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Epigenetic control of *PU.1* gene transcription during development of 5-Azacytidine resistance in acute myeloid leukemia

Epigenetická regulace genu PU.1 v rezistenci na léčbu 5-azacytidinem u akutní myeloidní leukémie

Diplomová práce

Vedoucí závěrečné práce:

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis

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Abstrakt

Hematopoéza je vysoce koordinovaný proces, ve kterém hematopoetická kmenová buňka dává vzniknout všem krevním buněčným elementům. Pro myeloidní a lymfoidní vývoj je nezbytná přísná regulace exprese transkripčního faktoru PU.1. Delece *PU.1* u myši je letální a jeho deregulace během vývoje hematopoetických buněk je spojena s hematologickými malignitami jako je akutní myeloidní leukémie (AML) a myelodysplastický syndrom (MDS). MDS a AML jsou závažné poruchy krvetvorby charakterizované expanzí nezralých krevních buněk a nedostatkem diferencovaných funkčních buněk. V patofyziologii leukemogeneze hrají významnou roli nejenom genetické, ale také epigenetické aberace. Deregulace PU.1 související s epigenetickými změnami na regulačních oblastech PU.1 genu představuje intenzivně studovaný mechanismus. Moderní terapie MDS a určité skupiny AML pacientů je založena na léčbě DNA hypometylačními látkami jako je 5-azacytidine (AZA), který ovlivňuje, mimo jiné, i regulaci PU.1 genu. Léčba AZA však často selhává a mechanismy rezistence nejsou příliš známy.

V této práci prezentujeme výsledky z klonů rezistentních na AZA připravených z MDS/AML buněčné linie OCI-M2. Analyzovali jsme DNA metylace a hydroxymetylace na klíčovém regulačním elementu genu *PU.1* (URE). Zjistili jsme, že epigenetické modifikace na URE značně ovlivňují expresi PU.1. V některých klonech rezistentních na AZA nebyl AZA schopen účinně demetylovat DNA v URE oblasti, což vedlo k nízké hladině PU.1. Dále jsme identifikovali protein TET3, jako hlavní enzym zodpovědný za konverzi DNA metylace na DNA hydroxymetylaci v OCI-M2 buněčné linii. Výsledky prezentované v této práci přinášejí nový pohled na epigenetické regulační mechanismy genu PU.1 ovlivněné léčbou AZA u AML a MDS.

Klíčová slova

Myelodysplastický syndrom (MDS), akutní myeloidní leukémie (AML), azacytidin (AZA), DNA metylace, DNA hydroxymetylace, PU.1, TET.

Abstract

Hematopoiesis is a highly orchestrated process, in which a single hematopoietic stem cell (HSC) gives a rise to all blood cellular components. For myeloid and lymphoid development precise controlled expression of the PU.1 transcription factor is needed. Deletion of PU.1 gene in mouse is lethal and its dysregulation during hematopoietic differentiation is associated with blood malignancies including acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). MDS and AML are serious blood disorders characterized by expansion of immature blood cells and lack of differentiated functional cells. Not only genetic but also epigenetic aberrations represent a very important field for studying pathophysiology of leukemia genesis and dysregulation of the PU.1 gene represents intensively studied candidate mechanism. Modern therapy of selected MDS and subset of AML patients is based on treatment with DNA hypomethylating agent Azacytidine (AZA) interfering in PU.1 gene regulatory mechanism. However, poor response or resistance to this therapy often occurs.

In this thesis we present data obtained from AZA-resistant clones of MDS/AML cell line OCI-M2. We analysed DNA methylation and DNA hydroxymethylation at the key regulatory element of the PU.1 gene (URE). We found that these epigenetic modifications at URE strongly influence the PU.1 gene expression. We found that in subset of AZA-resistant clones, AZA was not sufficient to demethylate DNA within URE, leading to low PU.1. Furthermore we identified an enzyme from TET protein family, TET3, responsible for DNA methylation to DNA hydroxymethylation conversion in OCI-M2. Collectively, data presented in my thesis bring a new insight into epigenetic regulatory mechanism of the PU.1 gene targeted by AZA therapy in AML and MDS.

Key words

Myelodysplastic syndrome (MDS), acute myeloid leukemia (AML), azacitidine (AZA), DNA methylation, DNA hydroxymethylation, PU.1, TET.

Abbreviations

5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5hmU	5-hydroxymethyluracil
5mC	5-methylcytosine
AID	Activation-induced cytidine deaminase
AML	Acute myeloid leukemia
AP2	Apetala 2
APOBEC	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ASXL1	Additional Sex Combs Like 1
AZA	Azacitidine
BaP	Basophil progenitor
B-cell	B-lymphocyte
BCL2L10	Bcl-2-like protein 10
BER	Base excision repair
bHLH	Basic helix-loop-helix
bHSH	Basic helix-span-helix
BMCP	Basophil-mast cell progenitor
bp	Base pair
BSA	Bovine serum albumin
bZIP	Basic leucine zipper
CD	Cluster of differentiation
CEBPα	CCAAT/enhancer-binding protein alpha
ChIP	Chromatin immunoprecipitation
CK1α	Cassein kinase 1 α
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
dNTPs	Deoxynucleoside triphosphates
DOC	2,5-Dimethoxy-4-chloroamphetamine
DSBH	Double-stranded β helix
E	Erythrocyte
EBF	Early B-cell factor
EDTA	Ethylenediaminetetraacetic acid
Egr	Early growth response protein
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EKLF	Erythroid Krüppel-like factor
Elf1	E74 Like ETS Transcription Factor 1
EoP	Eosinophil progenitor
EREBP	Ethylene-responsive element binding protein

ETS	E26 transformation-specific or E-twenty-six
EZH2	Enhancer of zeste homolog 2
FAB	French-American-British
FLI-1	Friend leukemia integration 1 transcription factor
FLT3	fms like tyrosine kinase 3
Gfi-1	Growth factor independent 1 transcriptional repressor
GMP	Granulocyte-macrophage progenitor
HEPES-KOH	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hMeDIP	Hydroxymethylated DNA immunoprecipitation
HMG	High mobility group box
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSF	Heat shock factor
IDAX	Inhibitor of dishevelled and axin complex
IDH	Isocitrate dehydrogenase
IMDM	Iscove's Modified Dulbecco's Medium
IPSS-R	International Prognostic Scoring System Revised
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
LEF	Lymphoid enhancer-binding factor
LMPP	Lymphoid-primed multipotent progenitor
Mac	Macrophage
MCP	Mast cell progenitor
MDP	Monocyte-dendritic progenitor
MDS	Myelodysplastic syndrome
MDS-EB	Myelodysplastic syndrome with excess blasts
MDS-MLD	Myelodysplastic syndrome with multilineage dysplasia
MDS-RD-MLD	Myelodysplastic syndrome with ring sideroblasts with multilineage dysplasia
MDS-RS	Myelodysplastic syndrome with ring sideroblasts
MDS-RS-SLD	Myelodysplastic syndrome with ring sideroblasts with single lineage dysplasia
MDS-SLD	Myelodysplastic syndrome with single lineage dysplasia
MDS-U	Myelodysplastic syndrome unclassifiable
MeDIP	Methylated DNA immunoprecipitation
Meg	Megakaryocyte
MEP	Megakaryocyte-erythrocyte progenitor
MLL	Mixed-lineage leukemia
MPP	Multipotent progenitor
Neut	Neutrophil
NK cells	Natural killer cell
NP	Neutrophil progenitor
Oct	Octamer-binding transcription factor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PEST	proline (P), glutamic acid (E), serine (S), and threonine (T)
Pro-B	Progenitor B-cell
Pro-NK	Progenitor natural killer cell
Pro-T	Progenitor T-cell
qPCR	Quantitative polymerase chain reaction
RA	Refractory anemia
RAEB	Refractory anemia with excess blasts
RCMD	Refractory cytopenia with multilineage dysplasia
RCUD	Refractory cytopenia with unilineage dysplasia
RN	Refractory neutropenia
rpm	Revolutions per minute
RT	Refractory thrombocytopenia
RT (methods)	Room temperature
RARS	Refractory anemia with ring sideroblasts
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
RUNX1	Runt-related transcription factor 1
S.O.C.	Super Optimal broth with Catabolite repression
SDS	Sodium dodecyl sulfate
SE	Standard error
SF3B1	Splicing factor 3B subunit 1
SFFV	Spleen focus forming virus
Sp1	Specifity protein 1
SRSF2	Splicing factor, arginine/serine-rich 2
STAT	Signal transducer and activator of transcription
TBPs	TATA-binding protein
T-cell	T-lymphocyte
TDG	Thymine-DNA glycosylase
TET	Ten-eleven translocation enzymes
URE	Upstream regulatory element
WHO	World Health Organization
WPSS	World Health Organization Prognostic Scoring System
MDAS	MD Anderson score
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Content

1. Literature review

1.1. Basic introduction to epigenetics and cell differentiation

Cell differentiation is a process by which a multipotent stem cell looses its ability to give a rise to many cell types and becomes a specialized cell with particular function. This process is accompanied by changes in gene expression. In mammals, with few exceptions, all cells contain the identical genetic information and changes in gene expression are performed without distortion in DNA sequence. Mechanisms by which cell regulates gene expression include covalent modifications of DNA or histone proteins, which affect chromatin structure, and non coding RNAs. Those processes are studied by epigenetics (Alberts *et al.,* 2005).

Main role in regulation of gene expression have transcription factors, which interact with particular nucleotides in DNA sequence and respond to covalent modifications of DNA and changes in chromatin structure. Differential activity of set of specific transcription factors determines a cell fate of particular stem cell, thus creating morphological and functional differences between cells in distinct tissues (Alberts *et al.,* 2005). Dysregulation of this system may lead to block in differentiation or aberrant development and initiate oncogenesis.

1.2. Chromatin structure and epigenetics

In eukaryotic cell, DNA is present in complex with proteins, forming chromatin. The fundamental unit of this structure is called nucleosome and consists of 4 types of histone proteins (H2A, H2B, H3, H4) creating octamer and approximately 146 bp of DNA wounded around them. This establishment enables the long DNA to be present in small nucleus and also, in cooperation with other proteins, regulates gene transcription. Genes in the sites, where DNA and histone interaction occurs, are not accessible for RNA polymerase. During cell cycle, histones undergo several posttranslational modifications such as acetylation, phosphorylation, methylation, ubiquitination and sumoylation, which change strength of their association with DNA and thus regulate gene transcription. Formation of particular histone modification depends on presence of appropriate protein. Recruitment of this protein is associated besides other things with DNA methylation (Alberts *et al.,* 2005; Lodish *et al.,* 2013).

