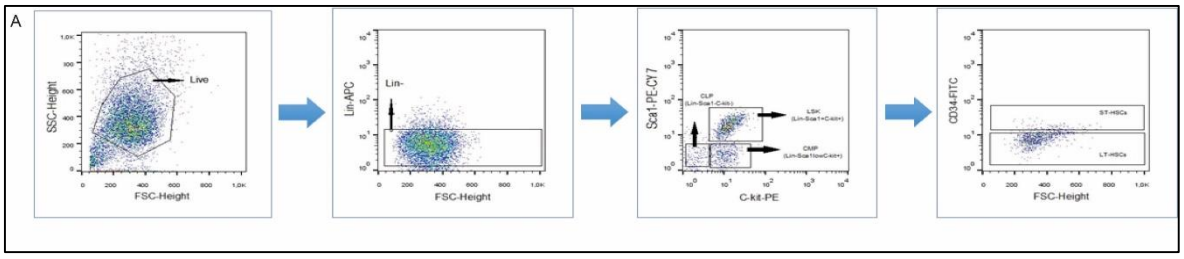


Figure 4.5. Flowchart shows that general strategy of flow analysis.

### 4.12.1. Flow Cytometry Analysis of Mouse HSPCs

Anti-Mouse CD16/32 Fc block, c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7 and mouse APC lineage cocktail (BD StemFlow, Cat.No. 560492) were used in order to identify LSK and LSKCD34low population after treated Meis inhibitors. First of all Fc Block was dissolved as a ratio of 4:100 (v/v) in DPBS and incubate on ice for 15 minutes. Then second mixture was prepared with lineage cocktail, Sca-1, c-Kit, CD34 FITC antibodies into DPBS as a ratio of 4:100 (v/v) and again incubate on ice for fifteen minutes. HSPC cells were concomitantly stained with two distinct mixture and incubate at room temperature for fifteen minutes after each staining steps. Finally the expressions of surface markers in the labelled cells were analysed by flow cytometry FACS Calibur (BD Bioscience, Cat.No.342975).

### 4.12.2. Flow Cytometry Analysis of Human HSPCs and Human BM-MSCs

Primarily isolated human UCB-HSPCs and BM- HSPCs treated with different concentration of MEISi-1 and MEISi-2 inasmuchas to determine effective dose of Meis inhibitors. Human derived HSPCs were concomitantly labelled with PE-conjugated anti-human CD34, APC-conjugated anti-human CD133 antibodies PE-Cyanine7-conjugated CD38 antibodies and FITC-conjugated CD90 antibodies which marker's companies and catalog numbers identified in Table 3.11. Surface marker expression of hematopoietic stem cells and incubated cells on ice for fifteen min. The expressions of surface markers in the labelled cells were analysed by flow cytometry via FACS Calibur (BD Bioscience, Cat.No.342975).

### 4.12.3. Flow Cytometry Analysis of AD-MSCs

Human derived AD-MSCs at passage two were used for small molecule treatment. 10.000 cells per well were seeded on 96 well plate in 200µl of expansion medium. The seeded cells were treated with three different doses (Final concentrations; 10 µM, 1 µM, 0.1 µM) of Meisi1 and Meisi2. After seven days of the treatment, the cells were labelled with human MSC markers; hCD73-APC, hCD90-FITC, hCD105-PerCP/Cy7, hCD45-PE which marker's companies and catalog numbers identified in Table 3 11 according to the manufacturer's protocol. After the labelling, the flow cytometry analysis was performed.

## 4.13. CELL CYCLE ANALYSIS (LSK VE HHSPC)

Lin-cells isolated from Balb-C mice and lin- cells population labeled with APC lineage cocktail, anti-mouse c-Kit (CD117) PE, Sca-1 PE-Cy7 and CD34 FITC and that were sorted by flow cytometry (FACSARIA III, BD Biosciences, Cat.No. 23-11539-00). Thus murine LSKCD34low (Lin-Sca1+c-Kit+CD34low) were obtained. On the other hand, UCB cells labelled with just CD34 and human CD34+ cells from UCB mononuclear cells were sorted by flow cytometry FACSARIA III, (BD Biosciences, Cat.No. 23-11539-00). The sorted cells were plated in supplemented SFEM media and seed 5,000 cells per well in 96 well-plate for murine and human cells. Then ells treated with the effective doses of MEISi-1 and MEISi-2 at 1 µM concentration for murine cells and 0,1 µM concentration of MEISi-1 and MEISi-2 for human cells. After four days of the treatment, the cells were stained with Hoechst 33342 (10µg/ml) (Sigma Aldrich, USA, Cat.No. 14533) and Pyronin Y (100µg/ml) (Sigma Aldrich, USA, Cat.No. P9172-1G) and incubate at cell culture incubator at 37°C and 5% $CO_2$ for thirty minutes after each staining steps and then analyzed by flow cytometry FACSARIA III, BD Biosciences, Cat.No. 23-11539-00).

## 4.14. ANNEXIN V-FITC APOPTOSIS DETECTION KIT

Lin- cells isolated from Balb-C mice and lin- cells population labeled with APC lineage cocktail, anti-mouse c-Kit (CD117) PE, Sca-1 PE-Cy7 and CD34 FITC and that were sorted by flow cytometry via FACSARIA III (BD Biosciences, Cat.No. 23-11539-00). Thus murine LSKCD34low (Lin-Sca1+c-Kit+CD34low) were obtained. On the other hand, UC cells labelled with just CD34 and human CD34+ cells from UCB mononuclear cells were sorted by flow cytometry FACSARIA III, (BD Biosciences, Cat.No. 23-11539-00). The sorted cells were plated in supplemented SFEM media and seed 5,000 cells/ well in 96 well-plate for both murine and human cells. Then cells treated with the effective doses of MEISi-1 and MEISi-2 at 1 μM concentration for murine cells and 0,1 μM concentration of MEISi-1 and MEISi-2 for human cells. After three days of the treatment, the cells were collected via centrifugation. Cells were resuspended in 500 μl of 1X Binding Buffer and then labelled with 5 μl of Annexin V-FITC and 5 μl of Propidium Iodide (PI). Labelled cells incubate in the dark for staining at room temperature for five minutes. In order to do quantification, Annexin V-FITC binding was analysed by flow cytometry via FACSARIA III, BD Biosciences, Cat.No. 23-11539-00).

## 4.15. CLONOGENIC ASSAY ANALYSIS

Primarily isolated HSPCs and seperated as lin- cells seed on 96 well plate as 30.000 cells / well just after seeding cells treated with MEISi-1 and MEISi-2 at efective concentration of 1 μM concentration. Cells cultured at the humidified incubator at 37°C and five percent $CO_2$ throughout seven days and then cells harvested and counted. According to counting these cells equal numbers of cells were resuspended in DMEM media which was not supplemented any growth factor antibyotics and serum, Then approximate 66.000 cells / well plated in methylcellulose-containing medium (MethoCult™ GF M3434, Stemcell Technologies) in 6-well plate by using special syringe (3cc syringes, Stemcell Technologies) and needles (16 gauge blunt-end needles, Stemcell Technologies). Colony formation was observed throughouts ten to twelve days, then colonies were classified as CFU-GEMM, CFU-G/M, BFU-E and B cell colonies and counted by inverted Carl Zeiss Inverted Microscopy (ZEISS, Cat.No.849000464) at a minimum magnification of 10X.

## 4.16. QUANTITATIVE REAL TIME ANALYSIS FOR DETECTING THE RELATIVE MRNA EXPRESSION LEVELS FOR DIFFERENT PRIMARIES

Lin- cells isolated from mouse bone marrow and cells were seeded on six well plate (Corning, Cat.No.CLS 3516) as one million cells /well. After four days expansion cells were collected for RNA isolation. Total RNA was isolated by using Zymogen Quick RNA Mini Prep (ZymoResearch, Cat.No. R1054S) according to the manufacturer's protocol. Isolated total RNA arranged as 2 μg total RNA and synthesized cDNA by following recommended protocol for SuperScript II Reverse Transcriptase Kits (Life Technology, 18080-051) and used spesific ratio was shown in Table 4.3. Reaction was performed using with GoTaq qPCR Master Mix (Promega, Cat.No6002) according to the manufacturer's protocol and their optimized ratio indicated in Table 4.4. In order to do quantitative analysis of determined HDR, HSC and CDKI genes, real time PCR was performed by Light Cycler 96 (Roche Health Care Thermalcycler 96, Cat.No: 12953) and their initial steps of experiment eloborated in Table 4.5. GAPDH was used as a housekeeping control to normalize gene expression using the ΔΔCt method and also β-Actin was used as as a housekeeping control for HSC gene pool analysis.

Table 4.3. The ratio of SuperScript II Reverse Transcriptase Kits that was synthesised cDNA from RNA.

| **Reagents** | **Volume** |
|---|---|
| RNA | 5μg |
| Random Primer | 2μl |
| Reaction Mix 2X | 15μl |
| Enzyme 30X | 1μl |

Table 4.4. The used ratio of GoTaq qPCR Master Mix reagents and primers

| **Reagents** | **Volume** |
|---|---|
| Syber Green 2X | 7,5µl |
| Primer Forward | 0,75µl |
| Primer Reverse | 0,75µl |
| Template | 2µl |
| Distilled Water | 4µl |

Table 4.5. Set-up conditions for RT-PCR in Lightcycler 96

| **Step** | **Temperature (ºC)** | **Time** | **Cycle** |
|---|---|---|---|
| Initial Activation Step | 95 | 120s | 1 |
| Quantification | 95 | 15 s | 55 |
| | 60 | 60 s | |
| Extansion | 72 | 45 s | 1 |
| Melting Curve | 95 | 10 | 1 |
| | 55 | 60 | |
| | 97* | 1 | |
| Cooling | 37 | 30s | 1 |

## 4.17. CELL PROLIFERATION ASSAY

In order to determine the effect of expansion of MEISi-1 and MEISi-2 small molecules on HSPCs and MSCs two different methods were used. First of all, Lin– cells which treated with different concentrations of MEISi-1 and MEISi-2 final concentrations; 10 μM, 1 μM, 0.1 μM), was stained with Hoechst 33342 (SigmaAldrich, Cat.No. 14533 ) at day one and day seven. Photos of stained cells were taken with inverted microscopy (Carl Zeiss Inverted Microscopy, Cat.No.3849000464) and these cells count with Scion Image Software. Other method of cell proliferation assay was colorimetric assay and water soluble tetrazolium salts (WST-1) was used. In order to observe how Meis inhibition affect other cells apart from hematopoetic stem cells, various cell types were used for proliferation assay.

L929 cell (mouse fibroblast cells, ATCC, Cat NO: ), Human umbilical vein endothelial cells (HUVECs, ATCC® CRL1730™) and primarily isolated BM-MSCs and AD-MSCs were used for cytotoxicity analysis and that were seeded in 96 well plates at a density of 2000 , 5000 cell and 10000 cell per well respectively. Because of all cell types are adherent, MEISi-1, MEISi-2 and DMSO (zero point five percent) treatment applied the day after cell seeding. 0.1 μM, 1 μM, 10 μM stocks were used for concentration gradient of Meis inhibitors. It is recommended to add 10 μl / well cell proliferation reagent WST-1 (Boster, AR1159) to the already treated cells (almost 36-48 after ). Cells were incubated with WST1 reagent throughout 2 hours in humidified cell culture incubator at 37°C and 5% $CO_2$ in dark. Absorbance of samples was taken measurements hourly at 450nm via Thermo Labsystem Multiskan Spektrum (Thermo, Cat.No.1500-176).

Furthermore, K562 (chronic myelogenous leukemia ) and U937 human lymphocyte cells were used for cytotoxicity analysis. 2000 -5000 cells / well seeded on 96 well-plate (Corning, Cat.No.CLS 3599 ). Cells were grown in 200 μl/culture media (RPMI media supplemented with ten percent fetal bovine serum (FBS), one percent (PSA) Antibiotic-Antimycotic (100X). MEISi-1 and MEISi-2 were added after cells adhere. 0.1 μM, 1 μM, 10 μM stocks were used for concentration gradient of Meis inhibitors. It is recommended to add 10μl / well cell proliferation reagent WST-1 (Boster, AR1159) to the already treated cells (almost 36-48 after ).

Cells were incubated with WST1 reagent for one to two hours in dark and humidified cell culture incubator. Absorbance of samples was taken measurements hourly at 450nm via Thermo Labsystem Multiskan Spektrum (Thermo, Cat.No.1500-176). Their absorbance was measured at day1- day2- day3-day4- day5 as a time dependent.

### 4.18. *IN VIVO* INJECTIONS OF MEIS INHIBITORS

1mM concentration of MEISi-1 and MEISi-2 inhibitors were diluted with DPBS and final concentration adjusted as 1 μM. 1 μM MEISi-1, 1 μM MEISi-2 and DMSO control (zero point one percent) were intraperitoneally injected to 4-5 week-old BALB/c mice at first, fourth and seventh day. After ten days, BALB/c mice sacrificed and whole bone marrow cells isolated from femur and tibia for flow cytometry analysis and total RNA isolation. WBM cells analyzed with surface marker which are APC lineage cocktail, c-Kit (CD117) PE, Sca-1 PE-Cy7, CD34 FITC and slam markers which are APC lineage cocktail, c-Kit (CD117) PE, Sca-1 PE-Cy7, CD150 FITC and CD48 APC shown in Table 3.9 and Table 3.10 analyzed with flow cytometry via FACS Calibur (BD Bioscience, Cat.No.342975).

### 4.19. STATISTICAL ANALYSIS

The statistics were analyzed by Dunnett's Multiple Comparison Test and p values was used. If the values were $p<0.05$, this results were considered statistically significant and if $p<0.01$ were measured statistically more significant. In addition to "2-tailed Student t test" was used to determine the level of significance.

# 5. RESULTS

## 5.1. IDENTIFICATION OF CONSERVED DNA LINKED AMINO ACIDS OF MEIS HOMEODOMAIN AND TARGETED AMINO ACIDS FOR VIRTUAL SECREENING

Meis1 homeodomain has a structure of sixty two amino acids in size that was recognized DNA. First of all, which amino acids sequences are interacting with DNA and subsequent analysis of protected amino acids have been determined. TGACAG nucleotides are known as Meis1 homeodomain binding sequences. Homologous / protected amino acids in the homeodomains of Meis1 and involved TALE proteins were identified. Thus protected amino acids for binding to DNA in their homeodomain was determined (see in Figure 5.1).

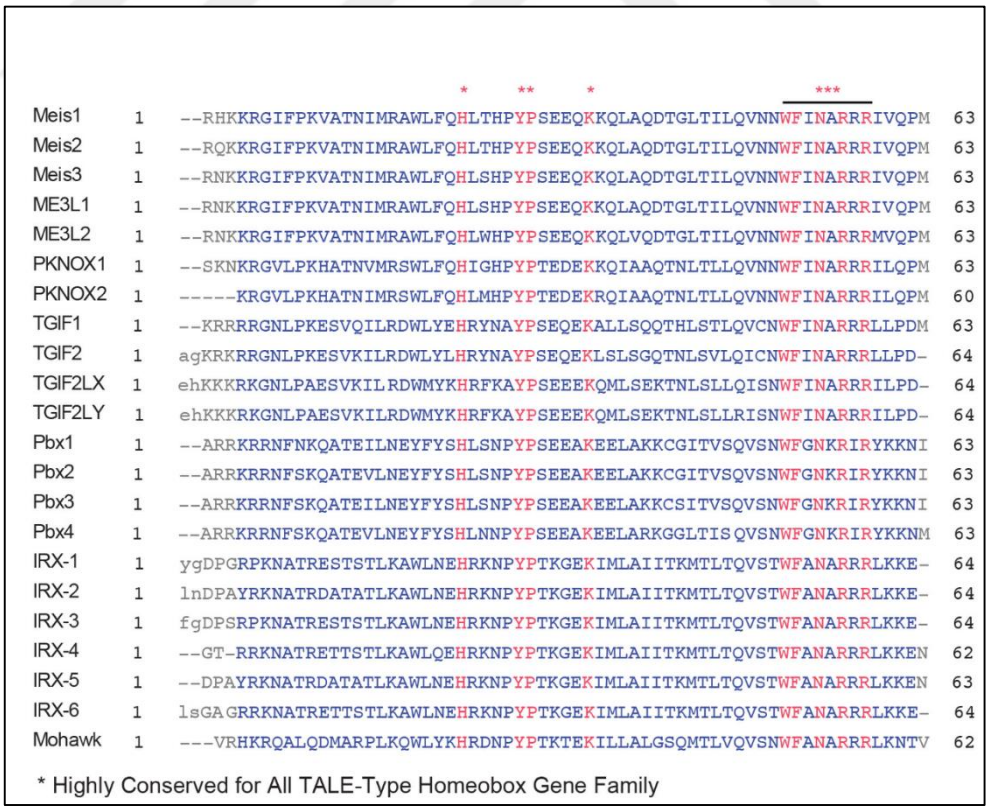


Figure 5.1. Multiple amino acid sequencing of the homeodomain regions of TALE family genes.

## 5.2. VIRTUAL SECREENING OF INHIBITORS RELATED TO MEIS HOMEODOMAIN

*In silico* detection of molecules with high binding energy that can prevent Meis1 from binding to DNA was completed by searching in 1,000,000 molecules. The molecules, whose binding energies smaller than -7 kcal/mol to the homeodomain of Meis1 were determined and subjected to specificity analysis as in IP3. Detection of Meis1 specific inhibitors was performed simultaneously with IP2 by screening and eliminating those with high binding value to other TALE family proteins. Those with less than 0.5-1 kcal/mol were eliminated. (For detailed strategy, see Figure 5.2). For virtual screening, a high-CPU (Center Processing Unit) was used. A multi-core computer, which has 12 cores, 24 CPUs, and 64 GB of RAM, was purchased as part of the project. Multi-core and 24 CPUs are ideal for multiple virtual screening at the same time, so that various scans were made quickly in a parallel manner. In order to test the feasibility of the studies proposed here, a small screening was carried out with a DNA binding region that we identified, containing primarily 10,000 molecules. After the identification of possible hits in this way, 1M molecules were scanned from the "drugs now, lead like" molecules in the ZINC database. This list contains the molecules, which have physicochemical properties that can be used as drugs, available on the market. In addition, Sigma`s 1280 molecule library was also included in the scans to make it easy to supply the small molecules identified after the drug screening.