1.2.1. DNA methylation

In mammals, cytosine in DNA is often modified by addition of methyl group to the carbon at 5' position of the pyrimidine ring (5mC). This modification occurs predominantly at sites, where cytosine is followed by guanine nucleotide, thus these regions are called CpG islands. Cytosine methylation is catalysed by DNA methyltransferases. In mammals there are 3 main types of DNA methyltransferases: DNMT1, DNMT3a and DNMT3b. DNMT3a and DNMT3b are also called "*de novo* methyltransferases" due to their function in establishment of new methylation patterns in embryo (Okano *et al.*, 1999). DNMT1 is primarily involved in maintenance methylation, after replication of DNA this enzyme copies methylation patterns of maternal DNA strand to newly emerged strand (Pradhan *et al.*, 1999). However this separation isn't strict, because DNMT1 can function also as a *de novo* methyltransferase and DNMT3 enzymes emerged to be involved in maintenance methylation too (reviewed in Jeltsch and Jurkowska, 2014).

DNA methylation on CpG islands in promoter regions is associated with repression of gene transcription (Antequera *et al.*, 1990; Herman *et al*., 1998). 5mC can physically block binding of transcription factors necessary for transcription initiation, or can indirectly cause modifications of histones, thus creating a closed chromatin structure and impair gene transcription. (Curradi *et al.*, 2002; Bird *et al*., 1998).

Aberrant DNA methylation was found in several types of cancer. Hypermethylation of tumor suppressors genes was found for instance in colorectal cancer, renal cancer and retinoblastoma (Herman *et al*., 1998; Prowse *et al*., 1997; Ohtani-Fujita *et al*., 1997). Abnormal DNA methylation has special importance in Myelodysplastic syndrome (MDS) and Acute myeloid leukemia (AML), serious blood malignancies with myeloid origin, characterized by expansion of immature blood cells and lack of differentiated cells. Promoter hypermethylations were found widespread over genome in MDS and AML patients affecting many genes, including genes involved in DNA repair, differentiation, cell cycle control and apoptosis (Figueroa *et al*., 2009; Jiang *et al*., 2009).

Although a lot is known about DNA methylation, DNA demethylation in somatic cells remains to be not fully understood. Methylation marks can be passively and non-specifically erased after replication by addition of DNMT inhibitors (Santi *et al.*, 1984). At specific sites demethylation occurs actively. Mechanisms of active demethylation most likely include base excision repair (BER). One proposed mechanism presumes that 5mC can be deaminated to thymine and T-G mismatch is then corrected by thymine-DNA glycosylase (TDG) and BER (Morgan *et al.*, 2004). The second mechanism involves oxidation of 5mC to 5-hydroxymethylcytosine (5hmC) (Grin and Ishchenko, 2016; Guo, *et al.*, 2011). Removal of 5hmC can be then mediated through production

of 5-hydroxymethyluracil (5hmU) or 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can be removed from DNA by same process as thymin from T-G mismatch (Fig. 1) (Grin and Ishchenko, 2016; Guo, *et al.*, 2011; Cortellino *et al*., 2011; Maiti and Drohat, 2011). Oxidation of 5mC to 5hmC can contribute to demethylation also passively, because DNMT1 doesn´t recognise 5hmC (Valinluck and Sowers, 2007). All oxidative derivates of 5mC - 5hmC, 5caC and 5fC are products of activity of Ten-eleven translocation methylcytosine dioxygenases (TET) (Ito *et al*., 2010; Ito *et al*., 2011).

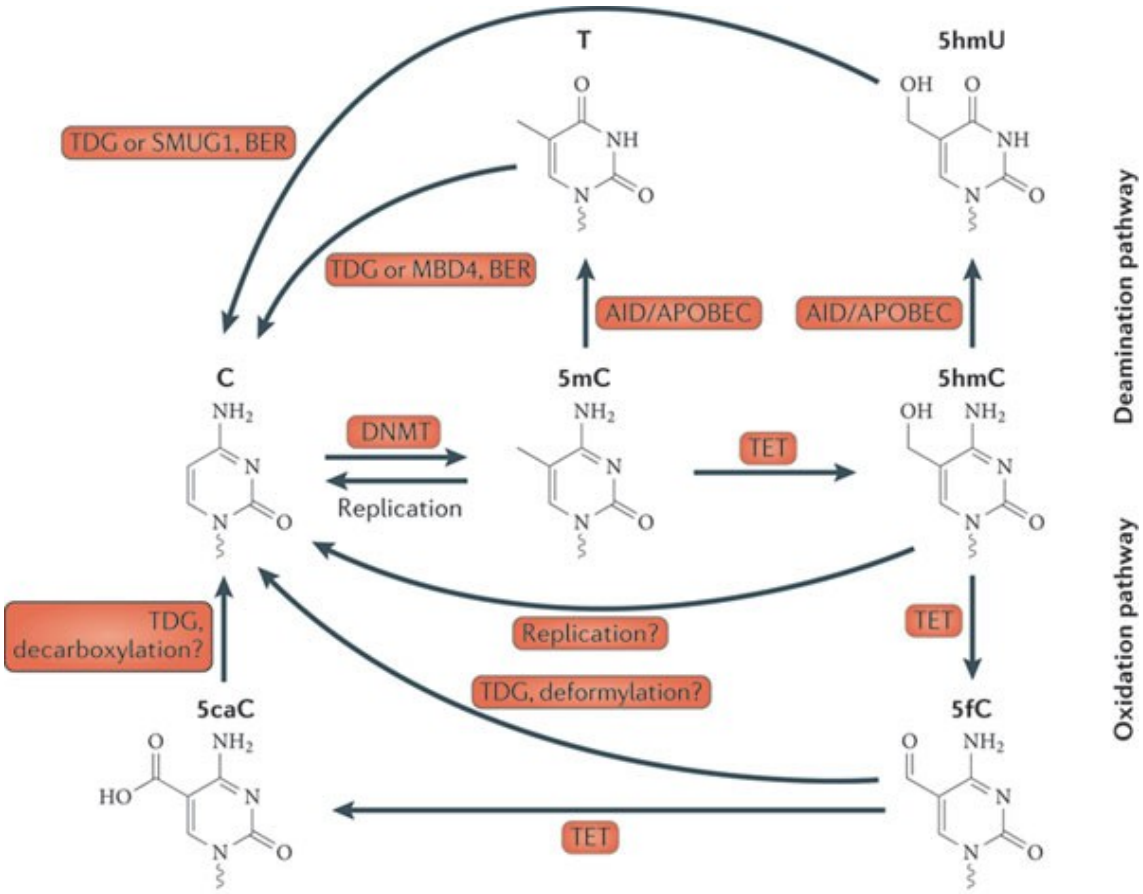


Figure 1: **Possible mechanisms of DNA demethylation**. 5mC and product of its oxidation 5hmC can be deaminated by activation-induced cytidine deaminase (AID) or Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like (APOBEC). Resulting mismatch can be then corrected by TDG followed by BER process. 5mC can be erased from genome also by passive demethylation due to inhibition of DNMT enzymes. 5hmC can contribute to this passive process too, because DNMT1 recognises 5hmC poorly. 5hmC can be further oxidized to 5fC and then to 5caC. Both bases are removed by TDG and BER. Mechanisms of direct removal of formyl or carboxyl group should be more investigated (taken from Branco *et al.*, 2011).

1.2.2. DNA hydroxymethylation and TET enzymes

Oxidation of 5mC to 5hmC may not be important only in demethylation process. According to recent observations, 5hmC is present in highly transcribed regions together with activating histone marks suggesting its role in activation of gene transcription (Stroud, *et al.*, 2011; Madzo *et al*., 2014).

As mentioned above, 5hmC is produced by TET proteins, α-ketoglutarate and Fe (II) dependent enzymes. TET family includes 3 proteins: TET1, TET2, TET3, each located on different chromosome. *TET1* is located on chromosome 10, *TET2* on chromosome 4 and *TET3* on chromosome 2. All enzymes share a cysteine rich domain, double-stranded β helix (DSBH) and binding sites for α-ketoglutarate and Fe (II). TET1 and TET3 enzymes also contain CXXC motif (2 cysteines separated by 2 other amino acids) capable of binding to unmethylated CpGs (Fig. 2) (Iyer *et al.*, 2009; Tahiliani *et al*., 2009; Lee *et al.*, 2001). CXXC motif of TET2 is not present in TET2 gene itself, but it is encoded by neighbouring gene IDAX (Iyer *et al*., 2009; Ko *et al*., 2013). IDAX is able to bind to TET2 and presumably recruit it to CpG rich regions. Moreover, IDAX function as a negative regulator of TET2, via activation of caspases, thus it is responsible for maintenance of proper TET2 level (Ko *et al.*, 2013). Although enforced expression of all mouse TET proteins in human HEK293T and U2OS cell lines led to generation of 5hmC, it seems, that TET proteins may not be fully interchangeable (Ito *et al.*, 2010). Experiment in human embryonic carcinoma cell line demonstrated, that due to differences in their structure, TET1 and TET2 may act on distinct sites. Authors also proposed, that TET2 and TET3 proteins, but not TET1 are primarily involved in conversion of 5hmC to 5fC and 5caC present in demethylation pathway (Putiri *et al.*, 2014). In mouse, deletion of TET2 together with TET3 led to complete loss of 5hmC in spleen and bone marrow, suggesting that these 2 enzymes alone are essential for production of 5hmC in mouse hematopoietic system (An *et al.*, 2015).

Mutations and dysregulations of TET proteins were found in distinct hematological malignancies, such as chronic myelomonocytic leukemia, myeloproliferative neoplasms, MDS and AML (Abdel-Wahab *et al.*, 2009). *TET2* mutation was found in 19 - 26% of patients with MDS and is considered to be an early event in disease development (Delhommeau *et al.*, 2009; Langemeijer *et al*., 2009). In AML, *TET2* mutation was identified in 7.6% of individuals (Gaidzik *et al.*, 2012).

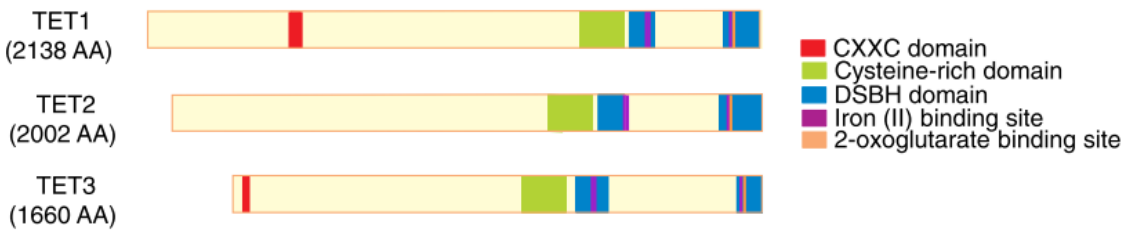


Figure 2: **Structure of human TET proteins** (taken from Scourzic *et al.*, 2015).

1.3. Hematopoiesis

Chromatin structure and DNA methylation play an important role in regulation of gene expression by influencing accessibility of DNA to transcription factors. For development of each cell type, specific set of transcription factors is needed. Good example demonstrating how differential expression of transcription factors influences cell decision, is hematopoiesis, a process in which a single hematopoietic stem cell (HSC) is able to give a rise to all blood cellular components. Self-renewal capacity of HSC helps to maintain appropriate number of undifferentiated cells which can generate blood cells whole life of an individual.

In human, primitive erythroblast starts to occur at third week of embryonic development. This hematopoiesis, called primitive, is mediated by extraembryonic mesodermal cells lining yolk sack. True HSC develop later, at 4-5 weeks of gestation, from intraembryonic cells in aorta-gonad-mesonephros region (Takashina, 1987; Tavian *et al*., 1996). Definite hematopoiesis appears next especially in livers of developing embryo with contribution of spleen, kidneys, thymus and lymph nodes. In the fifth month of fetal development, hematopoiesis starts to occur even in bone marrow. Bone marrow, especially in skull, scapulae, sternum, vertebrae, pelvis and ribs, remains to be the main hematopoietic organ even in adults (Rodak *et al.,* 2012).

Daily are in bone marrow produced approximately 10 billion blood cells. According to their origin, those cells can be divided in to two groups, myeloid and lymphoid. Myeloid lineage contains monocytes, granulocytes (neutrophils, eosinophils, basophils, mast cells), erythrocytes and megakaryocytes, while lymphoid lineage includes T and B lymphocytes (T-cell, B-cell), NK cells and dendritic cells (Fig. 3). According to their function we can divide blood components in to white blood cells (leukocytes), red blood cells (erythrocyte) and trombocytes. Leukocytes are important for immune response, erythrocytes deliver oxygen and trombocytes have role in blood coagulation (Hořejší *et al.,* 2009; Rodak *et al.,* 2012).

In myeloid lineage, two types of cells are involved in immune response, monocytes and granulocytes.

Monocytes are large (15-20 µm in diameter) cells representing 2-10% of leukocytes in bloodstream. Their main function is to differentiate into macrophages, osteoclast or myeloid dendritic cells after entering a tissue. Differentiation in to macrophages occurs not only in areas of inflammation, but even in healthy tissues e.g. in livers, skin, kidneys or brain. Macrophages are capable of phagocytosis, process by which any foreign particles such as pathogens are engulfed and destroyed (Rodak *et al.,* 2012; Hořejší *et al.,* 2009).

Granulocytes are the most abundant leukocytes in peripheral blood. They got their name due to presence of granules in their cytoplasm, which can be stained with Wright´s staining, mixture of eosin and methylene blue dyes. Granulocytes fight with pathogens by releasing cytokines and pathogen toxic content from their granules. They can also phagocyte foreign particles. There are four types of granulocytes, neutrophils, eosinophils, basophils and mast cells (Rodak *et al.,* 2012).

In contrast with monocytes and granulocytes, red blood cells and megakaryocytes aren't primarily involved in immune system. Erythrocytes are the most abundant blood components, they represent mostly half of the blood´s volume. They are well adapt to carry oxygen, they have biconcave shape giving them larger surface and they lack many organelles, thus they can contain more hemoglobin important for oxygen binding. Megakaryocytes are important for production of trombocytes (platelets). In healthy man there are 100 million megakaryocytes producing 100 billion trombocytes every day. Megakaryocytes are adjacent to endothelial cells in blood vessels and form pseudopodia going thought or between those cells. Platelets are made when a part of cytoplasm pinches off those pseudopodia. Platelets are necessary for blood coagulation, they are assembling around wound and stop blood flow (Wickrema *et al.,* 2009; Rodak *et al.,* 2012).

Lymphocytes represent a group of leukocytes with various functions. T-cells realize their role in immunity by secreting cytokines, stimulating B-cells or directly killing infected and tumor cells. They are also important for suppression of immune system in order to prevent autoimmune diseases. T-cells progenitors arise in bone marrow, but they become mature T-cells in thymus, where they undergo strict selection process. B-cells cooperate with T-cells and other leukocytes and are primarily involved in production of antibodies to specific antigen. B-cells emerge and also mature in bone marrow, but for their activation is essential spleen and lymph nodes, where they meet with antigens. NK cells have critical function in response to viral infection. They are subjects of interest also due to their toxicity for tumor cells. Dendritic cells are present in skin, pulmonary and digestive system where they engulf foreign particles. After activation by pathogen they are able to migrate in to lymph

nodes and in cooperation with other leukocytes stimulate immune response. (Hořejší *et al.,* 2009; Bartůňková *et al.,* 2007; Rodak *et al.,* 2012).

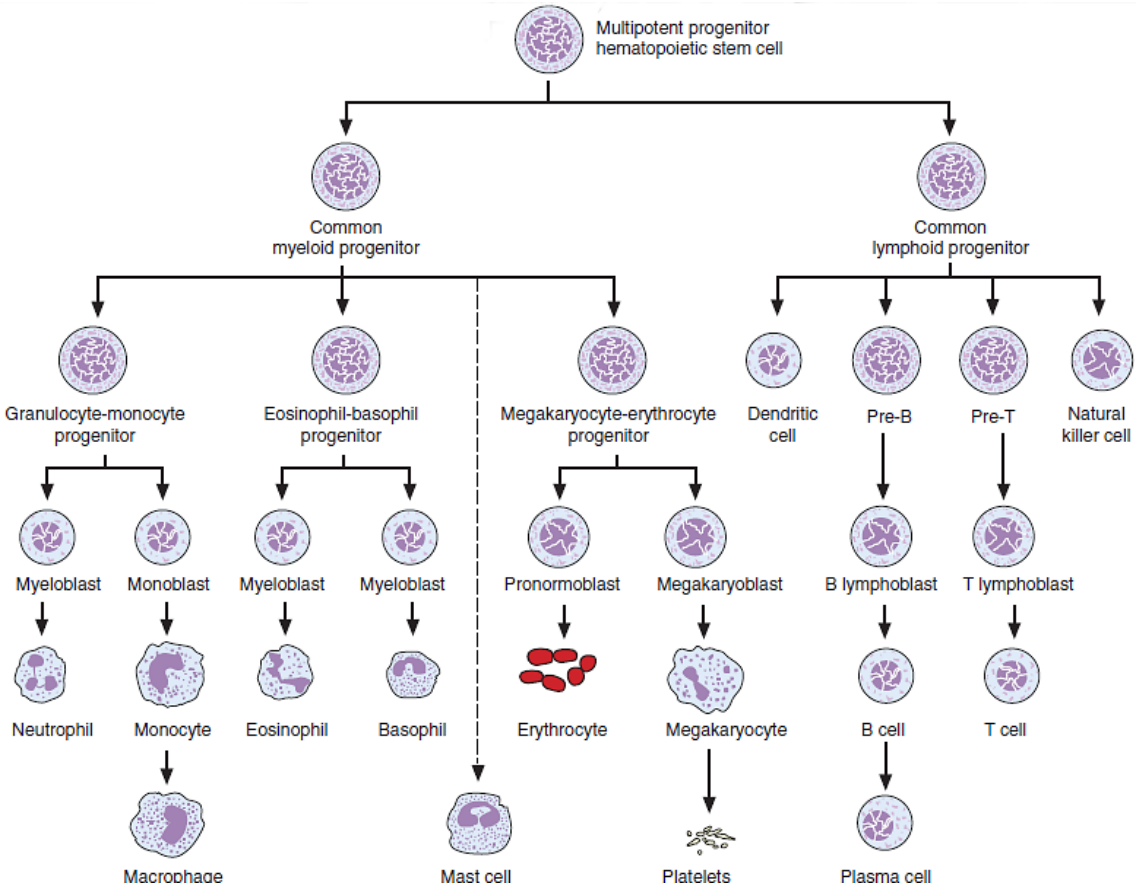


Figure 3: **Simplified view on hematopoiesis in human** (taken from Rodak *et al.,* 2012).

1.3.1. Transcriptional regulation of hematopoiesis, determinants of lineage specification

In the bone marrow, HSC divides and gives rise to two progenies. One, both or none of those daughter cells may undergo differentiation. This decision is probably determined by external signals coming from bone marrow niche (reviewed in Birbrair and Frenette, 2016). Two models of HSC differentiation exists. One of them declare that first differentiation step of HSC is commitment to myeloid or lymphoid lineage, when HSC divides and gives rise to common myeloid (CMP) and common lymphoid progenitor (CLP) (Akashi *et al.*, 2000; Kondo *et al.*, 1997). Those progenitors then

differentiate into particular lineages and through many other intermediates give rise to mature blood cell of specific type (Fig. 3). Second model proposed that first step of HSC differentiation may not be unilineage commitment. Other progenitors with specific differentiation abilities may exist first, such as lymphoid-primed multipotent progenitor (LMPP) with B and T-cell, but also granulocyte-macrophage potential and with no or low potential to differentiate in to erythrocytes and megakaryocytes (Fig. 4), (Adolfsson *et al.*, 2005).

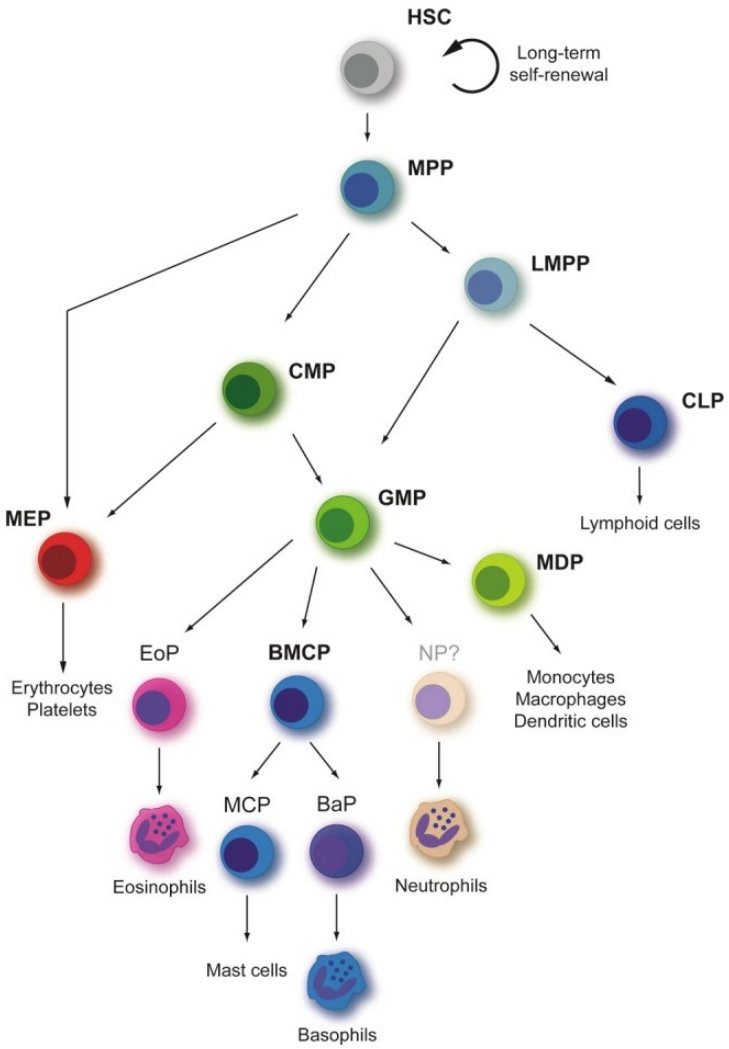


Figure 4: **Scheme of hematopoiesis proposed by Fiedler and Brunner 2012, including LMPP**. Abbreviations: HSC – hematopoietic stem cell, MPP – multipotent progenitor, LMPP – lymphoid-primed multipotent progenitor, CMP – common myeloid progenitor, CLP – common lymphoid progenitor, GMP – granulocyte-macrophage progenitor, MEP – megakaryocyte-erythrocyte progenitor, MDP – monocyte-dendritic progenitor, BMCP – basophil-mast cell progenitor, NP – neutrophil progenitor, EoP – eosinophil progenitor, BaP – basophil progenitor, MCP – mast cell progenitor.