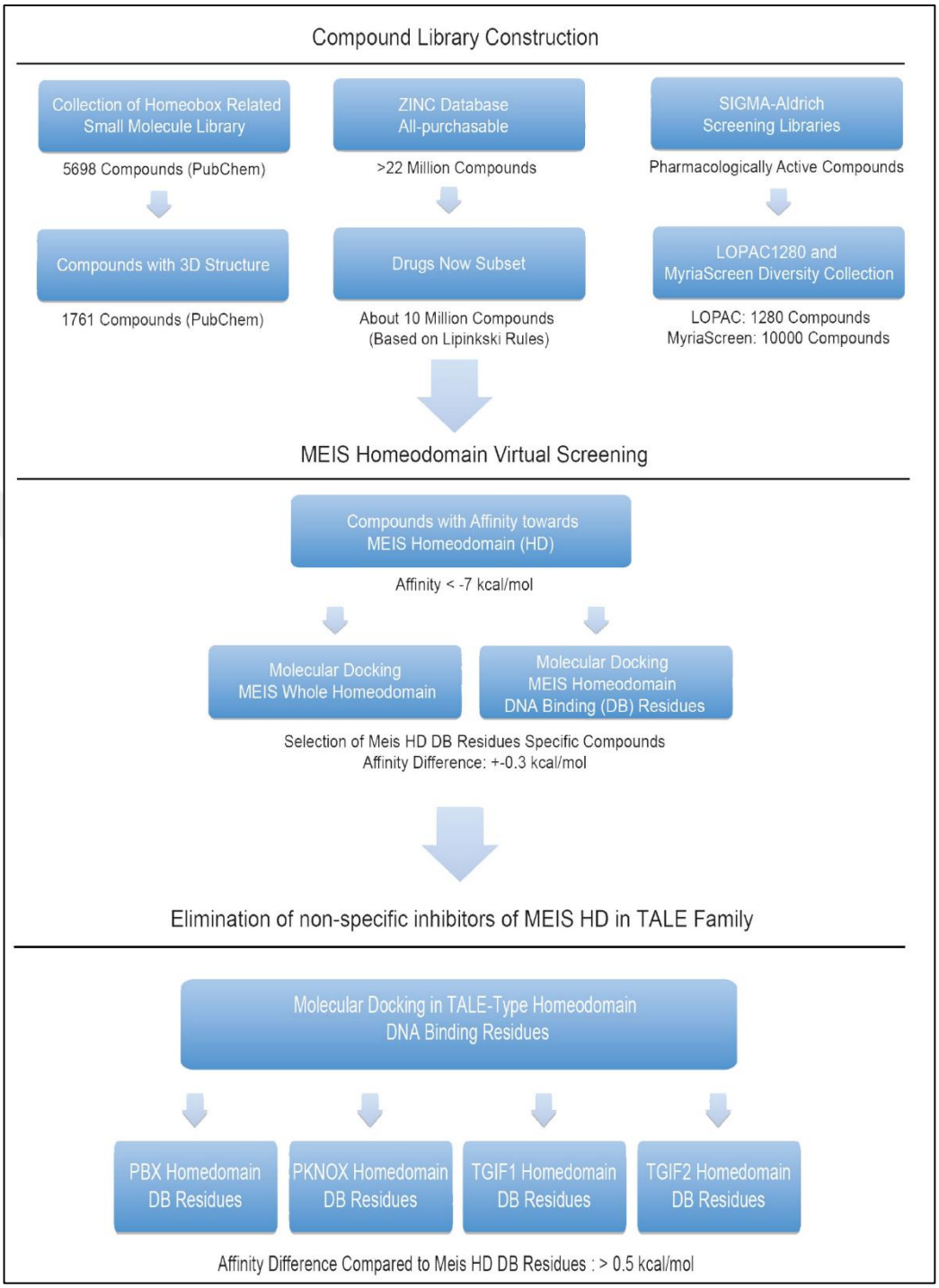


Figure 5.2. Virtural Secreening Strategy. This stretagy consist of three part. First part is in-silico library creating strategy, second part is Meis1 bridging strategy and final part is elimination strategy of non specific Meis inhibitors.

## 5.3. ANALYSIS OF KNOWN INHIBITORS WHICH BELONG HOMEOBOX FAMILY

In another study, a database for known inhibitors of TALE-type homeobox proteins that also contains the Meis1 gene was constructed by using the PubChem database. To achieve this, each of the 314 Homeobox genes was scanned in PubChem and a list of active substances was identified. In order to do virtual screening of these molecules in their three-dimensional formats, the appropriate formats (SDF) were downloaded and used to create the database. After the PubChem database analyses, it was shown that inhibitors were reported against 13 Homeobox genes (see Table 5.1). By using this approach, 5698 molecules, which inhibit or activate homeodomain-containing proteins, were identified. The list was also used in the searches of Meis1 inhibitors. Because any known inhibitor from such a list is known to be effective for another homeobox protein in an earlier *in vitro* experiment, also increases the likelihood of being effective *in vitro* for Meis1 as well as. In other words, the *in silico* database of 5698 molecules creates a physiological small molecule library for the screening drugs that may be the inhibitor of Meis1 homeodomain.

Table 5.1. The list of known genes belonging to homeobox family.

| Symbol | Name | Gene family description |
|---|---|---|
| EN2 | engrailed homeobox 2 | Homeoboxes / ANTP class: NKL subclass |
| GSC2 | goosecoid homeobox 2 | Homeoboxes / PRD class |
| MEOX1 | mesenchyme homeobox 1 | Homeoboxes / ANTP class : HOXL subclass |
| MEOX2 | mesenchyme homeobox 2 | Homeoboxes / ANTP class : HOXL subclass |
| MSX2 | msh homeobox 2 | Homeoboxes / ANTP class : NKL subclass |
| NANOG | Nanog homeobox | Homeoboxes / ANTP class : NKL subclass |
| NKX3-1 | NK3 homeobox 1 | Homeoboxes / ANTP class : NKL subclass |
| PAX2 | paired box 2 | Paired boxes, "Homeoboxes / PRD class" |
| PAX5 | paired box 5 | Paired boxes, "Homeoboxes / PRD class" |
| PAX8 | paired box 8 | Paired boxes, "Homeoboxes / PRD class" |
| PDX1 | pancreatic and duodenal homeobox 1 | Homeoboxes / ANTP class : HOXL subclass |
| PROX1 | prospero homeobox 1 | Homeoboxes / PROS class |
| SIX1 | SIX homeobox 1 | Homeoboxes / SINE class |

## 5.4. GRID BOX GENERATION AND DNA BINDING DOMAIN ANALYSIS FOR MOLECULAR DOCKING

During the docking procedure in AutoDock/Vina programme two different size range of scan boxes was used. Using AutoDockTools (Trott and Olson, 2009), pockets of F326, W327 and R331/333 residues were highlighted to determine the grid box locations for MEIS1 protein. Firstly, the grid size ( locations of scan boxes) was set to 40 x40 x40 xyz point covering the entire homeodomain with grid spacing of 1.000 Å and grid center was designed at dimensions (respectively x;y;z) 32.143; 39.869; 15.007. In order to determine the specific inhibitors of homeodomain binding to DNA a screening box was also identified with an approximate grid size 20 x 20 x 20 xyz point around the relevant amino acid that thought to interact the Meis homoedomain with DNA with grid spacing of 1.000 Å and grid center was designed at dimensions (respectively x;y;z) 24.251; 43.841; 15.007. Thus script file was prepared containing vina configuration (Figure 5.3).

Detection of specifically Meis1 inhibitors was performed by creating search boxes in a similar way compared to other TALE homeodomain. Pbx1, Pknox1, TGIF1, TGIF2 homeodomain that have known three-dimensional crystal structure of the TALE class homeobox proteins, was used. For PBX1 (PDB code: 1DU6) the grid size was set to 20 x24 x20 xyz point covering the entire homeodomain with grid spacing of 1.000 Å and grid center was designed at dimensions (respectively x;y;z) -5.986; 2.948; -20.788.

For PKNOX1 (PDB code: 1X2N) the grid size was set to 20 x24 x20 xyz point covering the entire homeodomain with grid spacing of 1.000 Å and grid center was designed at dimensions (respectively x;y;z) 7.253; 5.579; 14.508. For TGF2LX (PDB code: 2DMN) the grid size ( locations of scan boxes) was set to 20 x20 x20 xyz point covering the entire homeodomain with grid spacing of 1.000 Å and grid center was designed at dimensions (respectively x;y;z) -13.7; 0.918; -5.289. For TGF1 (PDB code: 2LK2) the grid size was set to 20 x 24 x 24 xyz point covering the entire homeodomain with grid spacing of 1.000 Å and grid center was designed at dimensions (respectively x;y;z) -8.817;-10.228; -6.963.

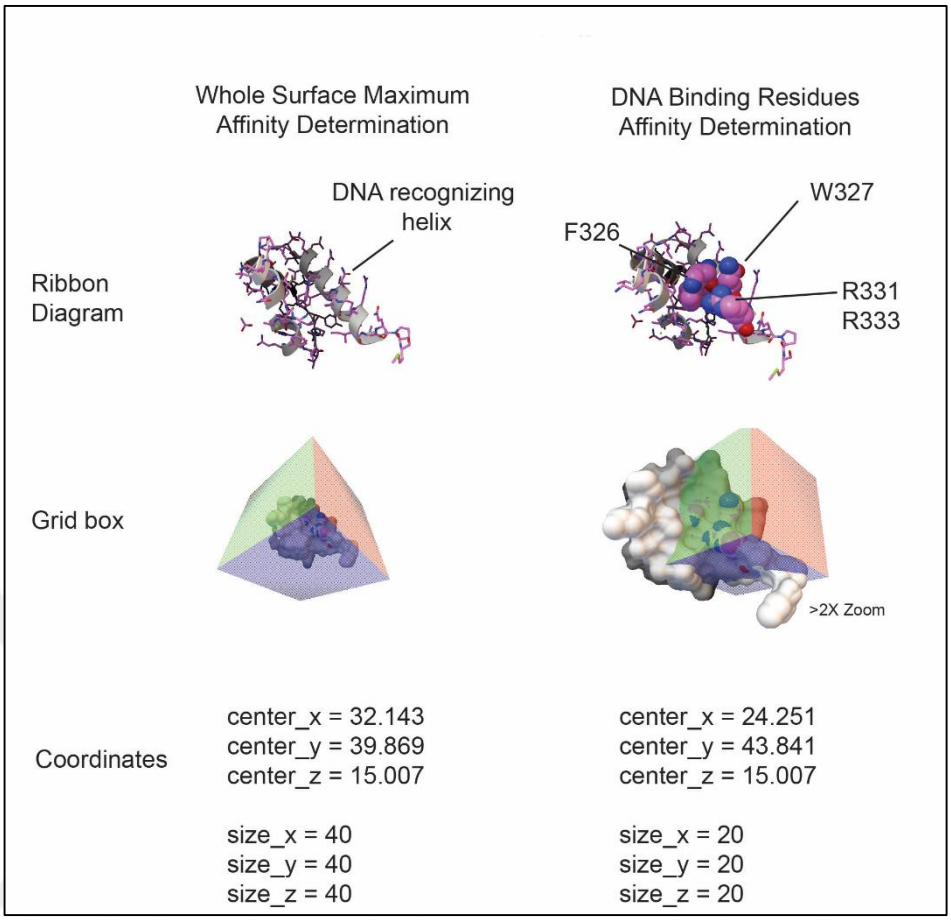


Figure 5.3. Molecular Docking of MEIS Homeodomain.

The binding energy of these molecules against with high affinity for Meis1 homeodomain, was determined and those with high affinity / binding energies to any of these four proteins were eliminated. To sum up, the conserved amino acids were identified by their scan boxes at about 20 Å x 20 Å x 20Å around their equivalents in the homeodomain of these four proteins and molecular docking was performed with the prototype molecules of Meis1 inhibitor. Both Meis1 and these proteins that would be have highly inhibition capacity were eliminated. Potential Meis1-specific molecules were classified in compliance with similarities of 2 dimensional (2D) and 3 dimensional (3D) structure. similarities. PubChem IDs were uploaded to the "PubChem Clustering" [79] server and clustering results obtained in 2D and 3D form.

## 5.5. AFFINITY ANALYSIS OF OF POTENTIAL MEIS INHIBITORS

After our PubChem database analyzes, we found that reported inhibitors were generally against 13 Homeobox genes. Meis1 homeodomain contains amino acid similarities with the homeodomains of other TALE family members. Three-dimensional crystal structures of the homeodomains of the four proteins in the homeobox TALE class (Pbx1, Pknox1, TGIF1, and TGIF2) are known. Those molecules have binding energies against these proteins identified here, and those with high affinity / binding energy to any of these four proteins were screened and eliminated. The affinity difference was calculated for the elimination, the ones which have at least zero point five or higher affinity to Meis1 rather than the other TALE homeodomains were selected. In addition, the collaborative study with Associate Professor Serdar Durdağı revealed those that may have possible cardiotoxicity from the identified hit molecules were eliminated by affinity calculation of the hERG channel. (see Table 5.2) Subsequently, potential molecules of Meis1 were classified according to their 2D and 3D similarity. The "PubChem Clustering" [79] server was used for this. In general, two main group hits were identified in detailed in Figure 5.4.

Table 5.2 Non- cardiotoxic small molecules inherit in MEIS homeodomain

| **Ligand ID** | | **Affinities (AutoDock Vina, kcal/mol)** | | | | | | **Glide XP** |
|---|---|---|---|---|---|---|---|---|
| **CID** | **ZINC** | **MEIS (PDB: 3K2A)** | **PBX (PDB: 1DU6)** | **PKNOX (PDB: 1X2N)** | **TGIF1 (PDB: 2LK2)** | **TGIF2LX (PDB: 2DMN)** | **ΔMEIS vs other TALES** | **hERG channel** |
| 51461848 | ZINC09442725 | -7,6 | -6,3 | -7,2 | -7,5 | -6,6 | 0,7 | -7,6 |
| 41065554 | ZINC09425201 | -7,3 | -5,6 | -6,2 | -5,2 | -5,2 | 1,8 | -7,2 |
| 49665604 | ZINC49406820 | -7,3 | -6,6 | -6,3 | -6,9 | -6,5 | 0,7 | -7,6 |
| 16252566 | ZINC16051182 | -7,3 | -6,7 | -6,9 | -7 | -6,5 | 0,5 | -6,3 |
| 1191314 | ZINC00954985 | -7,2 | -6,2 | -6,4 | -6,2 | -6,3 | 0,9 | -7,1 |
| 56340316 | ZINC71853541 | -7,2 | -6,5 | -6,8 | -6,8 | -6,3 | 0,6 | -7,5 |
| 26538558 | ZINC15354059 | -7,1 | -5,7 | -6,6 | -6,1 | -5,7 | 1,1 | -7,5 |
| 6251472 | ZINC13896602 | -7,1 | -6,5 | -6,8 | -6,4 | -5,7 | 0,8 | -7,2 |
| 1088469 | ZINC00807131 | -7,1 | -6,5 | -7 | -6,6 | -6 | 0,6 | -7,6 |
| 8023393 | ZINC06647669 | -7,1 | -6,4 | -6,7 | -6,1 | -7 | 0,6 | -7,7 |
| 24731800 | ZINC24880387 | -7,1 | -6,3 | -6,9 | -6,5 | -6,7 | 0,5 | -7,0 |
| 5717209 | - | -6,9 | -6,5 | -6,5 | -6 | -5,9 | 0,7 | -6,9 |

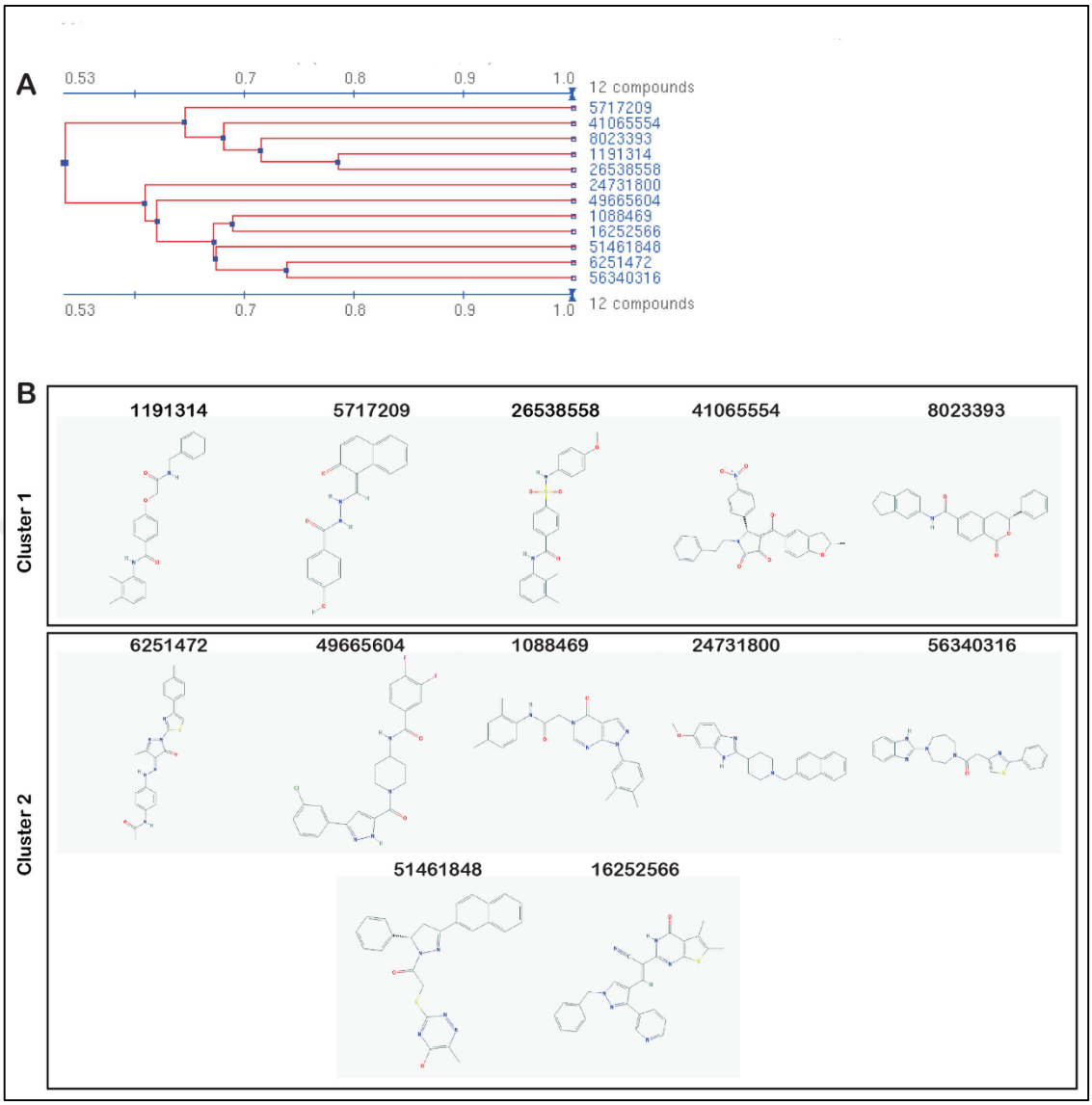


Figure 5.4. Hit small molecules determined as A) 2D categorization and B) two cluster of drug-like small molecules.

## 5.6. LUCIFERASE REPORTER ASSAY

Meis1 can activate luciferase reporter which comprise increasing ratio of p21, p16/p19 promoters [21, 30] pGL2 vectors with p21 promoter which were activated by Meis1 proteins, were used in trascriptional activation in luciferase reporter assay. p21-pGL2 was co-transfected with 400ng of the Meis1 expression vector pCMVSPORT6-Meis1 and 0.2 μg of pCMV-LacZ (internal control) into HEK293T cells. After the transfection cells treated with Meis1 inhibitors at 0.1, 1, 10μM concentration. After 48 hours, luciferase activity was measured using the luciferase reporter system. Luciferase reporter activity was resulted in gradually suppressive effect on p21-Luc MEIS reporter after treated with MEISi-1 as a dose dependent manner that was shown in Figure 5.5.A. On the other hand luciferase assay demonstrating dose dependent increase luciferase activity after treated MEISi-2 inhibitors and their result illustrated in Figure 5.5.B. Subsequent to dose dependent effect of luciferase activity was observed, the effective dose of Meis inhibitors analyzed. Luciferase measurement which absorbance determined at 420nm, normalized to β-Gal units. According to calculation, MEISi-1 inhibitors suppress about five folds increase compared to DMSO control. MEISi-2 reduced the expression of p21-Luc MEIS reporter even not as much as MEISi-1 .

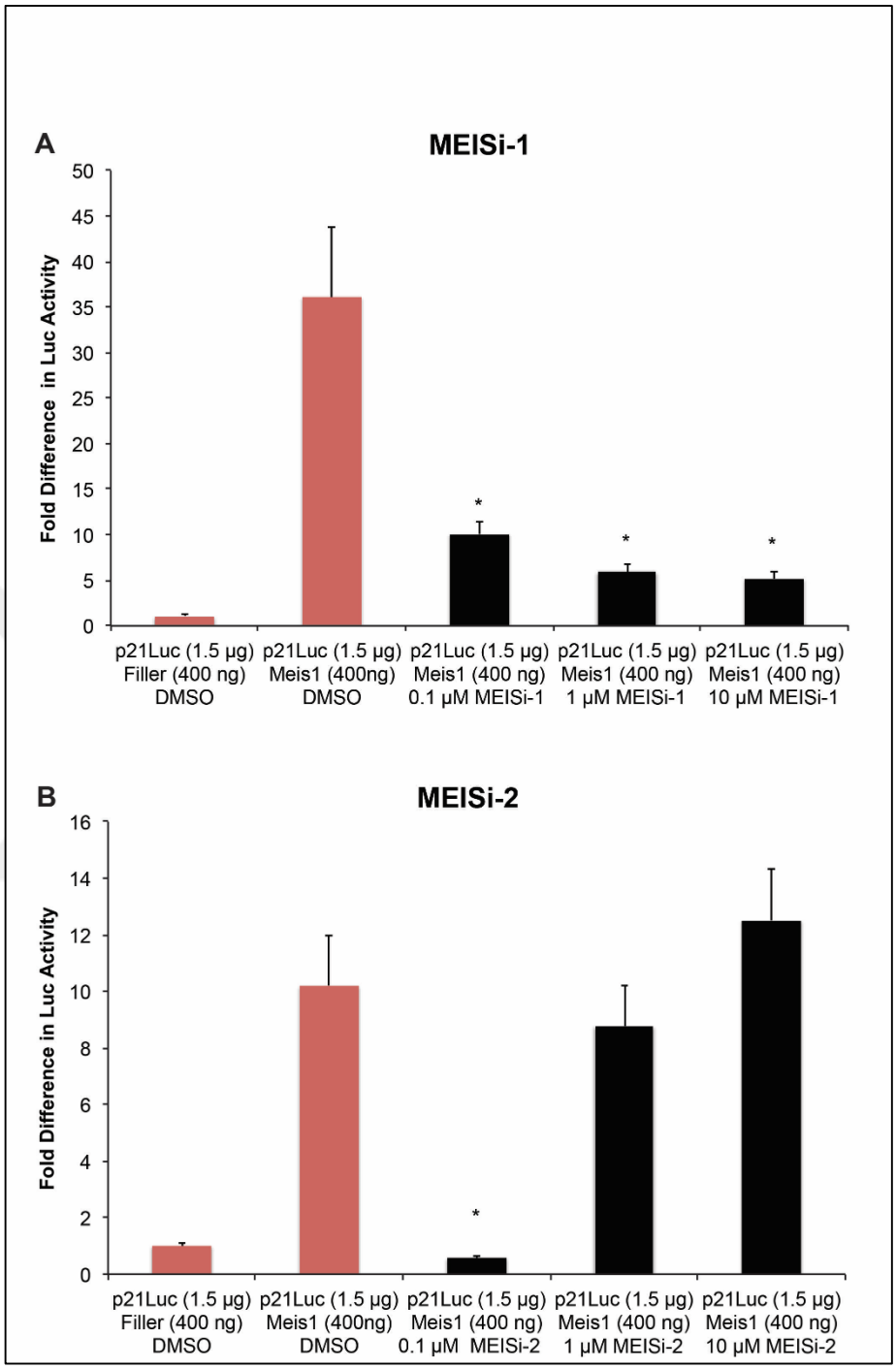


Figure 5.5. *In vitro* confirmation of Meis1 interaction with p21 promoters was done by Luciferase reporter assay. A) Luciferase reporter assay demonstrate dose depending effect of MEISi-1 inhibitors. B) Luciferase reporter assay demonstrate dose depending effect of MEISi-1 inhibitors compared to DMSO control.

(*p<0,05 compared with DMSOcontrol).

## 5.7. HEMATOPOIETIC STEM AND PROGENITOR CELLS EXPANSION

### 5.7.1. Lineage Negative HSPC Count Post Meis-I Treatments

After isolation of WBM, Lin cells obtained by using magnetic seperation. In order to determine effective dose of Meis inhibitors, Lin- cells were treated with MEISi-1 and MEISi-2 at 0.1, 1, 10μM concentration right after seeding. Hematopoietic cell expansion analysed with cell count. Lin- hematopoietic and stem cells which are treated different dosage of Meis inhibitors, stained with hoechest after one day and seven day incubation. The dyed cells were photographed by using the Carl Zeiss Inverted Microscopy (ZEISS, Cat.No.849000464) at a magnification of 4X objectives. Hematopoietic stem and progenitor cells proliferation can be clearly seen in Figure 5.6 irrespective of cell count. HSPCs density was observed less in a first day whereas cell population almost covered the entire plate in seventh day. In other respect, when we compare cell densities, Meis inhibitors treated cells showed that more cell confluency besides to DMSO.

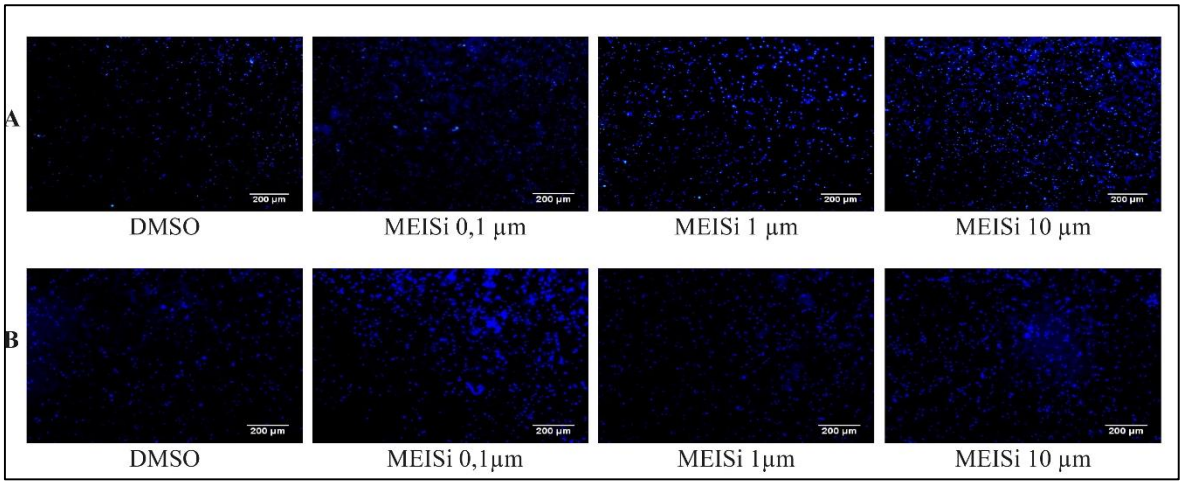


Figure 5.6. The analysis of immunofluorescence staining and image of HSC following treatment with two different small molecules targeting Meis1. A) and B) raws represented respectively 1.day and 7.day.

All pictures which were saved as jpeg form, performed batch conversion and resized by using Irfan view software and saved as a BMP form. After conversion, cells count with Scion Image software according to manufacturer's instructions. The result of cell counting by using Scion Image software prooved that expansion of HSPSCs.with increasing rate of cell number that was illustrated in Figure 5.7. Meis inhibitors treated cells showed that almost 2 fold increase compared to DMSO control in dose dependent manner.

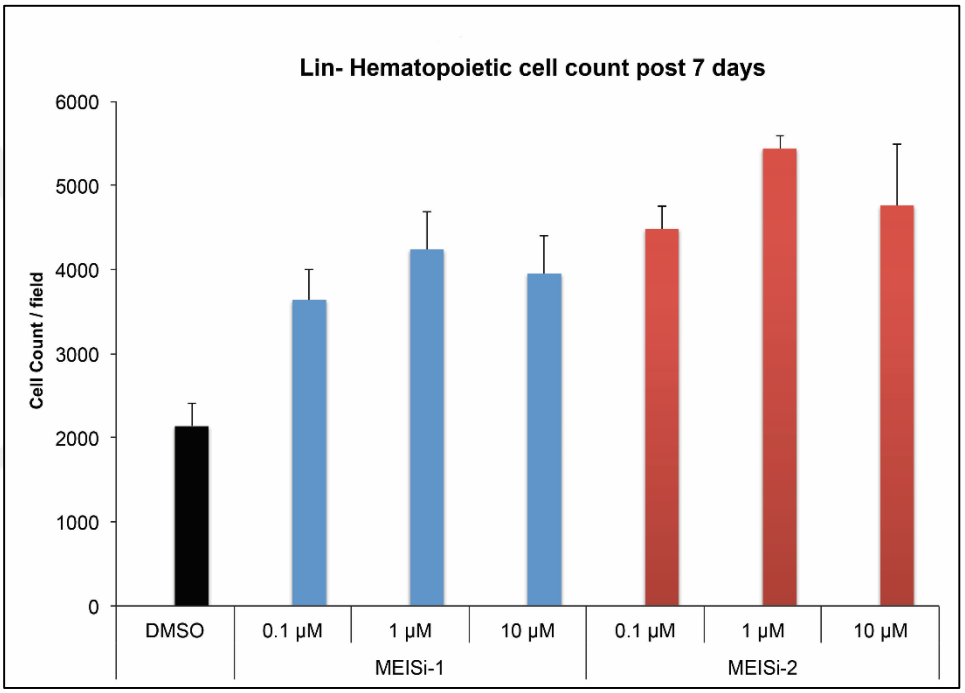


Figure 5.7. HSPCs cell count post seven days following treatment 0.1, 1, 10 µM concentration of MEISi-1 and MEISi-2 inhibitors and DMSO control on mouse lineage negative cells.

### 5.7.2. Cell Count of Lin - HSPCs Post Meisi Treatments with Flow Analysis

In order to determine effective dose of Meis inhibitors, lineage negative cells were treated with MEISi-1 and MEISi-2 at 0.1, 1, 10 μM concentration right after seeding. After seven days incubation, hematopoietic cell proliferation analyzed by flow cytometry. HSPCs labelled with surface markers which are c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7 and mouse APC lineage cocktail (BD StemFlow, Cat No. 560492). We examined the murine hematopoietic stem and progenitor cells characterized by lineage-, Sca-1+ and c-Kit+ (LSK) for LSK and HSC (LSKCD34low) content after seven days of the treatment with Meis inhibitors. While sample analyzed via flow cytometry, PE/ FSC dot plot were examined to observe c-Kit positive population. PE-Cy7/ FSC dot plot were examined to observe Sca1 positive population. Targeted population is LSK cell population for examined expansional effect of Meis inhibitors that consist intersection of C-Kit and Sca1. According to this flow analysis plot dot graph MEISi-1 inhibitor lead to more than 20 fold incresement in comparison with DMSO conrol. On the other hand MEISi-2 inihibitor increase the LSK population even if not MEISi-1 control. prooved that Meis inhibition take a part in expansion. This result revealed that Meis inhibition take a part in expansion of HSPCs (Figure 5.8).

While 100.000 cells event were analyzed and the effect of the doses of these molecules were observed. Three different dosages of MEISi-1 increase C-Kit+ and Sca1+ cell content almost 2 fold. LSK cell count was obtained from double positive population which were comprise of both c-Kit+ and Sca1+ cells and MEISi-1 enhance LSK cell content almost 2 fold compared to DMSO. Although MEISi-2 does not provide as much increase as the MEISi-1 inhibitor, MEISi-2 induces cell proliferation rely on c-Kit+, Sca1+ and LSK cell content. Finally, when we analyzed the accepted as a real HSCs content that was LSKCD34+Low cell content, showed that MEISi-1 and MEISi-2 inhibitors provide expansion. Different dosages of Meis inhibitors were testing on mouse lin- HSPCs and overall cell count proved that the efficient dosage of both Meisi- 1and MEISi-2 was 1 μM (see in Figure 5.9).

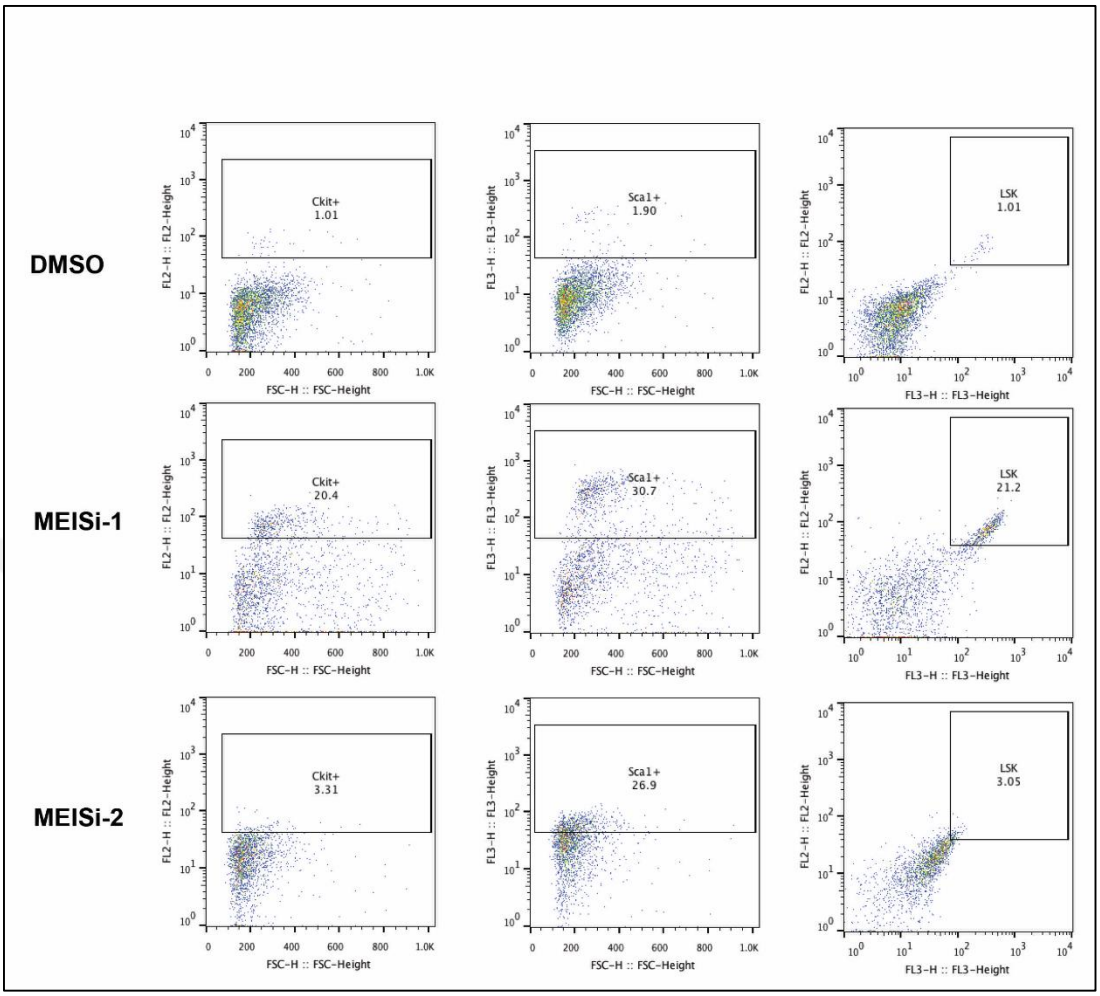


Figure 5.8. Analysis of Sca-1+ and c-Kit+ (LSK) for LSK cell population with dot plot analysis of flow cytometry.