Transcription factors are proteins regulating gene transcription. They are able to interact with specific DNA sequence via their DNA-binding domain. This interaction either enables binding of DNA-dependent RNA polymerase responsible for gene expression, or conversely prevent its recruitment, thus inhibiting mRNA production. Transcription factors can regulate gene expression by direct or mediated interaction with RNA polymerase or by modification of chromatin structure. In the cell, DNA is wounded around complex of histone proteins. Specific modifications of those proteins cause stronger or weaker bond to DNA. Relaxed DNA is more accessible for DNA polymerase than DNA strongly bound to histone proteins. Transcription factors can recruit histone modification proteins to specific sites and facilitate changes in chromatin structure (Alberts *et al.,* 2005).

Transcription factors are generally composed of trans-activating and DNA binding domain. Trans-activating domain is responsible for interaction with other proteins such as transcription coregulators, while DNA binding domain, as mentioned above, is necessary for interaction with DNA. DNA binding domains are non-covalently binding to specific sites called recognition sequences. Trans-activating and DNA binding domains are connected by flexible regions and are structurally independent on each other. The structural independence allows many combination possibilities of these domains with other protein modules. That´s why lot of various transcription factors may recognise same sequence on DNA. Transcription factors can also contain ligand binding domain responding to external signals (Lodish *et al.,* 2013).

Transcription factors can be classified according to a secondary structure of their DNA binding domain. One model divides transcription factors into 4 superclasses: *1.) Basic domains; 2.) Zinc-coordinating domains; 3.) Helix-turn-helix domains; 4.) Beta scaffold domains with minor groove contacts* (Stegmaier *et al.*, 2004), (Table 1). Newer model classifies transcription factors into 9 groups: *1.) Basic domains; 2.) Zinc-coordinating domains; 3.) Helix-turn-helix domains; 4.) Other all-α-helical DNA binding domains; 5.) α helices exposed by β structures; 6.) Immunoglobulin fold; 7.) β-harpin exposed by an α/β-scaffold; 8.) Sheet binding to DNA; 9.) β-Barrel DNA binding domains* (Wingender, 2013). Another classification of transcription factors is according to their function. General transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH) are part of transcription preinitiation complex and are necessary for transcription initiation. Specific transcription factors regulate transcription of particular genes (Alberts *et al.,* 2005).

Classification of transcription factors	
Superclass	**Class**
Basic domains	Basic leucine zipper (bZIP)
	Basic helix-loop-helix (bHLH)
	Basic helix-span-helix (bHSH)
Zinc-coordinating domains	Nuclear receptors
	C6 zinc clusters
	DM
	GCM
	WRKY
Helix-turn-helix domains	Homeo box
	Paired box
	Forkhead/ winged helix
	HSF domain
	Tryptophan clusters
	TEA domain
β-scaffold domains with minor groove contacts	RHR
	STAT
	p53-like
	MADS
	β-Barrel α-helix domains
	TBPs
	HMG
	Histone fold
	Grainyhead
	Cold-shock domain
	Runt-like domain
	SMAD/NF-1
	T-Box domain
Others	Cooper fist
	Pocket domain
	AP2/EREBP-related
	SAND domain

Table 1: Classification of transcription factors (made according to Stegmaier *et al.*, 2004)

Development of blood cells is a multistage process, in which HSC progeny becomes progressively restricted in differentiation ability and turn into a mature blood cell of specific type. At the molecular level, diversification is achieved by differential expression of specific transcription factors in HSC progenitors.

HSCs and multipotential progenitors are characterized by expression of transcription factors typical for multiple lineages. Those primary transcription factors prepare the cell for future differentiation in to specific lineage (Hu *et al*., 1997; Laslo *et al*., 2006). During unilineage commitment, expression of those transcription factors is reduced and is followed by expression of transcription factors specific for particular lineage (Miyamoto *et al.*, 2002).

Few transcription factors are considered to be master regulators necessary for elementary specification of blood cells: GATA-1, PU.1 and Ikaros. GATA-1 is an important determinant for development of erythroid and megakaryocytic cells (Pevny *et al*., 1991; Shivdasani *et al.*, 1997). PU.1 is a myeloid/lymphoid determinant (Scott *et al.*, 1994). Ikaros is necessary for commitment into lymphoid lineage (Georgopoulos *et al.*, 1994).

For further specification in to particular cell type are then necessary secondary transcription factors. For example, for commitment in to either erythroid or megakaryocytic lineage are required factors EKLF and FLI-1 respectively (Bouilloux *et al.*, 2008). Differentiation into B-cells is then guided by EBF factor, which represses genes for myeloid specification (Pongubala *et al.*, 2008). Development of T-cells is dependent on Notch signalling (Radtke *et al.*, 1999). For activation of macrophage programme are then important transcription factors Egr-1 and Egr-2. Transcription factor Gfi-1 leads cells to development into neutrophilic lineage and is suppressed by Egr-1 and Egr-2 in cells committed to macrophage lineage (Laslo *et al.*, 2006).

Disruption of hematopoietic regulatory network leads to hematologic diseases. This thesis is focused on blood disorders with myeloid origin, MDS and AML. Both illnesses are characterized by uncontrolled proliferation of malignant blood cells and decreased amounts of mature cells. Due to deficiency in functional blood cells (cytopenia) patients with MDS and AML often display fatigue, shortness of breath, increased bleeding and frequent infections. Depending on number and type of cytopenia and percentage of undifferentiated cells present in blood, median survival of patients with MDS is ranging from 9.6 months to 8.8 years (Greenberg *et al*., 2012). Therefore mechanisms responsible for development and pathogenesis of this illness are subjects of interest.

1.4. Transcription factor PU.1

One of the fundamental determinants necessary for proper hematopoiesis is a transcription factor PU.1, encoded by the *SPI-1* gene located in human on the short arm of chromosome 11 (11p11.2) (Nguyen *et al.*, 1990). Product of this gene is a 31kDa protein consisting of 3 main domains, ETS DNA binding domain, trans-activating domain and PEST domain. ETS domain is located in the C-terminus of the protein having a winged helix-turn-helix structure recognizing a purine-rich (GGAA) DNA sequence (Klemsz *et al.*, 1990; Kodandapani *et al*., 1996). Trans-activating domain occupies N-terminus of PU.1 and comprises 3 subdomains, 2 acidic and 1 glutamine rich. Between ETS and trans-activating domain is located PEST sequence, region rich in proline, glutamic acid, serine and threonine (Klemsz and Maki, 1996).

1.4.1. PU.1 in normal hematopoiesis

PU.1 is expressed in HSC and common myeloid and lymphoid progenitors and is considered to be a master regulator of their cell fate decisions. After commitment in to B and T-cells, its expression is attenuated in B-cells and completely repressed in T-cells. Within common myeloid progenitors, there are 3 populations of cells expressing *PU.1* on different levels. Population with high level of PU.1 gives rise to myeloid cells and dendritic cells, while cells expressing low levels of PU.1 appears to function as a progenitors for megakaryocytes and erythrocytes (Fig. 5) (Nutt *et al.*, 2005)

The crucial role of PU.1 as a primary lineage determinant was demonstrated by PU.1 knockdown experiments. Deletion of *PU.1* in mouse embryo led to loss of granulocytes, monocytes as well as elimination of B and T-cells resulting in lethality around 17.5 days of gestation. Some mutant embryos also displayed disrupted erythroblast maturation (Scott *et al.*, 1994). Similar *PU.1* knockdown experiment didn't lead to embryonic lethality, but *PU.1* null mice were born and were able to survive 17 days with antibiotic treatment. Mutant mice showed elimination of monocytes and B-cells and abnormal development of neutrophils, however they were able to generate T-cells (McKercher *et al.*, 1996). In cooperation with transcription factor GATA-2 is PU.1 necessary also for generation of mast cells (Walsh *et al.*, 2002).

In adult hematopoiesis, mutation of *PU.1* resulted in loss of myeloid and lymphoid progenitor cells and led to enormous production of granulocytes with disrupted maturation. This finding suggests, that in contrast with fetal hematopoiesis, in adult mice is PU.1 necessary for suppression of granulocyte production and is essential for their proper maturation (Dakic *et al.*, 2005).

PU.1 promotes its role via binding to promoters and enhancers of its target genes and by interaction with specific proteins. PU.1 can regulate expression of more than 3,000 genes in hematopoietic cells including genes involved in apoptosis, cell cycle, cellular metabolism, chromatin remodelling and signal transduction (Burda *et al.*, 2009). By direct interaction, PU.1 can also modulate function of other proteins (reviewed in Gangenahalli *et al*., 2005). Noteworthy is interaction with transcription factor GATA-1, responsible for triggering the erythroid programme (Pevny *et al.*, 1991). PU.1 and GATA-1 can bind together and repress each other´s activity, thus the ratio between those 2 transcription factors affects whether cell will differentiate to myeloid or erythroid lineage (Zhang *et al*., 2000; Rekhtman, *et al.*, 1999; Choe *et al*., 2003; Rao, *et al.*, 1997).