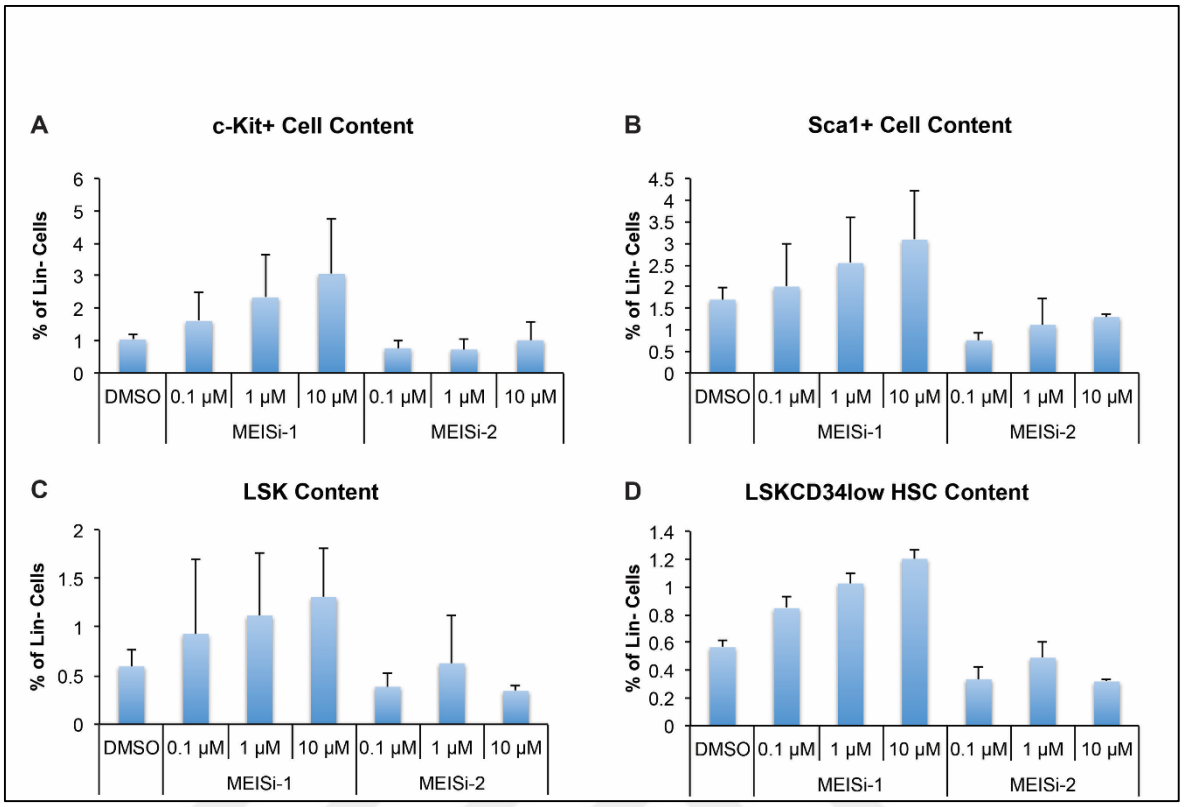


Figure 5.9. Determination of percentage of Lin- cells Content by flow cytometry post.Meis inhibitors treatment. A) the level of c-Kit+ cell content B) the level of Sca1+cell content C) LSK content D) LSKCD34Low content was shown 7 days of treatment with MEISi-1 and MEISi-2 compared to the cells treated with DMSO.

### 5.7.3. Human Umbilical Cord Blood Hematopoietic Cell Expansion Post Meis Inhibitors Treatment

Isolated mononuclear cells from UCB seeded and cells were treated with three different doses (final concentrations; 10 μM, 1 μM, 0.1 μM) of Meisi1 and Meisi2. After the seven days of the treatment, the cells were labelled with human MSC markers; hCD73-APC, hCD90-FITC, hCD105-PerCP/Cy7, hCD45-PE (Biolegend Cat no: 344006, 328108, 323216, 304008 respectively) according to the manufacturer's protocol. After the labelling, the flow cytometry analysis was performed. When we look at the totel mononuclear cell content was increased by Meis inhibiton. When eloborate on results, Meis inhibitors treated cells supply to increase four fold CD34+ and CD133+ population and also provide to two fold increase of CD34+CD133+ cell content. On the other hand Meis inhibitors treated cells supply to increase four fold targeted ALDHbr population that is known as important metabolic enzyme for HSC population. When we examine all result together, the effective dose determined as 1 μM for UCB-HSPCs. (Figure 5.10).

When we analyzed the Meis inhibition response on UCB-HSC content as dose dependently, we observed that effective doses of MEISi-1 and MEISi-2. While 100.000 cells event were analyzed and the effect of the effective doses of Meis inhbitiors were observed. Both MEISi-1 and MEISi-2 increased CD34+ cell count almost seven fold compared to DMSO. MEISi-1 increase the rate of CD133+ cell population more than twenty fold in comparison DMSO.When we compared the effect of MEISi-1 and MEISi-2, MEISi-2 raise the rate of CD133+ cell much more than MEISi-1. MEISi-1 inhibitors expanded CD34+CD133+ cell count more than two fold and ALDHbr cell count almost four fold compared to DMSO. However MEISi-2 provides less proliferation effect on CD34+CD133+ and ALDHbr cell population compared to the MEISi-1 (Figure 5.11).

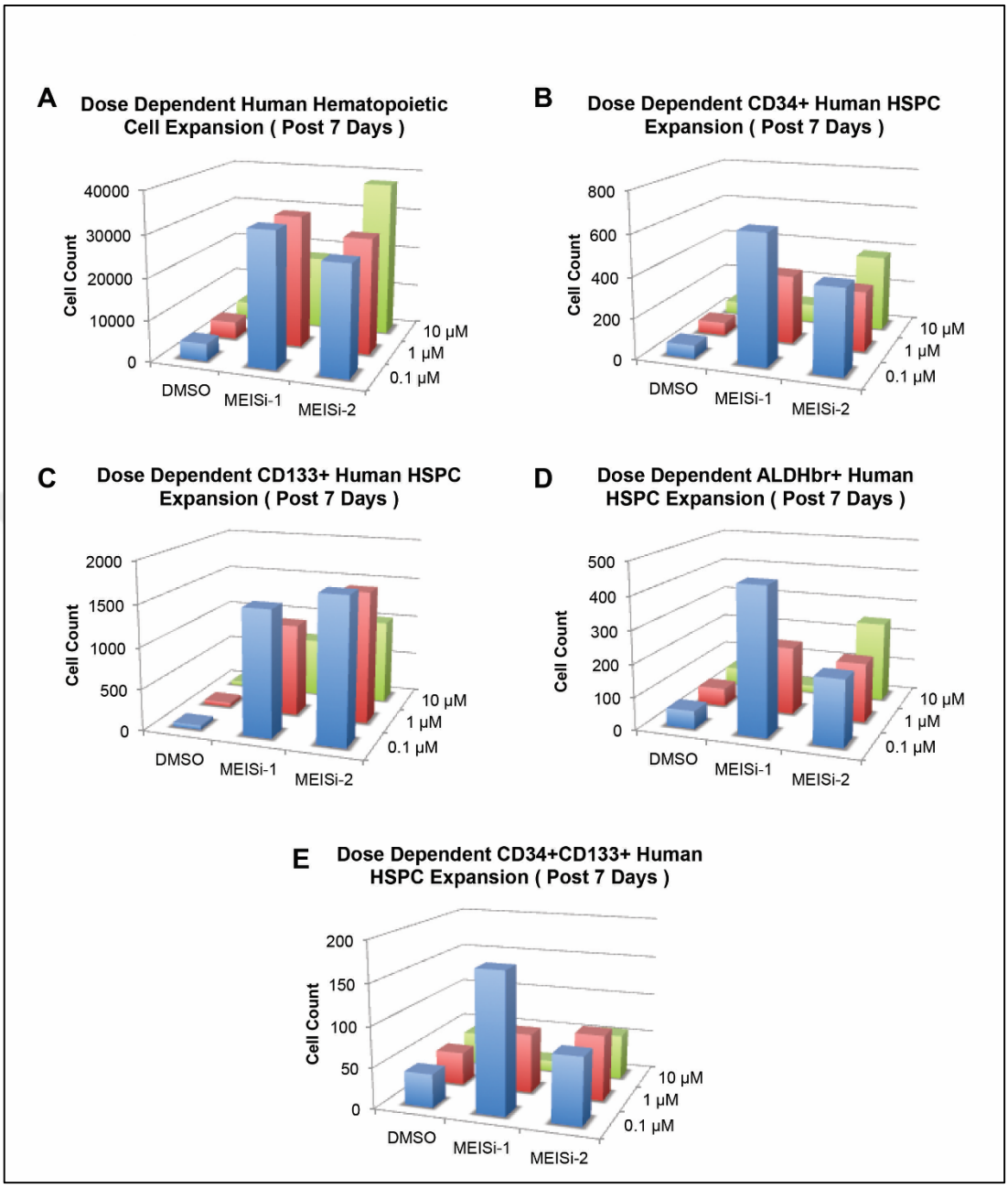


Figure 5.10. Determination of cell content of UCB- HSPCs by flow cytometry post Meis inhibitors treatment as a dose dependent manner. A) the total cell count of human hematopoietic cell count B) the cell count of CD34+ cell content C) the cell count of CD133+D) the level of ALDH br cell population E) the cell count of CD34+CD133+ cell content was shown 7 days of treatment with MEISi-1 and MEISi-2 compared to the cells treated with DMSO (0,5 percent control).

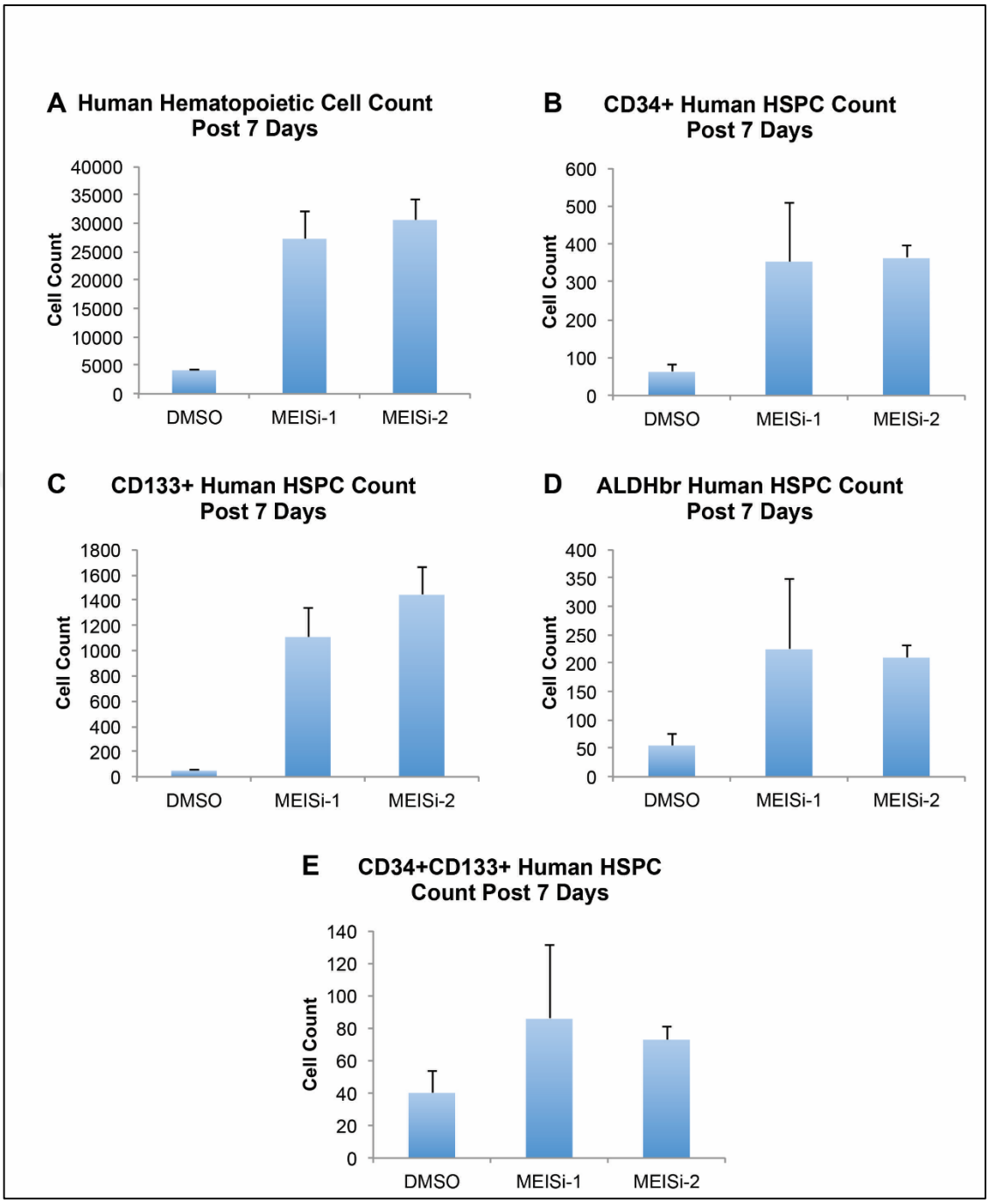


Figure 5.11. Determination of cell content of UCB-HSPCs by flow cytometry post Meis inhibitors treatment. A) the total cell count of human hematopoietic cell count B) the cell count of CD34+ cell content C) the cell count of CD133+D) the level of ALDH br cell population E) the cell count of CD34+CD133+ cell content was shown 7 days of treatment with MEISi-1 and MEISi-2 compared to the cells treated with DMSO (0,5% control).

### 5.7.4. Human Bone Marrow Derived Hematopoietic Cell Expansion Post Meis Inhibitors Treatment

Human bone marrow collected as a apheresis product and mononuclear cells isolated by using ficol and gradient centrifuge method. In order to analyse proliferation effect of Meis inhibitors on human bone marrow derived hematopoietic stem cells, cells treated with Meisi -1 and MEISi-2 just after seeding. Human bone marrow derived hematopoietic stem cells were labelled with CD34, CD133 , CD38 and CD90 post seven days and analysed with flow cytometry. The effect of MEISi-1 and Meisi2 inhbitiors differ from ach other for human bone marrow derived hematopoietic cells. When we analysed the percentage of CD34+ and CD34+CD38- cells MEISi-1 trigger proliferation and provides almost three fold increase compared with DMSO control. If we look at the CD133+ and CD90+ cell content MEISi-1 increase the cell population more than two fold. CD34+CD133+CD90+ and CD34+CD133+CD38- cell population targeted more specifically hematopoietic stem cell population and MEISi-1 could be raised these population almost 4 fold. However MEISi-2 could not be raised CD34+, CD90+, CD34+CD38-, CD34+CD133+CD90+ and also CD34+CD133+CD90+CD38-cell populations even if DMSO control, this inhbitors could rise just CD133+ cell population almost two fold compared with DMSO control (see Figure 5.12).

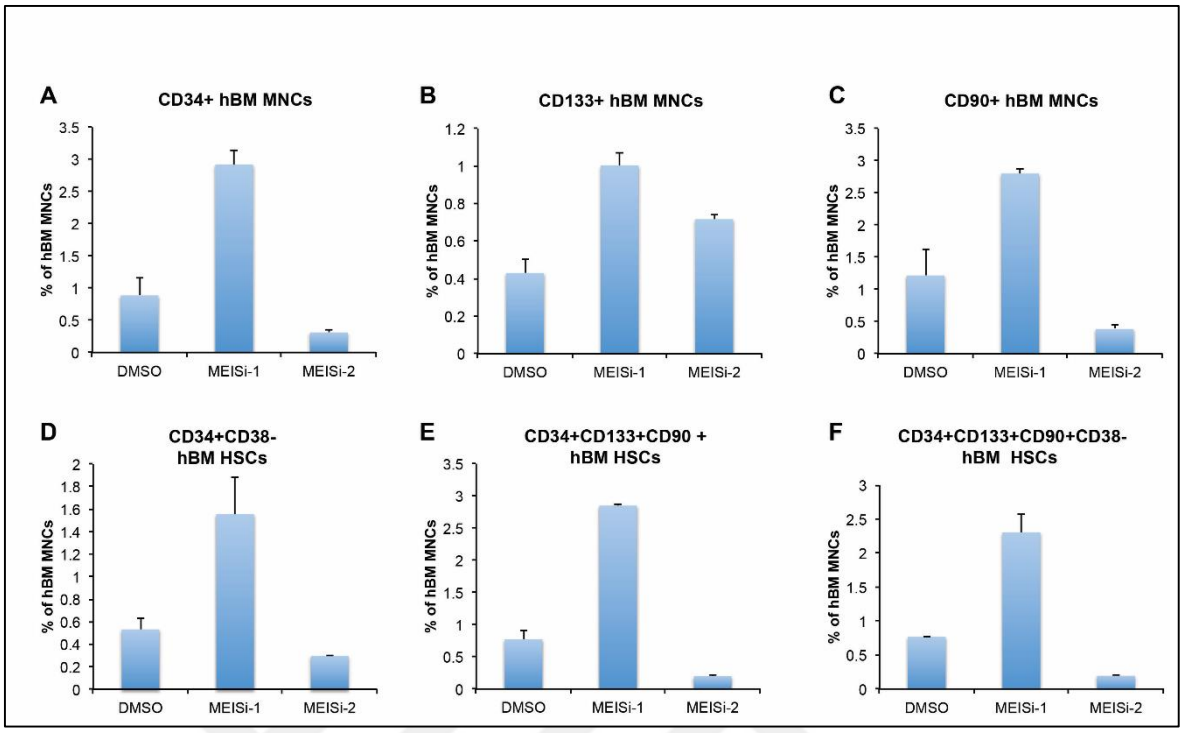


Figure 5.12. Determination of cell content of human BM- HSPCs by flow cytometry post Meis inhibitors treatment. A) the percentage of CD34+ cell content B) the percentage of CD133+ cell content C) the percentage of CD90+ cell content D) the percentage of CD34+CD38- cell content E) the percentage of CD34+CD133+CD90+ cell content F) the percentage of CD34+CD133+CD90+CD38- cell content was shown 7 days of treatment with MEISi-1 and MEISi-2 compared to the cells treated with DMSO.