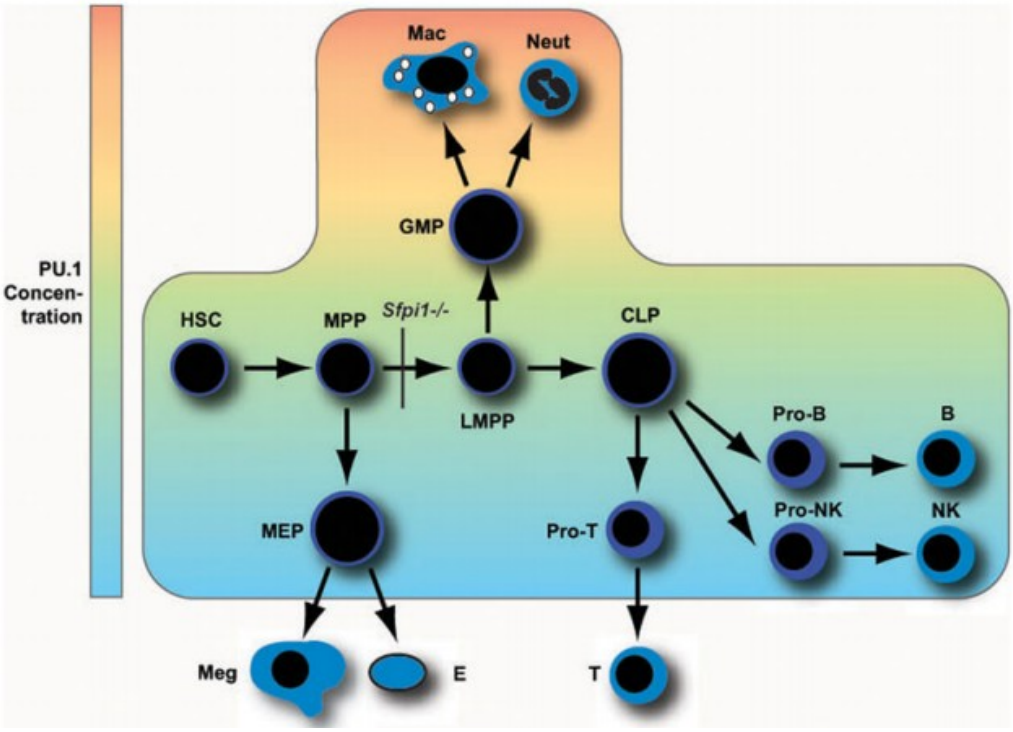


Figure 5: *PU.1* expression in hematopoietic cells: red color indicates higher concentration of PU.1, blue color lower. Mature T-cells, megakaryocytes and erythrocytes don't express *PU.1*. Abbreviations: HSC – hematopoietic stem cell, LMPP – lymphoid primed myeloid progenitor, CLP – common lymphoid progenitor, CMP – common myeloid progenitor, MPP - multipotential progenitor, pro-B - progenitor B cell, B - mature B cell, pro-NK - progenitor natural killer cell, NK - mature natural killer cell, pro-T - progenitor T cell, T - mature T cell, MEP - megakaryocyte-erythroid progenitor, Meg – megakaryocyte, E - erythrocyte, GMP - granulocyte-macrophage progenitor, Mac - macrophage, Neut – neutrophil (taken from Dahl and Simon, 2007)

1.4.2. Regulation of PU.1 expression

The regulation of *PU.1* expression is mediated by several mechanisms. At the transcriptional level, promoter and few regulatory elements of *PU.1* gene are involved in this control. Those sites are bound by specific transcription factors promoting differential PU.1 production in distinct cell types. Positive regulators binding to *PU.1* proximal promoter in mouse include Sp1, Oct-1, Oct-2 and PU.1 itself (Chen *et al*., 1996; Li *et al*., 2001). During erythro/megakaryocytic development, when is necessary to inhibit myeloid programme guided by PU.1, is promoter of *PU.1* gene bound by GATA transcription factors, which function as a *PU.1* repressors (Chou *et al.*, 2009).

The most important distal regulatory element of *PU.1* gene appears to be a site called URE (upstream regulatory element) located in mouse 14 kb and in human 17 kb upstream from transcription start site (Fig.6) (Li *et al.*, 2001; Tatetsu *et al.*, 2007). URE is crucial for proper *PU.1* expression, its deletion in mouse decreased PU.1 levels about 80% of wild type (Li *et al*., 2001; Okuno *et al*., 2005; Rosenbauer *et al.*, 2004). URE is bound by multiple transcription factors, depending on the cell type, which can either activate or repress *PU.1* expression. For instance, CEBPα binds URE in common myeloid progenitors, promoting PU.1 transcription, while binding of TCF/LEF factor in T-cells blocks *PU.1* expression (Yeamans *et al*., 2007; Rosenbauer *et al*., 2006). Negative effect on *PU.1* expression via binding to URE has also a transcription factor GATA-1 (Burda *et al.*, 2016). Other transcription factors binding URE include RUNX1, involved in expression of *PU.1* in early hematopoietic development, Elf1 binding URE in myeloid and lymphoid cells and Fli-1 binding URE in macrophages (Okuno *et al*., 2005; Hoogenkamp *et al*., 2007; Hoogenkamp *et al*., 2009). URE can be bound also by PU.1 itself creating a positive autoregulatory loop (Okuno *et al.*, 2005).

Additional regulatory elements of *PU.1* gene were found by DNase I hypersensitivity site mapping at regions 12, 10, 9 and 8 kb upstream from transcription start site in mouse myeloid lineage. Those elements were not detected in B-cells. In the myeloid cells, URE is bound by CEBPα resulting in formation of opened chromatin structure at -12 position. This site is then bound by PU.1 itself promoting its own expression (Leddin *et al.*, 2011).

PU.1 is regulated also post-transcriptionally via microRNA miR-155 (Vigorito *et al.*, 2007).

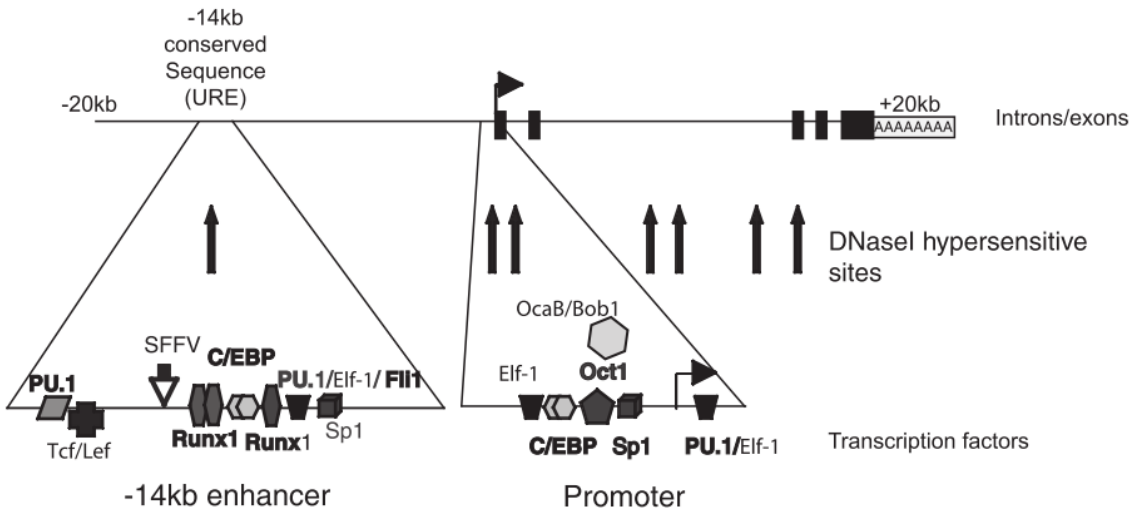


Figure 6: Structure of PU.1 locus in mouse. Binding of various transcription factors to URE and promoter are depicted. Arrow head indicate transcription start site, bold arrows mark DNaseI hypersensitive sites, black boxes indicate exons (taken from Hoogenkamp *et al.*, 2007).

1.4.3. PU.1 in malignant hematopoiesis

Due to its indispensable role in lineage specification, *PU.1* dysregulation has a dramatic impact on hematopoiesis and contributes to leukemogenesis. Decreased levels of PU.1 resulting from URE deletion led in mouse to development of AML, same as mutation of *PU.1* DNA binding domain, which disrupted its ability to bind to its target sites (Rosenbauer *et al.*, 2004 Cook *et al.*, 2004). Similar facts were observed even in human. 7% patients with AML carried mutations in *PU.1* gene and patients with MDS expressing lower levels of PU.1 displayed shorter survival than patients with intermediate or high expression (Mueller *et al*., 2002; Curik *et al*., 2012). Moreover, in patients with certain subtypes of AML (acute myelomonocytic and acute monocytic leukemia) were observed approximately four and half times higher levels of miRNA-155 than in healthy individuals (O'Connell *et al.*, 2008). Higher levels of miR-155 are associated even with human B-cell lymphomas (Huskova *et al.*, 2015).

Considerable role in downregulation of *PU.1* in hematological malignacies plays DNA methylation. Low levels of PU.1 correlated with high DNA methylation at URE in MDS patients as well as in human AML cell line OCI-M2. Treatment with demethylation agent resulted in upregulation of *PU.1* and promoted differentiation in OCI-M2 cell line (Curik *et al.*, 2012). High DNA methylation rate at URE and also at promoter of *PU.1* gene was detected even in several human myeloma cell lines and in a subset of myeloma patients (Tatetsu *et al.*, 2007).

The aforementioned facts suggest, that PU.1 acts as a tumor suppressor via inhibition of leukemogenesis. However, PU.1 plays a dual role. In myeloid leukemia, it serves as a repressor of leukemogenesis by promoting differentiation of myeloid cells, but in erythroid progenitors, PU.1 functions as an oncogene and its upregulation causes erythroleukemia (Rosenbauer *et al*., 2004; Moreau-Gachelin *et al.*, 1988). *PU.1* gene was originally described as a high-frequency integration site for Spleen focus forming virus (SFFV), an artificially prepared virus, which has potential to incorporate into specific genes and activate them. Integration of this virus into URE site of *PU.1* gene in murine erythroblasts caused *PU.1* upregulation resulting in inhibition of erythroid transcription factor GATA-1 and development of erythroleukemia (Moreau-Gachelin *et al*., 1988; Okuno *et al*., 2005; Rekhtman *et al.*, 1999)

The Information mentioned above demonstrate, that PU.1 is an essential master regulator of hematopoiesis and its up- or downregulation leads to blood disorders, such as MDS and AML. This dysregulation can be associated with epigenetic status of *PU.1* regulatory elements, especially DNA methylation at URE.

1.5. Myelodysplastic syndrome and acute myeloid leukemia

1.5.1. Myelodysplastic syndrome

Myelodysplastic syndrome (MDS) is heterogeneous group of blood malignancies with myeloid origin caused by mutation of HSC, resulting in uncontrolled proliferation of leukemic clones with blocked differentiation ability. Leukemic blasts are accumulated and display morphological abnormalities (dysplasia). Ineffective hematopoiesis leads to deficiency of mature blood cells from one or more lineages (cytopenias). MDS is diagnosed in more than 10,000 individuals annually in the United States with incidence rate higher in men than in women. Most of the patients is more than 60 years old (Ma *et al.*, 2007).

To diagnose MDS, patients usually undergo several examinations including complete blood count, peripheral blood smear and bone marrow biopsy (Šálek, 2012). Based on the results from these tests, MDS is categorized into specific subgroup. MDS classification is regularly updated due to new observations, thus particular subgroups of MDS were fused over the years or changed nomenclature and criteria. First used classification system, called FAB (French-American-British) divided MDS into 5 subgroups depending on the percentage of myeloblasts in bone marrow and peripheral blood and presence of ring sideroblasts, erythroblasts with iron filled mitochondria surrounding the nucleus (Bennett *et al*., 1982; Mufti et al., 2008). Nowadays is used WHO (World Health Organization) classification system, which takes

into account even cytogenetic abnormalities and numbers of lineages with morphological aberrations (Vardiman *et al.*, 2009). This classification system was established in 2001, but in 2008 and 2016 was revised (Table 2 and 3) (Vardiman *et al*., 2009; Arber *et al*., 2016).

To determine prognosis of patients with MDS, several prognostic systems can be used. Similar as classification systems, even prognostic systems has evolved with new discoveries. Commonly used prognostic scoring include WPSS (WHO Prognostic Scoring System), MDAS (MD Anderson Score) and IPSS-R (International Prognostic Scoring System Revised), which presents updated version of scoring system established in 1997 (Malcovati *et al*., 2007; Kantarjian *et al*., 2008; Greenberg *et al*., 2012; P. Greenberg *et al*., 1997). Prognostic scoring estimates risk of disease progression (low, intermediate, high), thus helps doctors to choose appropriate treatment for particular patient and predict his survival.

MDS patients often display cytogenetic abnormalities and mutations. In approximately half of MDS patients cytogenetic aberrations such as 5q deletion, trisomy of 8 chromosome and 20q deletion occurs (Gangat *et al.*, 2015). Mutations in MDS are most frequent in genes involved in RNA splicing (*SF3B1, SRSF2*), DNA methylation and hydroxymethylation (*DNMT3A, TET2*) and histone modifications (*EZH2, ASXL1*) (Papaemmanuil *et al.*, 2013). Less frequent, but important are mutations in *IDH1/2* genes encoding isocitrate dehydrogenase enzymes catalyzing production of α-ketoglutarate. Mutation of *IDH1/2* impairs function of these enzymes, thus affecting even activity of TET proteins, which are α-ketoglutarate dependent (Papaemmanuil *et al*., 2013; Patnaik *et al*., 2012; Iyer *et al*., 2009). As a result of frequently observed mutations of genes involved in epigenetic control of gene expression, it was proposed that MDS is an epigenetic disease (Issa, 2013). In agreement with this suggestion are aberrant DNA methylation found at the specific sites and also across whole genome in MDS by many research groups *(Figueroa et al*., 2009; Valencia *et al*., 2011; Hofmann *et al*., 2006; Potapova *et al*., 2010). As mentioned in previous chapter, DNA hypermethylation was found in MDS also at URE site of *PU.1* gene (Curik *et al.*, 2012).

WHO 2008 MDS classification	
Refractory cytopenia with unilineage dysplasia (RCUD), including refractory anemia (RA), refractory neutropenia (RN), refractory thrombocytopenia (RT)	
Refractory anaemia with ring sideroblasts (RARS)	
Rerfractory cytopenia with multilineage dysplasia (RCMD)	
Refractory anemia with excess blasts (RAEB)	RAEB-1
	RAEB-2
MDS unclassified (MDS-U)	
Refractory cytopenia of childhood *(Provisional entity)*	

Table 2: Until recently used WHO classification system. Refractory cytopenia of childhood is a provisional entity, because additional research is needed to confirm its categorization (made according to Vardiman et al., 2009).

WHO 2016 MDS classification	
MDS with single lineage dysplasia (MDS-SLD)	
MDS with multineage dysplasia (MDS-MLD)	
MDS with ring sideroblasts (MDS-RS)	with single lineage dysplasia (MDS-RS-SLD)
	with multilineage dysplasia (MDS-RS-MLD)
MDS with isolated del(5q)	
MDS with excess blasts (MDS-EB)	MDS-EB-1
	MDS-EB-2
MDS unclassifiable (MDS-U)	with 1% blood blasts
	with single lineage dysplasia and pancytopenia
	based on defining cytogenetic abnormality
Refractory cytopenia of childhood *(Provisional entity)*	

Table 3: **Current WHO classification of MDS after 2016 revision.** Refractory cytopenia of childhood is a provisional entity, because additional research is needed to confirm its categorization (made according to Arber et al., 2016).

1.5.2. Acute myeloid leukemia

Approximately third of patients with MDS develops with time Acute myeloid leukemia (AML) (Shukron *et al.*, 2012). This leukemia is also called secondary, but AML can occur even *de novo*, without previous diagnosis of MDS or other myeloid disorder. Characterization of AML is very similar to the characterization of MDS, dysplastic leukemic blasts incapable of differentiation are expanding to the detriment of normal hematopoiesis. However, according to WHO classification, difference between AML and MDS is in number of blasts present in bone marrow or peripheral blood, 20% or more blasts is considered to be an AML. AML is diagnosed even if percentage of blasts is lower than 20% but patients display specific cytogenetic abnormalities (Vardiman *et al.*, 2009).

As well as MDS, even AML is categorized into subgroups according to FAB and WHO classification systems. FAB system separates AML into 8 subgroups (M0-M7) depending on morphology and cytochemistry (Table 4) (reviewed in Pui *et al*., 2003). WHO system takes into account even cytogenetic and genetic aberrations (Table 5) (Arber *et al.*, 2016). Both systems are still widely used. Prognosis of patients with AML is poor, especially in older individuals. In untreated patients is median survival only 2 months, in patients receiving treatment 6 months (Oran and Weisdorf, 2012).

Genetic and epigenetic abnormalities in AML are similar to those found in MDS. Frequently mutated genes include *DNMT3A, TET2, IDH1/IDH2* and *ASXL1* (Thol et al., 2011; Gaidzik et al., 2012; Paschka et al., 2010; Schnittger et al., 2013). Secondary AML, same as MDS, displayed high levels of promoter DNA hypermethylation and it was proposed, that abnormal DNA methylation has major impact on progression of MDS to AML (Figueroa *et al.*, 2009; Jiang *et al.*, 2009).

Even though it seems, that difference between MDS and AML is just in number of blasts, the interpretation shouldn't be that simplified. In MDS, hematopoietic cells display higher rate of apoptosis than in AML (Boudard *et al*., 2000; Albitar, 2002). The excessive apoptosis is possible explanation why bone marrow of MDS patients is usually hypercellular or normocellular, while in peripheral blood cytopenia occurs (Boudard *et al.*, 2000; Kerbauy and Deeg, 2007). As MDS progresses to AML, apoptosis decreases, probably due to selection of clones less sensitive to apoptotic signals (Parker *et al.*, 2000). Gain of specific mutations may be involved in this process. Mutations suspected of transition from MDS to AML include for instance mutations of *RUNX1, MLL* and *FLT3* genes (Dicker *et al.*, 2010). Important role in AML transformation plays even DNA methylation. In high risk MDS and AML patients was observed higher rate of promoter DNA methylation than in low risk MDS patients (Jiang *et al.*, 2009).

FAB AML classification	
M0	Undifferentiated
M1	Myeloblastic without maturation
M2	Myeloblastic with maturation
M3	Promyelocytic
M4 and M4 (Eo)	Myelomonocytic and myelomonocytic with bone marrow eosinophilia
M5	Monocytic
M6	Erythroleukemia
M7	Megakaryoblastic

Table 4: Classification of AML according to FAB system (made according to Tenen, 2003).

WHO 2016 AML classification	
AML with recurrent genetic abnormalities	AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
	AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
	APL with t(15;17)(q22;q12); PML-RARA
	AML with t(9;11)(p21.3;q23.3);MLLT3-KMT2A
	AML with t(6;9)(p23;q34.1);DEK-NUP214
	AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
	AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
	Provisional entity: AML with BCR-ABL1
	AML with mutated NPM1
	AML with biallelic mutations of CEBPA
	Provisional entity: AML with mutated RUNX1
AML with myelodysplasia-related changes	
Therapy-related myeloid neoplasms	
AML, not otherwise specified (NOS)	AML with minimal differentiation
	AML without maturation AML
	AML with maturation
	Acute myelomonocytic leukemia
	Acute monoblastic/monocytic leukemia
	Pure erythroid leukemia
	Acute megakaryoblastic leukemia
	Acute basophilic leukemia
	Acute panmyelosis with myelofibrosis

Table 5: Current classification of AML after 2016 revision. AML with mutated RUNX1 is a provisional entity, because additional research is needed to confirm its categorization (made according to Arber et al., 2016).

1.5.3. Treatment of MDS and AML

The only option of cure for patients with MDS and AML is hematopoietic stem cell transplantation (HSCT). Unfortunately this procedure carries risk of serious complications, which may be fatal. Thus HSCT isn't suitable for most of patients, because majority is more than 60 years old and transplantation is hazardous for them (de Witte *et al.*, 2000).

Patients who are not able to undergo transplantation receive supportive care and disease modifying agents. The main goal in MDS is to delay progression into AML. The supportive care is used to reduce symptoms and includes blood transfusions and stimulating factors to support hematopoiesis (reviewed in Steensma, 2011). The only approved disease modifying drugs for treatment of MDS include Azacitidine, Decitabine and Lenalidomide. Azacitidine and Decitabine, both DNA hypomethylating agents, are used for treatment of high-risk MDS patients, while Lenalidomide, inhibitor of casein kinase 1A1 (CK1α), is used specifically for MDS with 5q deletion (Fenaux *et al*., 2009; Kaminskas *et al*., 2005; Kantarjian *et al*., 2006; Krönke *et al*., 2015; List *et al*., 2006). High risk MDS patients can receive also therapy used for AML (see below) (Kantarjian, Beran, *et al.*, 2006).

In AML, the main target is to achieve complete remission. Strong chemotherapy with combination of several drugs is usually used. In most cases of AML, patients are treated with Cytarabine and Anthracycline (Idarubicine, Daunorubicine) (Wiernik *et al.*, 1992). Elderly patients with bone marrow blasts counts more than 30% can receive treatment also by Azacitidine (Huls, 2015). The acute promyelocytic leukemia is specifically treated by retinoic acid and arsenic trioxide (Lo-Coco *et al.*, 2013). Therapy for relapsed disease usually consists of three drug combinations: Mitoxantrone, Etoposide, Cytarabine or Fludarabine, Cytarabine, Idarubicine, Filgrastim or Mitoxantrone, Cladribine, Cytarabine, Filgrastim (Trifilio *et al*., 2012; Pastore *et al*., 2003; Robak, *et al.*, 2000).

Patients with poor prognosis are encouraged to join clinical trials. Several novel agents for treatment of MDS and AML are currently tested. They include for instance histone deacetylase inhibitors, kinase inhibitors or new DNA hypomethylating agent (Selina *et al*., 2013; Garcia-Manero *et al*., 2016; Issa *et al*., 2015). Additional testing is still needed to reveal potential beneficial effects of these drugs.

1.5.3.1. Azacitidine

Azacitidine (AZA) (4-amino-1-β-D-ribofuranosyl-s-triazin-2(1H)-one) is an analogue of cytidine synthetically prepared more than 50 years ago in Czechoslovakia. It is distinguished from cytosine by presence of nitrogen atom in the 5 position of the pyrimidine ring (Fig. 7). AZA was initially described as a cytostatic agent, but later also appeared to have an inhibitory effect on DNA methylation (Cihák, 1974; Jones and Taylor, 1980). Due to its positive effects on survival and quality of life, AZA became the main option of treatment for high-risk MDS patients, which are not able to undergo HSCT (Kaminskas *et al.*, 2005).