## 5.8. CELL CYCLE ANALYSIS

Lin- cells isolated from Balb-C mice and lin- cells population labeled with APC lineage cocktail, anti-mouse c-Kit (CD117) PE, Sca-1 PE-Cy7 and CD34 FITC and that were sorted via FACS-Flow. Thus murine LSKCD34low (Lin-Sca1+c-Kit+CD34low) cells population which were treated with MEISi-1 and MEISi-2 at 1 µM concentration, were examined cell cycle by using Hoechst 33342 (10 µg/ml) (Sigma Aldrich, USA, Cat.No. 14533) and Pyronin Y (100µg/ml) (Sigma Aldrich, USA, Cat.No. P9172-1G) and analyzed via flow cytometry FACSARIA III, BD Biosciences.

LSKCD34low cell were obtained in order to examine the effect of Meis inhibitors on cell cycle arrest .When murine HSCs were analyzed both post four and seven days, the HSC content of LSKCD34low cells were not significant increment in G0 phases of the cell cycle in comparison to DMSO control. On the other hand the HSC content of LSKCD34low cells were inappreciably increase G1 phases post seven days compared to post four days that was shown in Figure 5.13.

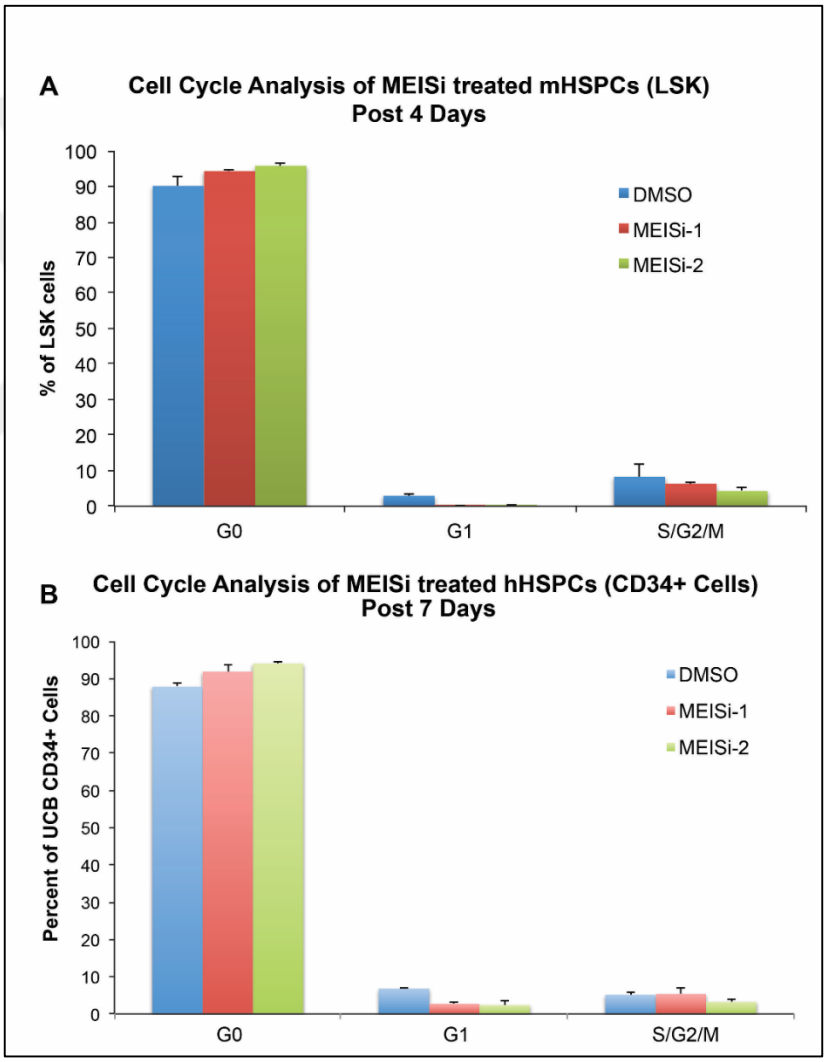


Figure 5.13. Cell cycle analysis of murine HSCs after treated with DMSO (control,0,5%), MEISi-1 (1 µM), MEISi-2 (1 µM). A) following treatment Meis inhibitors with control post four days and B) following treatment Meis inhibitors with control post 7 days. Murine HSCs were examined as $G_0$ , $G_1$ and S/ $G_2$ / M followed by staining with Hoechst 33342 and Pyronin Y.

## 5.9. ANNEXIN—V APOPTOSIS ANALYSIS

Isolated lin- cells population labeled with Sca-1, c-Kit and Lin coctails and and that were sorted by flow cytometry (FACSARIA III, BD Biosciences, Cat.No. 23-11539-00). Thus murine LSK cells (Lin- Sca1+C-Kit+) were obtained. Sorted cells were seed as 5000cells/well and treated with Meis inhbitors just after seeding. After three day incubation, cells were collected via centrifugation and resuspended 1X Binding Buffer and then labelled with Annexin V-FITC and Propidium Iodide. Labelled cells incubate in the dark for staining at room temperature in order to do quantification of Annexin V-FITC binding cells via flow cytometry. Apoptotic and necrotic cell behavior were examined after healthy cells treated with Meis inhibitors. LSKCD34Low cells after treated with 1 µM MEISi-1 and MEISi-2 indicated that cell population were not necrotic. Besides, MEISi-1 and MEISi-2 treated LSKCD34Low cells were examined as late apoptosis this is not significant increment in comparison with DMSO control that was shown in Figure 5.14..

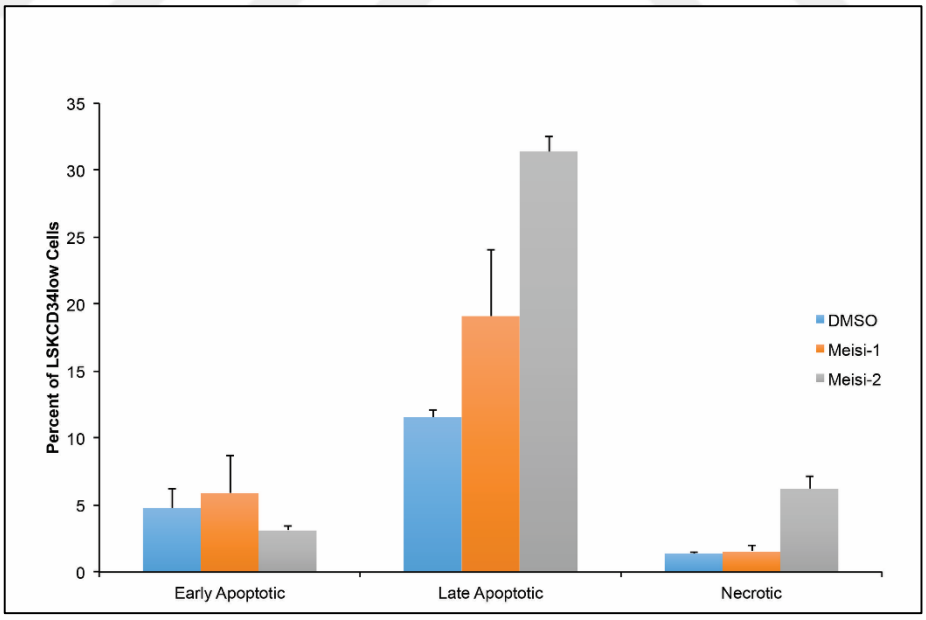


Figure 5.14. Apoptosis analysis of murine HSCs after treated with DMSO (control,0,5%), MEISi-1 (1 µM), MEISi-2 (1 µM) post three days. Murine HSCs were examined asearly apoptotoic, late apopototoic and necrotic followed by staining with Annexin V- FITC and PI.

## 5.10. COLONY FORMING UNIT ASSAY

Colony forming unit assay take an important part among *ex vivo* experiments. CFU assay revealed that the potential expantional profile of the molecules on HSCs. Lin- cells were incubate and then HSPCs were seed by using methoult media. Different kind of colonies were eventuated throughout 12 days of incubation at humidfying cell culture incubator. Colonies were observed with naked eye as a knoll on the media but that were analyzed via Carl Zeiss Inverted Microscopy (ZEISS, Cat.No.849000464) at a minimum magnification of 10X in order to identify and count colonies.

The most important point of this experiment was pink colonies which are BFU-E colonies and that was a sign to erythroid progenitor cells. We illustrated that in Figure 5.15. MEISi-1 inhbitor treated cells had pink BFU-E and mix colonies. Also MEISi-2 had increased number of CFU-GEMM colonies and erythroid progenitor cells (BFU-E colonies) compared to DMSO control but there is no pink colonies ( see in Figure 5.15).

Overall results demonstrate that both MEISi-1 and MEISi-2 inhibitors treated cells had higher number of primitive progenitor and hematopoietic stem cells by extension of increased number of CFU-GEMM and BFU-E colonies compared to DMSO control. However there was no significant change in the number of myeloid progenitor cells (CFU-G/M/GM colonies). In addition, number of CFU-GEMM were six fold higher in MEISi-1 treated hematopoietic cells compared to DMSO control and three fold higher compared to MEISi-2treated hematopoietic cells and also BFU-E colonies were two fold higher in MEISi-1 treated hematopoietic cells compared to DMSO control (Figure 5.16).

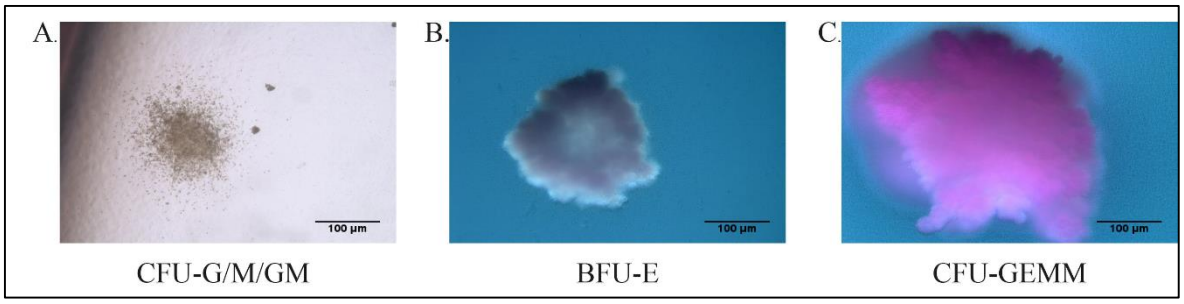


Figure 5.15. CFU analysis of HSCs post Meis inhibitors treatment. Thecolony image of A) CFU-G/M/GM B) BFU-E C) CFU- GEMM was illustrated.

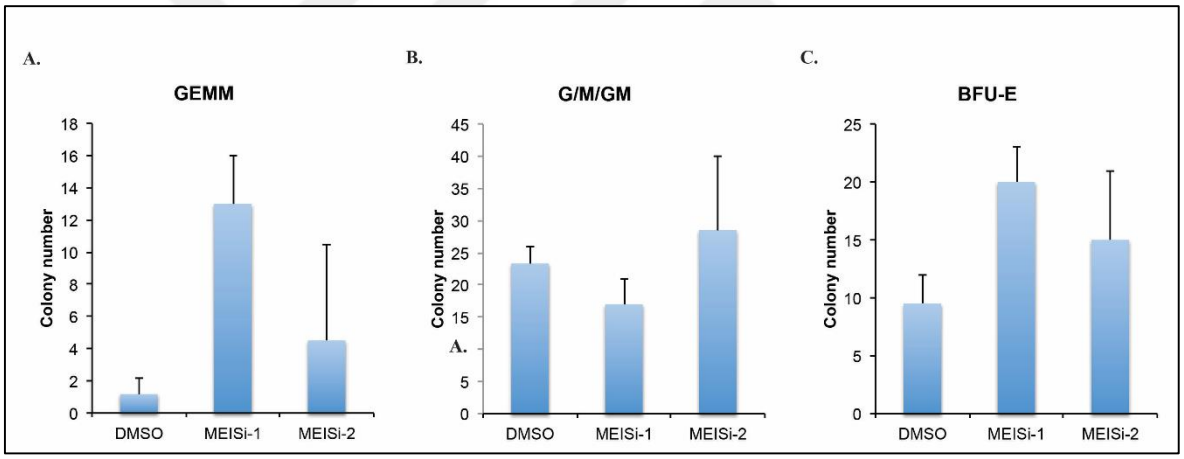


Figure 5.16. CFU analysis of HSCs post Meis inhibitors treatment. The numbers of colony were gathered post 12 day incubation and illustrated as A) CFU- GEMM, B) CFU-G/M/GM and C) BFU-E colonies formed.

## 5.11. QUANTITATIVE ANALYSIS OF CDKI GENE EXPRESSION

We try to identify Meis inhibitiors that responsible for MEIS inhibition. In order to determine the effect on expansion of HSPCs, Relative mRNA expression levels were examined for Meis target genes, hypoxia inducible factor (HIFs) and cyclin-dependent kinase inhibitors (CDKI). As a result of which data was analyzed using the ΔΔCt method. Fold change was calculated as difference in gene expression and that normalized based on DMSO control and also GAPDH was used as a housekeeping control.

*In vitro* gene expression were determined with Lin- cells by using real time PCR (Lightcycler 96, Roche CatNo: 12953 ). Lin- cells treated with 1 μM MEISi-1 and MEISi-2 and DMSO control (zero point five percent) just after seeding and incubate for four days at humidified cell culture incubator at 37°C and 5% $CO_2$. When transcriptional activation was analyzed, Meis inhibitors induce significant downregulation of CDKIs including CDKN1A (p21), CDKN2B (p15), CDKN2A (p16), p19 (CDKN2D), p19ARF, CDKN2C (p18), and CDKN1C (p57). On the other hand, we found that that expression level of HIfs including Hif1α, Hif2α, Hif3α down regulated by Meis inhibition.

The CDKIs which are p15, p16, p21 expressions were increased more than 5 fold compared to DMSO control, especially p15 expression was decreased almost 10 fold after the MEISi-1 treatment, but we found that there were no meaningful changes on p18, p19ARF, p27 and p57 expression after MEISi-1 treatment. (see Figure 5.17). Especially MEISi-1 inhibitors reduced more than 10 folding to Hif2α expression compared to DMSO control. Besides, MEISi-1 treatment resulted in reduced Hif2α, Hif3α gene expression.

The HIFs including Hif1α, Hif2α, Hif3α were downregulated after MEISi-2 treatment. However, when Hif2α reduction was more than 5 fold, Hif1α, and Hif3α were not that much. The CDKIs which are p16, p21 expressions were increased approximately 2 fold, p19, p57 expressions were decreased about 5 fold, p18, p27 expressions were reduced more than 10 fold compared to DMSO control, especially p19ARF expression was decreased almost 20 fold after the MEISi-2 treatment, but we found that there were no meaningful changes on p15. (Figure 5.17).

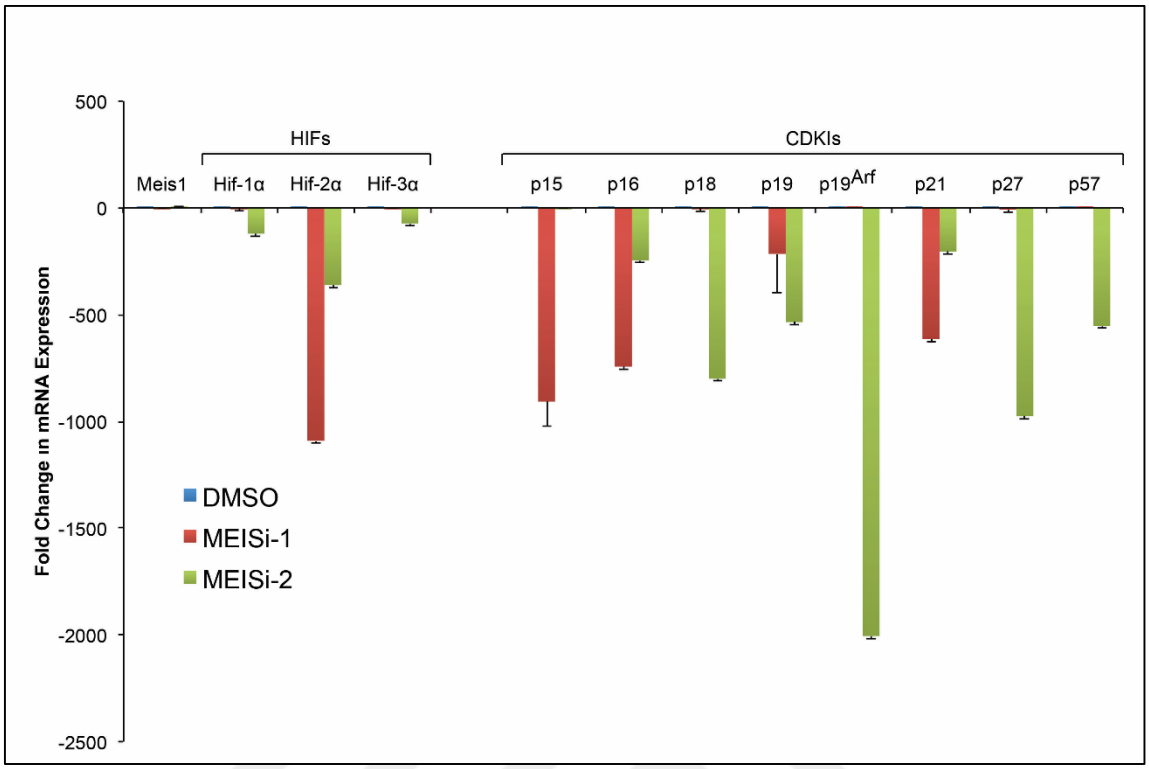


Figure 5.17. Gen expression analysis of Meis inhibitors applied Lin- cells. After lin- cells treated with 1 µM MEISi-1 and MEISi-2 and DMSO control were analyzed for expression of CDKIs including CDKN1A (p21), CDKN2B (p15), CDKN2A (p16), p19 (CDKN2D), p19ARF, CDKN2C (p18), and CDKN1C (p57) and expression level of HIfs including Hif1α, Hif2α, Hif3α.

## 5.12. CELL VIABILITY ANALYSIS

In this research we aimed to induce HSPCs expansion by using small molecules. Most ex-vivo experiment prooved that Meis-1 and MEISi-2 inhibitors can raise the raito of HSPCs .In order to examine the Meis inhibiton whether spesicif to hematopietic stem cells or not we used various type of stem cells. Bone marrow derived mesencyhmal stem cells and adipogenic mesencyhmal stem cells were used and treated with effective dose of MEISi-1 and MEISi-2 (as so final concentration was 1 µM ) and DMSO (zero point five percent, as a control).

After 3 days incubation cell viability was measured with WST1 assay. As a result there is no remarkable difference on Meis inhibitors treated bone marrow derived mesencyhmal stem cells and adipogenic mesencyhmal stem cells compared to DMSO control. In addition to analysis stem cells, human vascular endothelial cells (HUVEC) was examined at the same condition and their result same as MSCs there is no considerable alteration compared to DMSO control.(see in Figure 5.18).

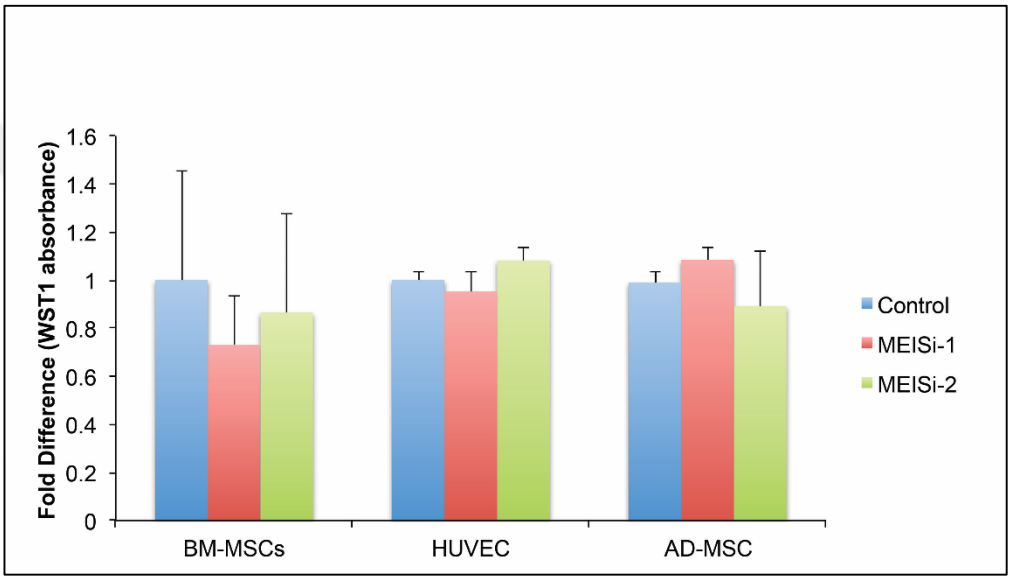


Figure 5.18. Cell viability effect of MEISi-1 and MEISi-2 on BM- MSCs, HUVEC and AD-MSCs. BM-MSCs, HUVECs, and AD-MSCs, were treated with 1 µM MEISi-1, MEISi-2 inhibitor and DMSO (control, 0.5%). Fold difference in WST1 absorbance at 450nm was determined after 3 days.

L929 is murine fibroblastic cells an we used L929 cell line to identify the cytotoxic effect of Meis inhibitors. Firs of all we used the specific three dose which are 0,1 µM, 1 µM, 10 µM and these concentration range were used for determination of efficient concentration. If we look at the percentage of cell viability via WST1 assay, results prooved that curren dosage were not cytotoxic. (Figure 5.19.A).

In order to determine cytotoxic line, we prefer to wide concentration range and 0,001 µM, 0,1 µM, 1 µM, 10 µM, 20 µM, 50 µM and 100 µM MEISi-1, MEISi-2 inhibitor were used. Besides wide concentration range of Meis inhibitors, positive and negative control were used. Negative control were comprised of just expansion media without cells. Positive control were comprised of just expansion media with cells. According to measured WST1 absorbance cell viability was not be affected until 1 µM concentration. (Figure 5.19.B). However when used concentration were increased more than 1 µM, cell viability reduced dramatically. Nevertheles used concentration of Meis inhibitors were not greater than 1 µM.

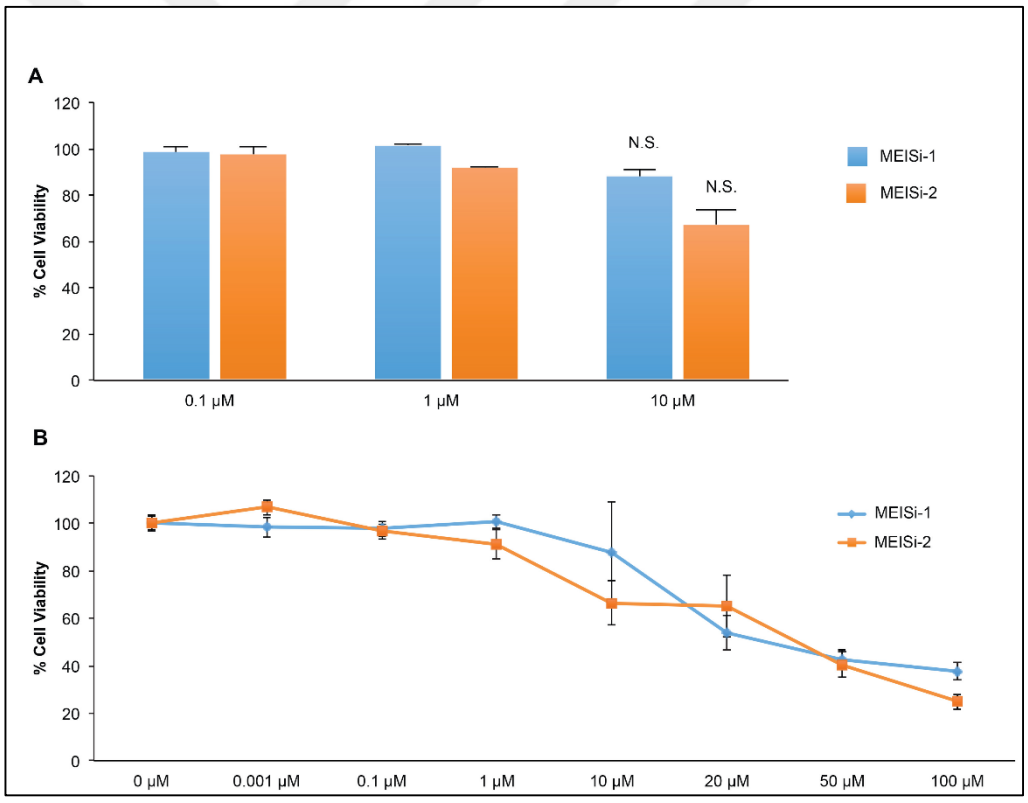


Figure 5.19. Cell viability effect of MEISi-1 and MEISi-2 on L929 murine fibroblast cells dose dependently. A) L929 were treated with 0,1 µM, 1 µM, 10 µM MEISi-1, MEISi-2 inhibitor and DMSO (control, 0.5%) B) L929 were treated with 0,001 µM, 0,1 µM, 1 µM, 10 µM, 20µM, 50µM and 100µM MEISi-1, MEISi-2 inhibitor. Fold difference in WST1 absorbance at 450nm was determined after three days

The relationship between Meis1 and cancer were known in publication until this time. For this reason we try to determine proliferaiton kinetics on K562 myelogenous leukemia cell line and U937 histiocytic lymphoma with Meis inhibiton. Their proliferation effect were analyze with WST1 assay and as time dependent manner. K562 and U937 were seed and treated with MEISi-1, MEISi-2 inhibitor on separately plate as day0, day1, day2, day3, day4 and day5. Each days cell proliferation kinetics were measured via WST1 absorbance at 450nm and their absorbance measurements were taken every half and hour. Second day absorbance measurement showed that Meis inhibition reduce the cell proliferation on both K562 and U937 cell line. However tthis result cannot be have noteworthy meanings. Since cell proliferation kinetics acceptable as stabile during ongoing process. Meis inhibiton with using meis inhibitors may give a result with detailed research for cancer treatment but not now. As a result fold differences absorbance values showed that proliferation kinetics admittable as stable especially MEISi-1 and MEISi-2 treated K562 myelogenous leukemia cell line ( seeFigure 5.20.A). If we examine MEISi-1 and MEISi-2 treated U937 histiocytic lymphoma, even second day absorbance measurement reduced with Meis inhibition, cell proliferation kinetics were induced in the ongoing process but untill starting level ( see Figure 5.20.B).

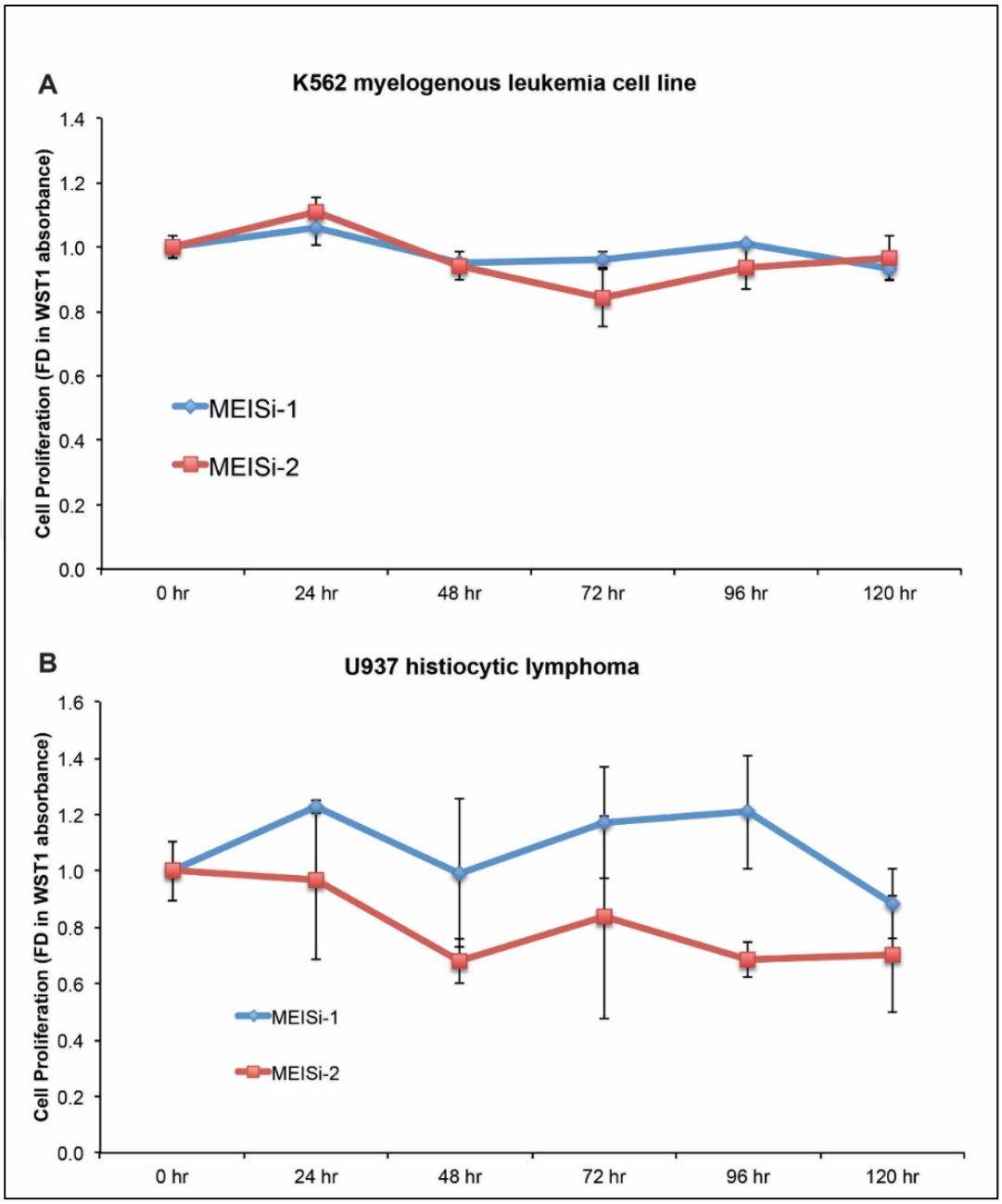


Figure 5.20. Cell proliferation effect of MEISi-1 and MEISi-2 on cancer cell line as a time dependent manner. A) K562 myelogenous leukemia cell line were treated with MEISi-1, MEISi-2 inhibitor and B) U937 histiocytic lymphoma were treated with MEISi-1, MEISi-2 inhibitor. Fold difference in WST1 absorbance at 450nm was determined throughout one week.

## 5.13. MUTAGENITY ANALYSIS (AMES TEST)

In order to examine mutagentiy of MEISi-1 andMEISi-2, first of all anti-microbial test were applied ( Figure 5.21). Anti-microbial activity were analysed with disc diffusion test by using 200µg of MEISi-1 and MEISi-2 were added onto the two separate disks on uniformly swabbed of Salmonella Typhimurium (ATCC Cat.No. 29629). After inoculation with this bacterium, there is no zone formation. Thereby histidine dependent mutagenity test become feasible. Ames test were applied with negative and positive control, positive control involve mutagens andnegative control comprise just buffer. Following AMES test prooved that both MEISi-1 and MEISi-2 has no mutagen capacitty according to counted number of His+ revertant of Salmonella Typhimurium ( Table 5.3).

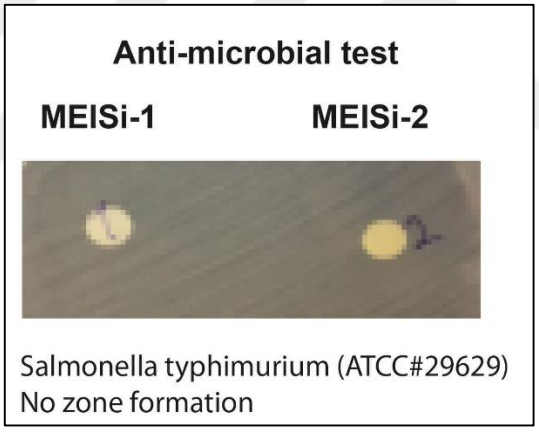


Figure 5.21. Anti-microbial test for Meis inhibitors that was initial step of AMES test.

Table 5.3. Colonies Number of His+ Revertant of *Salmonella typhimurium.*

| **AMES Test** | **Positive Control ($NaN_3$)** | **Negative Control** | **Meisi-1** | **Meisi-2** | **Positive Control + Meisi-1** | **Positive Control + Meisi-2** |
|---|---|---|---|---|---|---|
| # Colonies | 361±29 | 24±3 | 75±11 | 80±4 | 402±43 | 343±36 |
| p-value | | | 0.0066 | 0.0003 | 0.4790 | 0.7520 |

## 5.14. IN VIVO ANALYSIS OF POST MEISI TREATMENTS HEMATOPOIETIC CELL COMPARTMENT

1 μM MEISi-1, 1 μM MEISi-2 and DMSO control (zero point one percent ) were intraperitoneally injected to 4-5 week-old BALB/c mice at day 1st, 4th and 7th . After 10 days of following injections, BALB/c mice sacrificed and whole bone marrow cells isolated and WBM cells analyzed with APC lineage cocktail, c-Kit (CD117) PE, Sca-1 PE-Cy7, CD34 FITC and slam markers which are APC lineage cocktail, c-Kit (CD117) PE, Sca-1 PE-Cy7, CD150 FITC and CD48 APC shown in Table. The repopulated HSC compartment were analyzed via flow cytometry. According to flow cytometric analysis, when MEISi-1 induced more than 3 fold inreasec-Kit+ and Sca-1+ hematopoetic cell content, MEISi-2 provided 2,5 fold increase c-Kit+ and almost 1 fold Sca-1+ hematopoetic cell content compared with DMSO control (see Figure 5.22.A and Figure 5.22.B). MEISi-1 and MEISi-2 increase LSK cell content (Figure 5.22.D) but meaningful increment was observed in LSKCD34low cell content.(see.Figure 5.22.E) When Meis inhibition examined with slam markers, MEISi-1 supplied to more than 5 fold increase CD150+ hematopoetic cell content and also MEISi-2 more than 7 fold increase CD150+ hematopoetic cell content in comparison to DMSO control. (Figure 5.22.C). Finally the MEISi-1 increased LSKCD15+CD48- expression approximate two fold and the MEISi-2 raised LSKCD150+CD48- expression approximate 5 fold in proportion to DMSO control.