After entering the cell via nucleoside transporters, AZA is phosphorylated by uridine-citidine kinases, thus it can be incorporated into nucleic acids (Damaraju *et al.*, 2012; Li, *et al.*, 1970; Van Rompay, *et al.*, 2001) Because AZA is a ribonucleoside, it is primarily incorporated into RNA (Li *et al.*, 1970; Choi, *et al.*, 2007). However ribonucleotide reductase is able to convert AZA in to its deoxy analogue, which can be integrated even in to DNA (Aimiuwu *et a*l., 2012; Choi *et al*., 2007). Incorporation in to nucleic acids leads to disruption of their structure and impairment of protein synthesis, which can result in apoptosis (Kiziltepe *et al*., 2007; Schneider-Stock *et al*., 2004). Moreover, integration of AZA in to DNA leads to erasure of DNA methylation due to inhibition of DNMT enzymes. After replication, maintenance DNMT covalently binds to cytosines of the newly emerged strand via their carbon-6 atom and methylates them according to the maternal strand. DNMTs bind to 5-azacytosines similarly like to cytosines, however due to presence of nitrogen atom in the 5 position of the AZA cytosine ring, the covalent bond can´t be resolved. (Santi *et al*., 1984; Jüttermann, *et al.*, 1994; L. Chen *et al*., 1991; Ghoshal *et al*., 2005). Trapped DNMT isn't able to finish replication of DNA methylation patterns and is degraded (Jones and Taylor, 1980; Ghoshal *et al*., 2005). This results in progressive erasure of DNA methylation after each DNA replication cycle (Fig. 8) (Jones and Taylor, 1980).

Role of AZA in DNA demethylation was confirmed even directly in MDS patients. Treatment with AZA led to changes in DNA methylation patterns and loss of DNA methylation at tumor suppressor genes was associated with clinical response (Stresemann, *et al.*, 2008; Tran *et al*., 2011). AZA is also capable of DNA demethylation at regulatory sites of *PU.1* gene in MDS patients as well as in acute myeloid leukemia and myeloma cell lines (Curik *et al*., 2012; Tatetsu *et al*., 2007). These facts suggest, that AZA may be efficient drug due to upregulation of tumor suppressor or differentiation genes.

Treatment with AZA can significantly prolong survival of MDS patients, however outcome of these patients is still poor. Overall survival of MDS patients treated with AZA is 24.5 months compared with 15 months survival of patients treated only by

supportive care or chemotherapy (cytarabine) (Fenaux *et al.*, 2009). Moreover approximately 40% of AZA treated MDS patients don´t respond to this treatment or their treatment fails (Fenaux *et al*., 2009; Silverman *et al.*, 2002). Mechanisms of the resistance aren´t fully understood. However few studies revealed interesting facts. Some patients with poor response to AZA displayed lower expression of uridine-cytidine kinase which is involved in AZA phosphorylation (Valencia *et al.*, 2014). Resistance to AZA also correlated with high levels of anti-apoptotic protein BCL2L10 and high expression of ribosomal genes (Cluzeau *et al*., 2012; Belickova *et al*., 2016). It was also proposed that poor AZA response may be due to higher levels of DNMT1 caused by downregulation of miRNAs targeting DNMT1 mRNA (Solly *et al.*, 2016).

To complete our understanding of poor response to AZA, additional researches should be made. The findings may be used in prediction of treatment response and can help us to establish different therapeutic strategies.

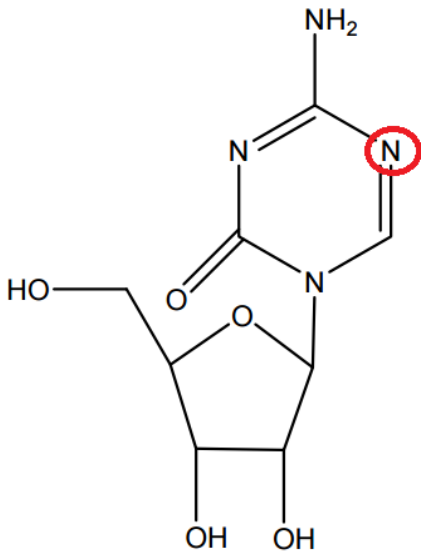


Figure 7: **Molecular structure of AZA.** Nitrogen atom in the position 5 is highlighted by red circle (taken and modified from Celgene, 2016)

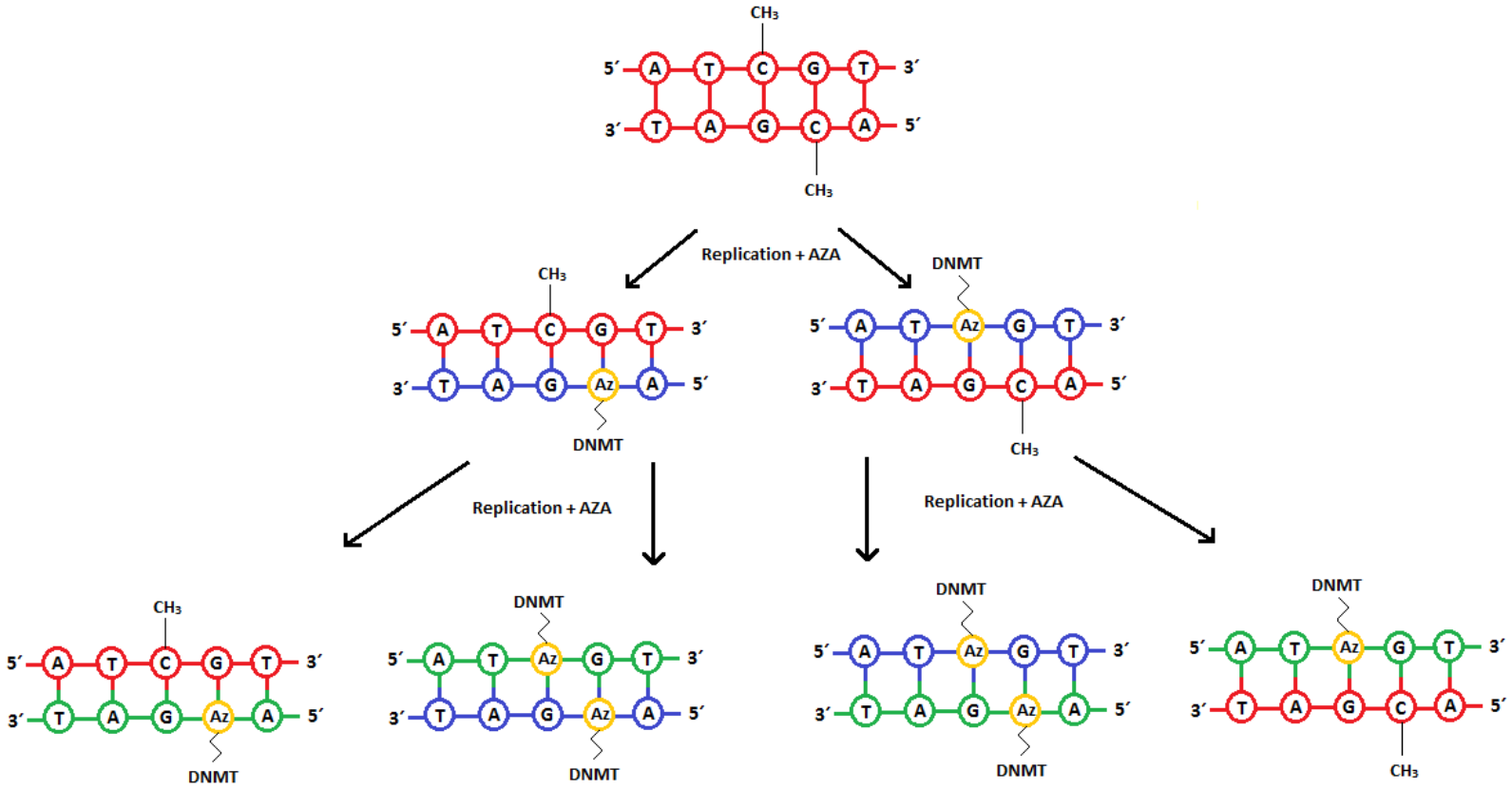


Figure 8: Mechanism of AZA action: AZA (yellow circle) is incorporated into DNA forming a covalent bond with DNMT (zigzag line). This results in gradual loss of DNA methylation after each synthetic phase.

Abbreviations: Az – Azacytidine, A – adenine, T – thymine, C – cytosine, G - guanine

2. The aims of the thesis

To reveal epigenetic modifications at *PU.1* gene contributing to AZA resistance in acute myeloid leukemia and myelodysplastic syndrome.

- To determine the expression profile of PU.1 and putative PU.1 gene epigenetic regulatory proteins in AZA-resistant AML/MDS.

- To analyze DNA methylation and DNA hydroxymethylation profiles at URE in AZA-resistant AML/MDS.

- To identify proteins responsible for DNA hydroxymethylation generation in AML/MDS cell line OCI-M2.

3. Materials

3.1. Organisms

3.1.1. OCI-M2 cell line

Human AML cell line. Established from MDS transformed in to AML (M6-erythroleukemia) of a 56 years old patient in 1984. (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, DSMZ no.: ACC 619)

3.1.2. Healthy bone marrow CD34+ cells

Fresh CD34+ cells isolated from bone marrow of healthy donors by immunomagnetic separation. Obtained from Lonza (Bone Marrow CD34+ 1 million cells, fresh, no: 1M-101C, clones #8 and #9)

3.1.3. Subcloning Efficiency DH5α Competent Cells

Chemically competent cells of *Escherichia Coli*, strain DH5α obtained from Invitrogen (no: 18265017), suitable for blue-white screening. Genotype: F- Φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-

3.2. Chemicals and reagencies

Agarose I (Amresco)

Ampicillin (Sigma-Aldrich)

Antibody 5-hydroxymethylcytosine (from hMeDIP Kit (Abcam, no: ab117134))

Antibody 5-methylcytosine (from MeDIP Kit (Abcam, no: ab117133))

Antibody IgG (from MeDIP Kit (Abcam, no: ab117133))

AZA (Vidaza - Celgene Europe)

Betaine solution 5M (Sigma-Aldrich)

Boric acid (Amresco)

BSA (Sigma-Aldrich)

Control siRNA (Dharmacon)

DMSO (Carl Roth)

DNA Loading Dye 6x (Thermo Scientific)

dNTPs 10mM (Biogen)

DOC (Sigma-Aldrich)

EDTA (Amresco)

EGTA (Amresco)

Ethanol (Penta)

Ethidium bromid (Amresco)

Fetal Bovine Serum (Sigma-Aldrich)

Formaldehyd (Electron Microscopy Sciencies)

GeneRuler 1kb SM0313 (Thermo Scientific)

Glycerol (Sigma-Aldrich)

Glycine (Amresco)

Glykogen (Ambion)

HEPES-KOH (Amresco)

Chloroform (Penta)

IMDM (Sigma Aldrich)

IPTG (Thermo Scientific)

Isopropyl alcohol (Penta)

JumpStart Taq DNA polymerase (Sigma-Aldrich)

LB Broth with agar (Sigma-Aldrich)

LB Broth, Miller (Amresco)

LiCl (Amresco)

NaCl (IPL Uhersky Brod)

$NaHCO_3$ (Chemapol Praha)

NP40 (Sigma-Aldrich)

PBS - Dulbecco's PBS 10x (Sigma-Aldrich)

PCR Buffer JumpStart 10x (Sigma-Aldrich)

Penicilin-Streptomycin 100x (Gibco)

Phenol (Penta)

Primers (Sigma-Aldrich)

Probes (Roche)

Protease Inhibitor Cocktail (Sigma-Aldrich)

Protein A agarose (Sigma-Aldrich)

Protein G agarose (Sigma-Aldrich)

Proteinase K (Thermo Scientific)

RNase (Sigma-Aldrich)

RNase-free water (Qiagen)

S.O.C. medium (Invitrogen)

SDS (Amresco)

Sodium acetate (Amresco)

Sybr Green PCR Master Mix (Applied Biosystems)

TaqMan Multiplex Master Mix (Applied Biosystems)

TET siRNA (Dharmacon)

Tris base (Sigma-Aldrich)

Tris-HCl (Amresco)

Triton-X-100 (Amresco)

TRIzol reagent (invitrogen)

X-Gal (Thermo Scientific)

3.3. Commercial kits

DNeasy Blood and Tissue Kit (Qiagen, no: 69506)

EpiTect Bisulfite Kit (Qiagen, no: 59104)

Gel/PCR Fragments Extraction Kit (Geneaid, no: DFL100).