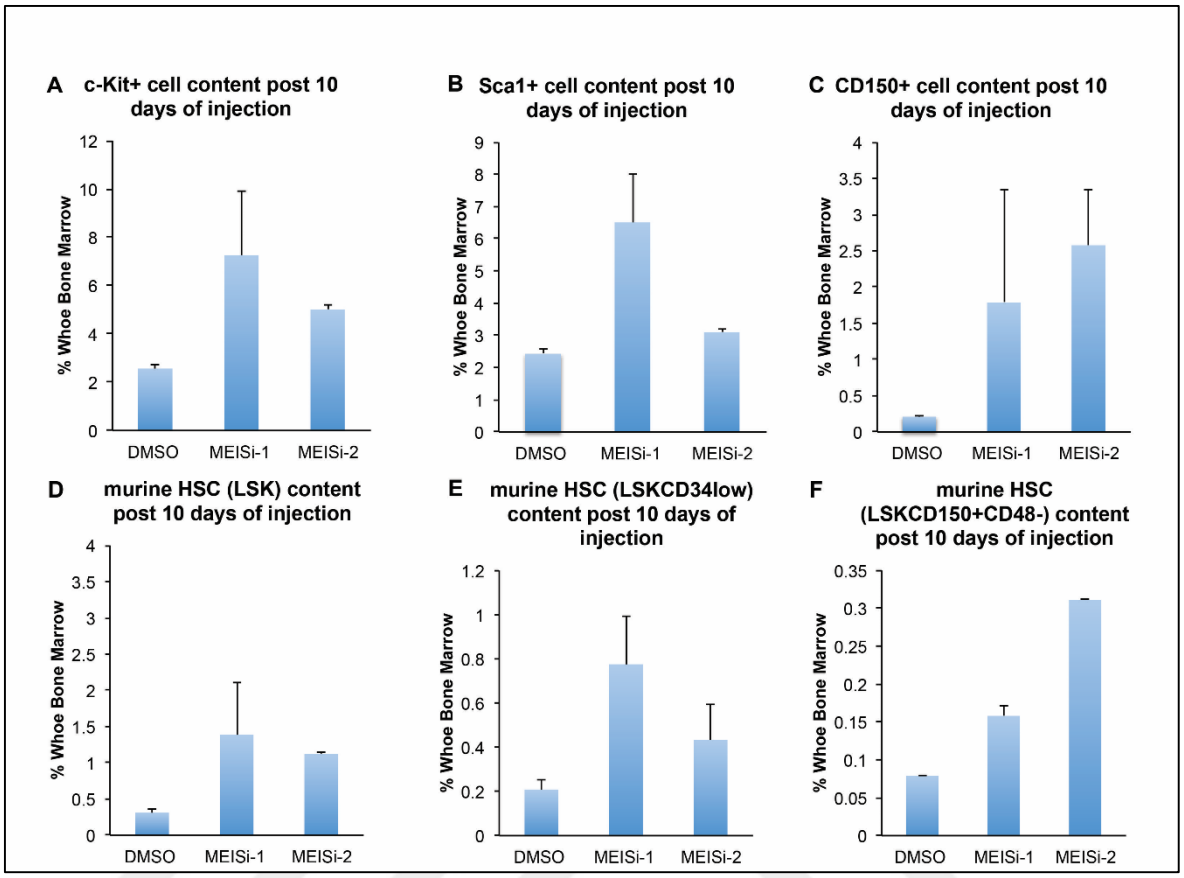


Figure 5.22. *In vivo* analysis of HSC compartment post MEISi treatment. A) c-Kit+ cell content in the whole bone marrow following injection of MEISi-1, MEISi-2 and DMSO contol. B) Sca-1+ cell content in the whole bone marrow following injection of MEISi-1, MEISi-2 and DMSO contol. C) CD150+ cell content in the whole bone marrow following injection of MEISi-1, MEISi-2 and DMSO contol. D) Percent of LSK cell content post ten days injection of MEISi-1, MEISi-2 and DMSO contol. E) Percent of LSKCD34low cell content post ten days injection of MEISi-1, MEISi-2 and DMSO contol. F) LSKCD48-CD150+ HSCs in the whole bone marrow following injection of MEISi-1, MEISi-2 and DMSO contol.

## 5.15. *IN VIVO* QUANTITATIVE ANALYSIS OF CDKI GENE EXPRESSION

Expression level of cyclin dependent kinase inhibitors were detected in whole bone marrow by using quantitative polymerase chain reactions (qPCR) following *in vivo* injection. B-actin was used as the house keeping gene. As a result of which data was analyzed using the ΔΔCt method. Fold change was calculated as difference in gene expression and that normalized based on B-actin. When transcriptional activation of entreated Meis inhibitors was analyzed, CDKIs including CDKN1A (p21), p27, CDKN2A (p16), p19 (CDKN2D), p19ARF, CDKN2C (p18), and CDKN1C (p57) and HIfs including Hif1α, Hif2α, Hif3α were used ( see Figure 5.23).

Meis-1 inhibiton lead to downregulation of CDKIs including p16, p19, p19ARF and p57; 3 fold, 5fold, 11 fold, 3 fold respectively. On the other hand, Meis1 gene was downregulated 3 fold on the presence of MEISi-1. When we observed that gene expresssion of HIFs, while MEISi-1 downregulated the Hif2α gene 4 fold, intriguingly upregulated the Hif1α. However there was no remarkable effect of MEISi-1 on Hif3α gene expression. Inhibition with MEISi-2 provides downregulation on CDKIs gene expression except p27 and p21. There is no significant effect of MEISi-2 on P21 gene expression. If MEISi-2 inhbition effect on HIFs gene expression were examined, while Hif1α and Hif2α were downregulated 2 and 5 fold but Hif3α were upregulated as 2fold. When we analyze overall results, Meisi1, p16 , p19, p19ARF geen expression down regulated more thad 5 fold in both MEISi-1 and MEISi-2 inhibtion.

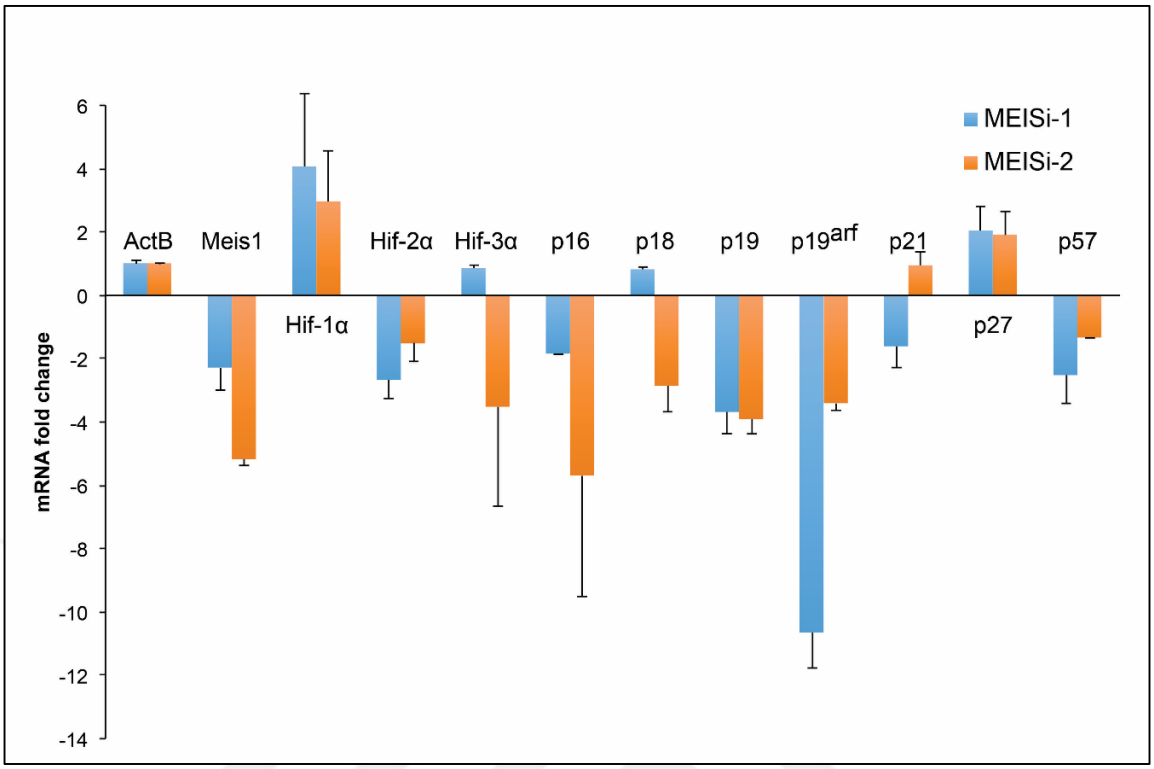


Figure 5.23. Gen expression analysis of CDKIs and HIFs following in vivo injected Meis-1 and MEISi-2 inhibitors. After injection 1 µM MEISi-1 and MEISi-2 and DMSO control were analyzed for expression of CDKIs including CDKN1A (p21), CDKN2B (p15), CDKN2A (p16), p19 (CDKN2D), p19ARF, CDKN2C (p18), and CDKN1C (p57) and expression level of HIfs including Hif1α, Hif2α, Hif3α.

# 6. DISCUSSION

Hematopoietic Stem Cells (HSCs) are recognized by properties of self-renew and differentiation to all blood cell types. HSCs are responsible for generation of billions of mature blood cell lines throughout life of an adult.[68, 80] However, hematological diseases get damaged the rutin process. In this case, bone marrow transplantation is primary treatment method for hemataological diseases. HSC transplantation has been used for treatment of leucemia, lymphoma, some solid cancers, autoimmune diseases and genetic diseases such as sickle cell anemia or mediterranean anemia [67]. While the cells achieve basis of the bone marrow transplantation, also, have a promising future for gene therapy studies [80]. Genetically modified HSCs could be used for treatment of genetic diseases such as sickle cell anemia or mediterranean anemia, in recent years.[61, 66-68]

Recent studies carry out on single cell expansion after the genetic arrangement on the cells. The insufficient on the number of HSCs because of lack of Human Leukocyte Antigen (HLA)-matched cells obtained from the donors leads to major problem for the transplantation. HSC transplantation efficiency depends on the presence of HLA-matched donor and obtaining of sufficient number of HSC from the donor for the engraftment. [65]

The insufficient of the HSC number obtained from donor has decreased the bone marrow transplantation success. Therefore, the problem has been solved by inducing *ex vivo* HSC expansion. It is possible that mouse and human HSCs could be isolated and identified by staining of surface antigens with fluorescence activated cell sorting (FACS), and expanded by treatment with TPO, Flt-3l, SCF cytokines. While the most of the method related to HSC expansion depends on cytokines and growth factors, usage of small molecules which target to HSC quiescence factors is not highly prevalent. Thus, therapeutical approaches which could be beneficial for *ex vivo* HSC expansion have been possible by targeting of HSC quiescence regulators with small molecules. [66-68, 80]

In this project, the identification of effective small molecules on *ex vivo* HSC expansion has been aimed by determination of Meis1 inhibitors and functional effect of the determinated small molecules, and investigation of possible effects on the *ex vivo* HSC expansion.

Therefore, we think that small molecules which inhibit Meis1 could be helpful to improve the alternative HSC expansion strategies. According to studies, identified Meis1 as important modulator of hematopoietic stem cell (HSC). This provided basis for identification of hematopoietic inhibitors of Meis1. Identified Meis1 as one of the key regulators of hematopoietic stem cells (HSCs) This provided basis for identification hematopoietic inhibitors of Meis1as an additionally a potential therapeutical target. To this end, we have employed " *in silico* "and " *in vitro* " drug screening approaches. We will test known homeobox inhibitors in order to determine hematopoietic inhibitors of Meis1 that could induce proliferation. HSCs. Meis1-/- HSCs were indicated to be much less quiescent and prone to apoptosis.

Meis1 homeodomain has a known aminoacids sequences and DNA binding nucleotides. In order to be able to perform *in silico* drug secreening in this region, first of all, which amino acids sequences are interacting with DNA and subsequent analysis of protected amino acids have been determined. Two approaches were followed to determine which amino acids binds to known TGACAG nucleotides of Meis1 homeodomain. First of all, homologous / protected amino acids in the homeodomains of Meis1 and involved TALE proteins were identified and protected amino acids was determined Then crystal structure of Pbx1 homeodomain together with DNA was designated. This finding is important due to Pbx1 and Meis1 belong to same protein family. The Meis1 homeodomain protein binds to DNA with which of the possible amino acids was determined by making three dimensional comparison with crystal structure of Meis1 homeodomain on this structure. *İn silico* drug screenings could be done whereby profiling of the crystal structures.

To this end, we have established a library of relevant homeobox family inhibitors and developed a high-throughput *in silico* screening, We have established an *in silico* library of small molecules inhibitors of homeobox proteins. We screened up to druggable small molecules from PubChem and ZINC databases against crystallized Meis 1 homeodomain with the Autdock-vina platform. Subsequently, inhibitor screening was performed with AutoDock Vina in libraries (ZINC database). In this study, 1 million molecules were scanned for the detection of high binding molecules.

During the docking procedure in AutoDock/Vina programme two different size range of scan boxes was used. Molecules with a high binding value to Meis1 homeodomain of the DNA-binding amino acids eliminated by comparing to the binding values in the whole homeodomain which binding values of scanning in 40 Åx 40 Å x 40Å scan box. Furthermore in order determine the specific inhibitors of homeodomain binding to DNA a screening box was also identified with an approximate grid size 20 x 20 x 20 xyz point around the relevant amino acid that thought to interact the Meis homoedomain with DNA.

Throughout *in silico* analysis small molecules have been prefferred because having lower than 900 dalton molecular weight and high penetration capacity through cells or organs. These small molecules do not result in immun response due to dispersal through target destination. Thus small molecules can be used as inhibitors that play a key role in some cellular pathways.[81].

Briefly, detection of small molecules which were preferably bind to DNA-linked amino acids, eliminated depending on the binding value differences (affinity of the entire homeodomain protein-bound amino acids) was ± 0.3 kcal / mol and the binding energy of -7 kcal / mol was taken and used in subsequent verification studies. According to the hERG channel analysis ( the collaborative study were completed by Associate Professor Serdar Durdağı), if predictive inhibitors had the binding energy more than -7 kcal / mol , all of these were eliminated because of accepting as cardiotoxic [76].

As a result. twelve predicted small molecules were selected but just two of them have been obtained. 4-[2-(benzylamino)-2-oxoethoxy]-N-(2,3-dimethylphenyl) was first used small molecules that denominated as MEISi-1 and other one was 4-hydroxy-N'-[(Z)-(2-oxonaphthalen-1-ylidene)methyl] benzohidrazit benzohidrazit that denominated as MEISi-2. Both molecules are novel druggable molecules never used in any experiment before.

This is followed by " *in vitro* " validation of candidate according to *in silico* analysis, putative highly conserved Meis1 binding sites motifs which consist of TGACAG nucleotides on p21 promoter was defined and their functionality proved that luciferase assay. We have utilized transient transfection of Meis1 expression vector and corresponding luciferase reporters into HEK cells, which is followed by treatment with identified small molecules. PGL2 vectors which includes the promoter of the p21 gene activated by Meis1 protein were used in luciferase assays.

It has been observed that MEIS protein activates luciferase reporters including increasing amounts of p21 promoters. As a result of these experimental studies, MEIS1 were determined as a specific hits with *in silico* study in comparison to MEIS homeodomain and other TALE homeodomain.These studies warrant identified Meis1 inhibitors to be considered as a potential therapeutics to be tested in HSC expansion.

We hypothesized that Meis inbitiors provide expansion of HSPCs, for this reason we prefer to apply of Meis inhibitors on isolated HSPCs derived from various sources. First of all we analyzed Meis inhibitors on lineage negative cells. Lineage negative cells isolated mouse whole bone marrow by using magnetic seperation. HSPCs labelled with c-Kit (CD117) PE, CD34 FITC, Sca-1 PE-Cy7 and mouse APC lineage cocktail. Each antigens are member of HSPCs markers. For this reason we targeted LSKCD34Low population especially examined native HSCs. According to flow analysis, MEISi-1 and MEISi-2 inhibitors significantly induced HSC expansion and increased murine LSK and LSKCD34low HSC population. On the other hand PMBCs are most important source of HSC that were isolated from umbilical cord blood. In order to examine HSC compartment, cells labelled with hCD73-APC, hCD90-FITC, hCD105-PerCP/Cy7, hCD45-PE. Targeted human CD34+ and CD133+ HSC population proved that Meis inhibitors induced HSC expansion. Intriguingly, while MEISi-1 induce murine LSK and LSKCD34low HSC population more than MEISi-2, MEISi-2 led to more increasing rate of CD34+ and CD133+ HSC compartment than MEISi-1.

Throughout long-term HSC expansion supported with growth factors and cytokines which including TPO, FL3, IL3, IL6, IL11 and SCF for both murine bone marrow and UCB derived HSPSs.Alongside of LSK and LSKCD34low and CD34+ and CD133+ population, HSC characterized by ALDH compartment. ALDH is metabolic enzyme that is known as a cytosolic enzyme and used as primitive HSPSCs marker for both human and murine. All population showed that Meisi inhibition increase ALDH activity. That is the one of the most important sign of expansion of target population [82].

On the other hand the proliferation capacity of Meis inhibitor were examined on human bone marrow derived and adipose derived mesenchymal stem cells and also human umbilical vascular cells (HUVEC) were examined and there is no effect on expansional effect.Meis1 plays a critical role in homeostasis by maintaining the epidermal stem cells in the bulge region of the epidermis. Meis1 is highly expressed in the bulge region of the hair follicle, which is one of the stem cell niches.

The number of these adult stem cells is decreased by the disruption of Meis1 in the mouse epidermal tissue [10]. Interestingly, epidermal disruption of Meis1 is involved in tumor development and malignant conversion, suggesting a multifunctional model for Meis1 in the epidermis [10].

In addition, Meis1 have been shonw in several cancer types such as, ovarian carcinomas, prostate cancer and leukemia etc which suggests oncogenic potential of Meis1.[11-13]. Although correlations have been made regarding Meis1 and tumorigenesis, the molecular mechanism behind it remains undetermined. Nevertheless K562 (chronic myelogenous leukemia) and U937 human lymphocyte cells were used for examine cytotoxic effect of Meis inibitors. There is no meaningful cytotoxic or proliferative effect of Meis inhibitors on these ceancer cell line. Cell proliferation approximately stayed stabile but this is no answer that Meis inhibitor may use as drug on lymphoma. This analyze need to more metabolic activation and pathway analysis

Previously Kocabaş et all were declared that Meis1 has a characteristic of activating cyclin dependent kinase inhibitors (CDKIs) on the cardiac muscles. According to their genetic experiments, Meis1 protein can control CDKI genes which are p21, p16, p19. Parallel to indications when meis1 knock-out genetically from/on the mouse heart, the ratio of CDKI p21,p16 and p19 were increased. These methods provide comformable approach to test Meis1 inhibitors as one of the key regulator of cardiomyocyte cell cycle arrest and transcriptional activator of two synergistic cyclin dependent kinase inhibitors p16 and p21 provided a new platform for the development of therapeutics targeting cardiomyocyte cell cycle.

In the present study demonstrated that Meis inhibiton stimulate HSC expansion. MEISi-1 and MEISi-2 inhibitors showed lower apoptotic characteristcs. Thus Meis inhibiton reinforced regulation of stemness and axpansion of HPSCs. On the other hand significant increased number of CFU-G/M/GM colonies and observed pink erythroid progenitor cells (BFU-E colonies) have provided evidence of HSCs expansion.

# 7. CONCLUSION

Inhibition of Meis has been indicated to promote the proliferation of quiescent hematopoietic stem and progenitor cells demonstrated with the present research. Thereby Meis 1 inhibitors could have far reaching impacts on hematological therapeutics. To this end, we have established a library of relevant homeobox family inhibitors and developed a high-throughput *in silico* screening, luciferase reporter assays, *ex vivo* and *in vivo* studies to discover cardiogenic and hematopoietic inhibitors of Meis1. These studies identify a number of putative small molecule modulators of Meis1. Intriguingly, several of these small molecules *in vitro* were reported to inhibit other homeobox proteins. Further analysis demonstrate that they are much more specific to Meis1 as determined by lower affinity towards other members of TALE-type homoebox proteins. Thus demonstrating that Meis inhibitors can be targeted as a potential therapeutic agent that is effective in the proliferation of hematopoietic cells and may increase transfusion yield.

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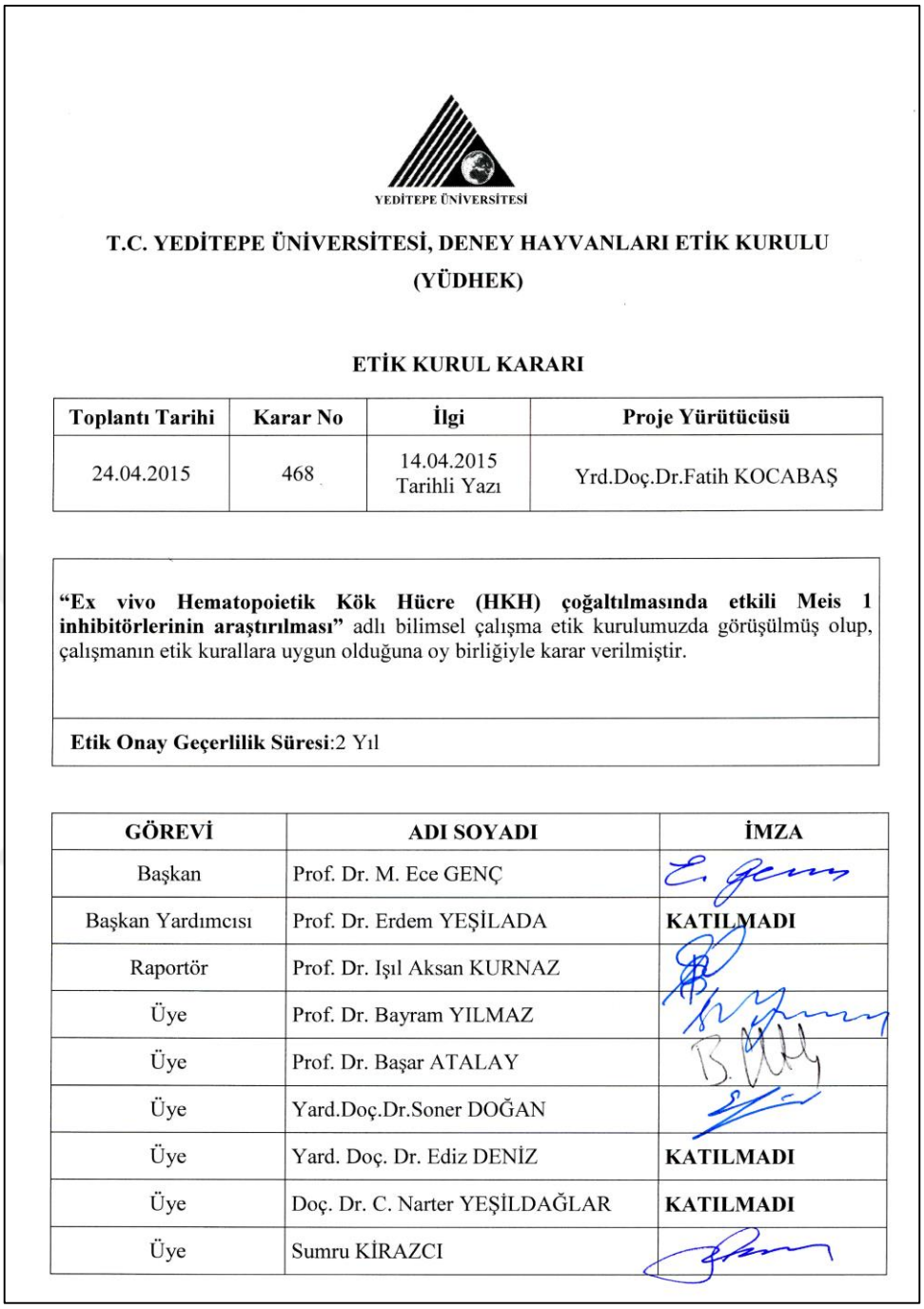
# APPENDIX A: ETHICAL APPROVAL

All animal studies were approved by the Institutional Animal Care and Use Committee of Yeditepe University, Istanbul, Turkey in 2015 (YUDHEK, Decision Number: 468). It was shown inFigure A. 1. All human were approved by the Institutional Clinical Studies Ethical Committee of Yeditepe University, Istanbul, Turkey in 2015 (Decision Numbers: 547 and 548). It was shown in Figure A. 2 and Figure A. 3.



YEDİTEPE ÜNİVERSİTESİ

**T.C. YEDİTEPE ÜNİVERSİTESİ, DENEY HAYVANLARI ETİK KURULU (YÜDHEK)**

**ETİK KURUL KARARI**

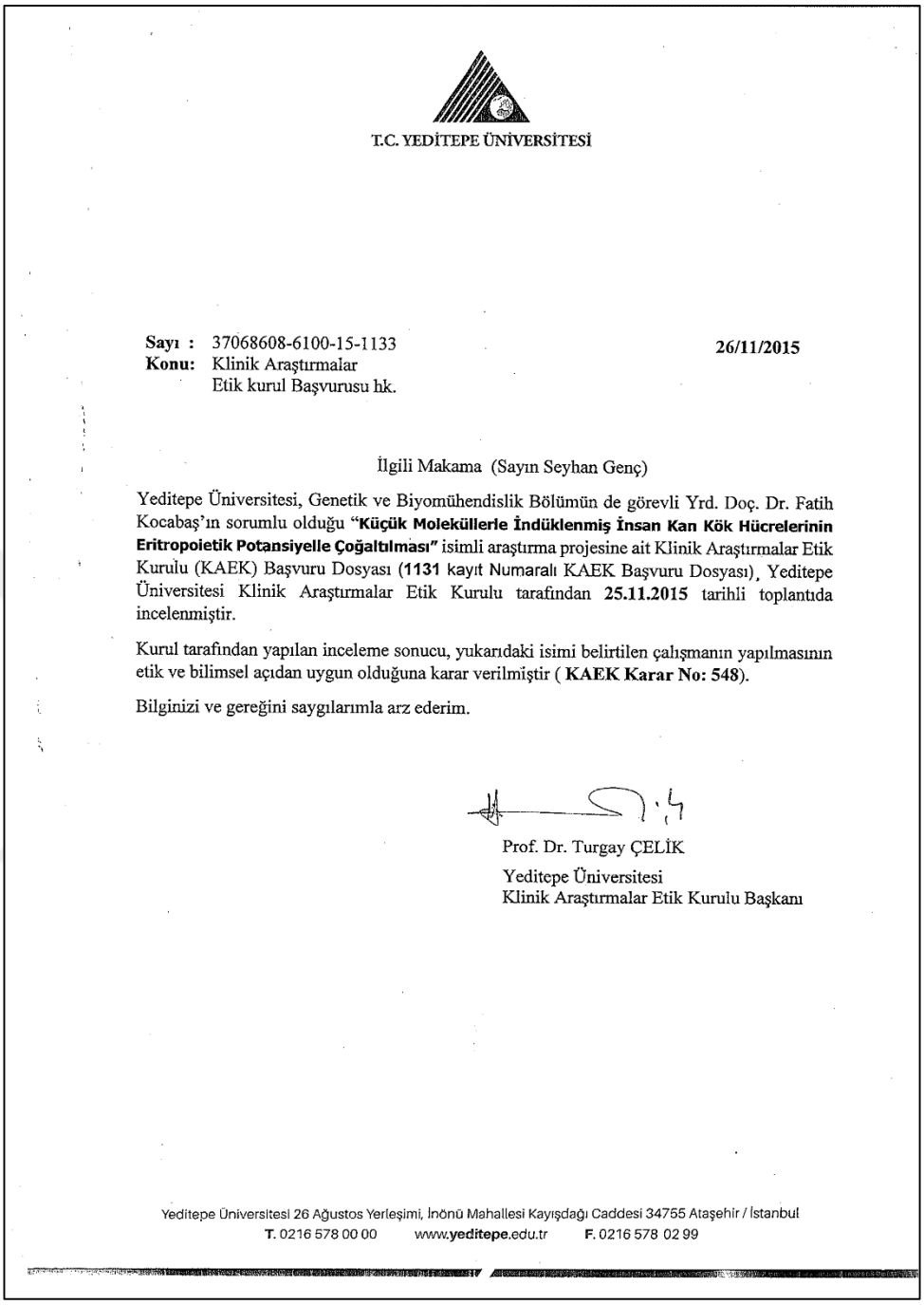
| Toplantı Tarihi | Karar No | İlgi | Proje Yürütücüsü |
|---|---|---|---|
| 24.04.2015 | 468 | 14.04.2015 Tarihli Yazı | Yrd.Doç.Dr.Fatih KOCABAŞ |

**"Ex vivo Hematopoietik Kök Hücre (HKH) çoğaltılmasında etkili Meis 1 inhibitörlerinin araştırılması"** adlı bilimsel çalışma etik kurulumuzda görüşülmüş olup, çalışmanın etik kurallara uygun olduğuna oy birliğiyle karar verilmiştir.

**Etik Onay Geçerlilik Süresi**:2 Yıl

| GÖREVİ | ADI SOYADI | İMZA |
|---|---|---|
| Başkan | Prof. Dr. M. Ece GENÇ | |
| Başkan Yardımcısı | Prof. Dr. Erdem YEŞİLADA | **KATILMADI** |
| Raportör | Prof. Dr. Işıl Aksan KURNAZ | |
| Üye | Prof. Dr. Bayram YILMAZ | |
| Üye | Prof. Dr. Başar ATALAY | |
| Üye | Yard.Doç.Dr.Soner DOĞAN | |
| Üye | Yard. Doç. Dr. Ediz DENİZ | **KATILMADI** |
| Üye | Doç. Dr. C. Narter YEŞİLDAĞLAR | **KATILMADI** |
| Üye | Sumru KİRAZCI | |

Figure A. 1. Ethical approval for animal use in this experimental research of *in vivo* and *in vitro* studies.



T.C. YEDİTEPE ÜNİVERSİTESİ

**Sayı :** 37068608-6100-15-1133 **26/11/2015**
**Konu:** Klinik Araştırmalar
Etik kurul Başvurusu hk.

İlgili Makama (Sayın Seyhan Genç)

Yeditepe Üniversitesi, Genetik ve Biyomühendislik Bölümün de görevli Yrd. Doç. Dr. Fatih Kocabaş'ın sorumlu olduğu **"Küçük Moleküllerle İndüklenmiş İnsan Kan Kök Hücrelerinin Eritropoietik Potansiyelle Çoğaltılması"** isimli araştırma projesine ait Klinik Araştırmalar Etik Kurulu (KAEK) Başvuru Dosyası (**1131** kayıt Numaralı KAEK Başvuru Dosyası), Yeditepe Üniversitesi Klinik Araştırmalar Etik Kurulu tarafından **25.11.2015** tarihli toplantıda incelenmiştir.

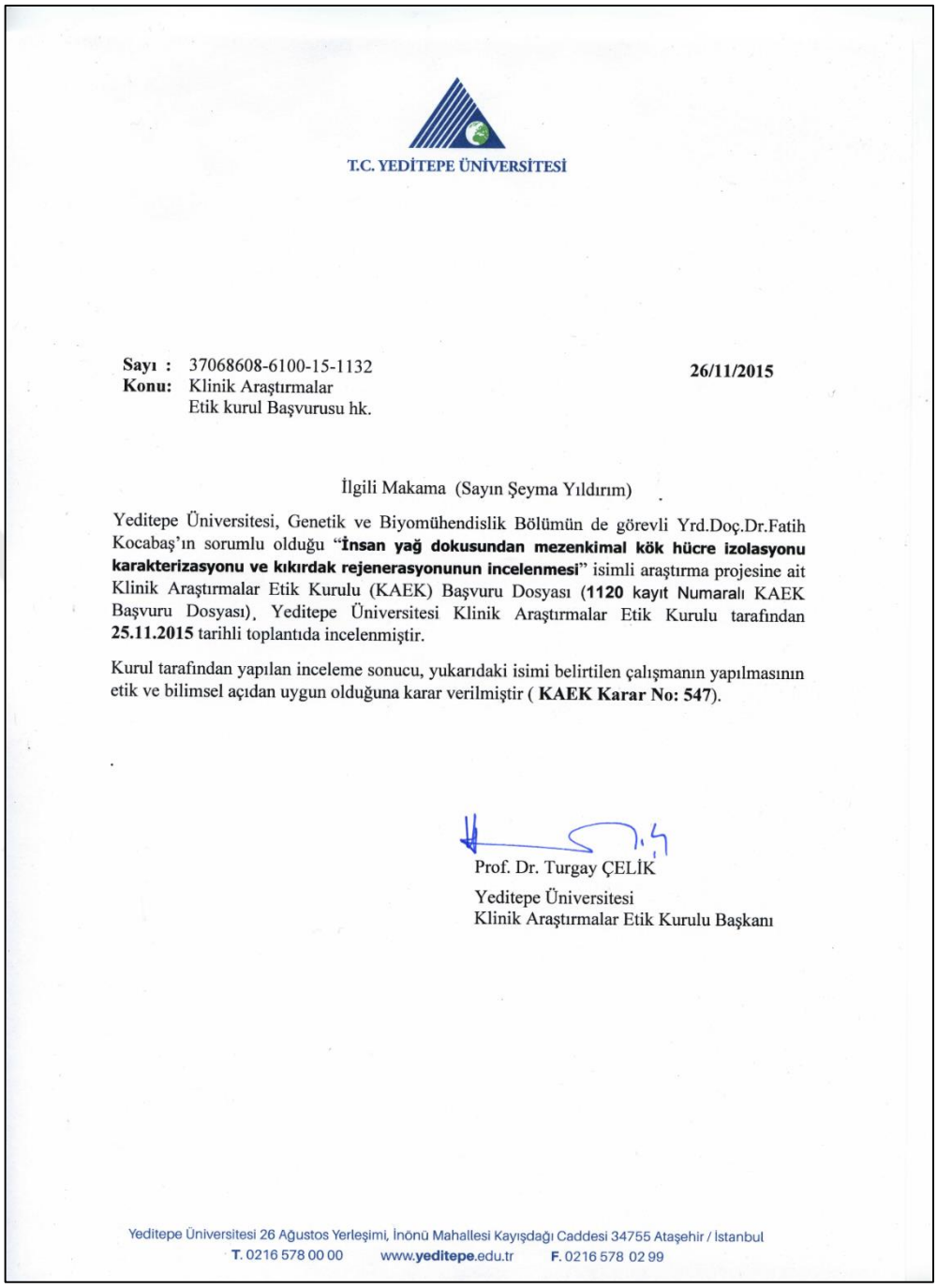
Kurul tarafından yapılan inceleme sonucu, yukarıdaki isimi belirtilen çalışmanın yapılmasının etik ve bilimsel açıdan uygun olduğuna karar verilmiştir ( **KAEK Karar No: 548**).

Bilginizi ve gereğini saygılarımla arz ederim.

Prof. Dr. Turgay ÇELİK
Yeditepe Üniversitesi
Klinik Araştırmalar Etik Kurulu Başkanı

Yeditepe Üniversitesi 26 Ağustos Yerleşimi, İnönü Mahallesi Kayışdağı Caddesi 34755 Ataşehir / İstanbul
T. 0216 578 00 00 www.yeditepe.edu.tr F. 0216 578 02 99

Figure A. 2. Ethical approval for human bone marrow use in this experimental research of *in vitro* studies



T.C. YEDİTEPE ÜNİVERSİTESİ

**Sayı :** 37068608-6100-15-1132 **26/11/2015**
**Konu:** Klinik Araştırmalar
Etik kurul Başvurusu hk.

İlgili Makama (Sayın Şeyma Yıldırım)

Yeditepe Üniversitesi, Genetik ve Biyomühendislik Bölümün de görevli Yrd.Doç.Dr.Fatih Kocabaş'ın sorumlu olduğu "**İnsan yağ dokusundan mezenkimal kök hücre izolasyonu karakterizasyonu ve kıkırdak rejenerasyonunun incelenmesi**" isimli araştırma projesine ait Klinik Araştırmalar Etik Kurulu (KAEK) Başvuru Dosyası (**1120** kayıt Numaralı KAEK Başvuru Dosyası), Yeditepe Üniversitesi Klinik Araştırmalar Etik Kurulu tarafından **25.11.2015** tarihli toplantıda incelenmiştir.

Kurul tarafından yapılan inceleme sonucu, yukarıdaki isimi belirtilen çalışmanın yapılmasının etik ve bilimsel açıdan uygun olduğuna karar verilmiştir ( **KAEK Karar No: 547**).

Prof. Dr. Turgay ÇELİK
Yeditepe Üniversitesi
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Figure A. 3. Ethical approval for human bone marrow use in this experimental research of *in vitro* studies.

# APPENDIX B: FUNDING ACKNOWLEDGEMENTS

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