High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, no: 4368814)

High-Speed Plasmid Mini Kit (Geneaid, no: PD300)

Human Monocyte Nucleofector Kit (Lonza, no: VPA-1007)

Hydroxymethylated DNA Immunoprecipitation (hMeDIP) Kit (Abcam, no: ab117134)

InsTAclone PCR Cloning Kit (Thermo Scientific, no: K1213)

Methylated DNA Immunoprecipitation (MeDIP) Kit (Abcam, no: ab117133)

3.4. DNA vector pTZ57R/T

pTZ57R/T vector is a component of InsTAclone PCR Cloning Kit (Thermo Scientific, no: K1213). The cloning principle of this kit is based on amplification of PCR product with

Taq polymerase, which adds a single deoxyadenosine triphosphate overhang at 3´ends of PCR fragment. pTZ57R/T is a linearized vector with cloning site containing single ddT overhangs, thus PCR product can be easily and efficiently cloned into the vector (Fig. 9, 10). The vector contains Ampicillin resistence and multiple cloning site within lacZ gene encoding β-galactosidase. Insertion of PCR product disrupts production of functional β-galactosidase, thus successful ligation can be measured by blue-white screening.

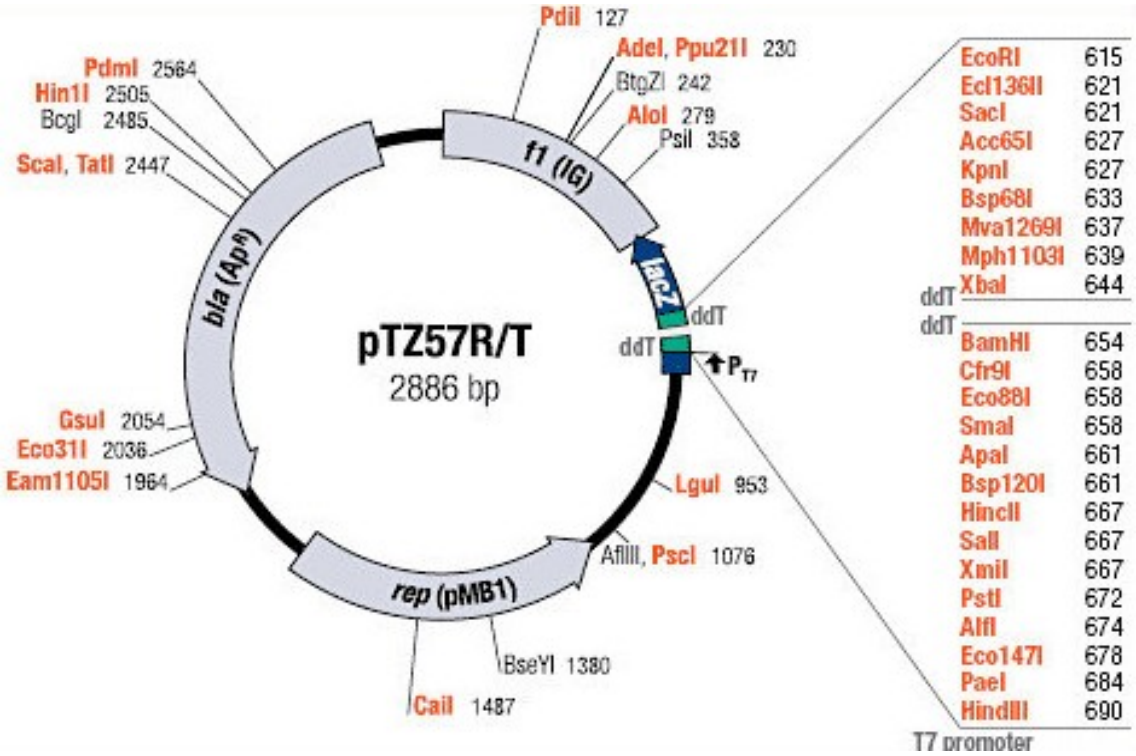


Figure 9: **Map of the pTZ57R/T vector** (Thermo Scientific, InsTAclone PCR Cloning Kit User guide).

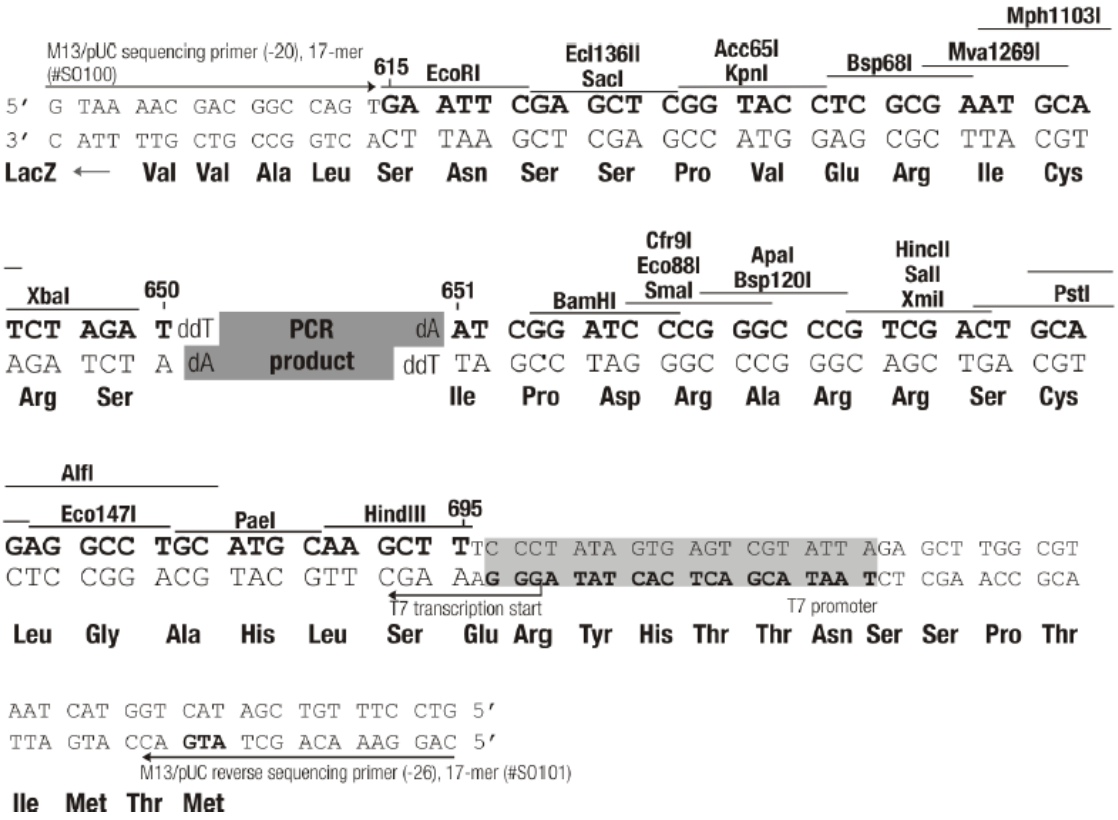


Figure 10: Detail of pTZ57R/T vector cloning site (Thermo Scientific, InsTAclone PCR Cloning Kit User guide).

3.5. Primers and probes

3.5.1. Primers for bisulfite treated DNA

PU.1 - URE - F	GAGAAATGGTTTTTTTGTGATTT
PU.1 - URE - R	ACAACTACCCCTATTTCCACAT

Table 6: Primers used for amplification of URE in *PU.1* locus. F – forward primer, R – reverse primer

URE site and position of primers are depicted in Fig. 12 in chapter 3.5.4. below.

3.5.2. Primers and probes for analysis of gene expression

GAPDH mRNA - F	GCCCAATACGACCAAATCC	#60
GAPDH mRNA - R	AGCCACATCGCTCAGACAC	#60
TET1 mRNA - F	TCTGTTGTTGTGCCTCTGGA	#57
TET1 mRNA - R	GCCTTTAAAACTTTGGGCTTC	#57
TET2 mRNA - F	GAAAAAGATGAAGGTCCTTTTTATACC	#68
TET2 mRNA - R	TTTACCCTTCTGTCCAAACCTT	#68
TET3 mRNA - F	CGCCTCTATCCGGGAACT	#25
TET3 mRNA - R	TCCCCGTGTAGATGACCTTC	#25
PU.1 mRNA - F	CCACTGGAGGTGTCTGACG	#1
PU.1 mRNA - R	CTGGTACAGGCGGATCTTCT	#1
DNMT1 mRNA - F	CCCAAGTAACTGGGATTAGAGC	#45
DNMT1 mRNA - R	GGTTTGCCTGGTGCTTTTC	#45

Table 7: Primers used for analysis of gene expression by qPCR. F – forward primer, R – reverse primer. Numbers on right indicate numbers of Roche probes.

3.5.3. Primers for colony PCR

M13 - F	GTAAAACGACGGCCAG
M13 - R	CAGGAAACAGCTATGAC

Table 8: Primers used for amplification of cloned product in pTZ57R/T vector. F – forward primer, R – reverse primer.

3.5.4. Primers for analysis of DNA fragments after ChIP, MeDIP and hMeDIP

PU.1 (-16.6kb) - F	CCTGACCCCACATTCTGATT
PU.1 (-16.6kb) - R	CTTCTTCTGGGCTCTCAGC

Table 9: Primers used for analysis of DNA methylation and hydroxymethylation by qPCR after ChIP and after MeDIP and hMeDIP. F - forward primer, R – reverse primer. Number -16.6kb indicate PCR amplicon of *PU.1* DNA locus upstream from transcription start site of *PU.1* gene.

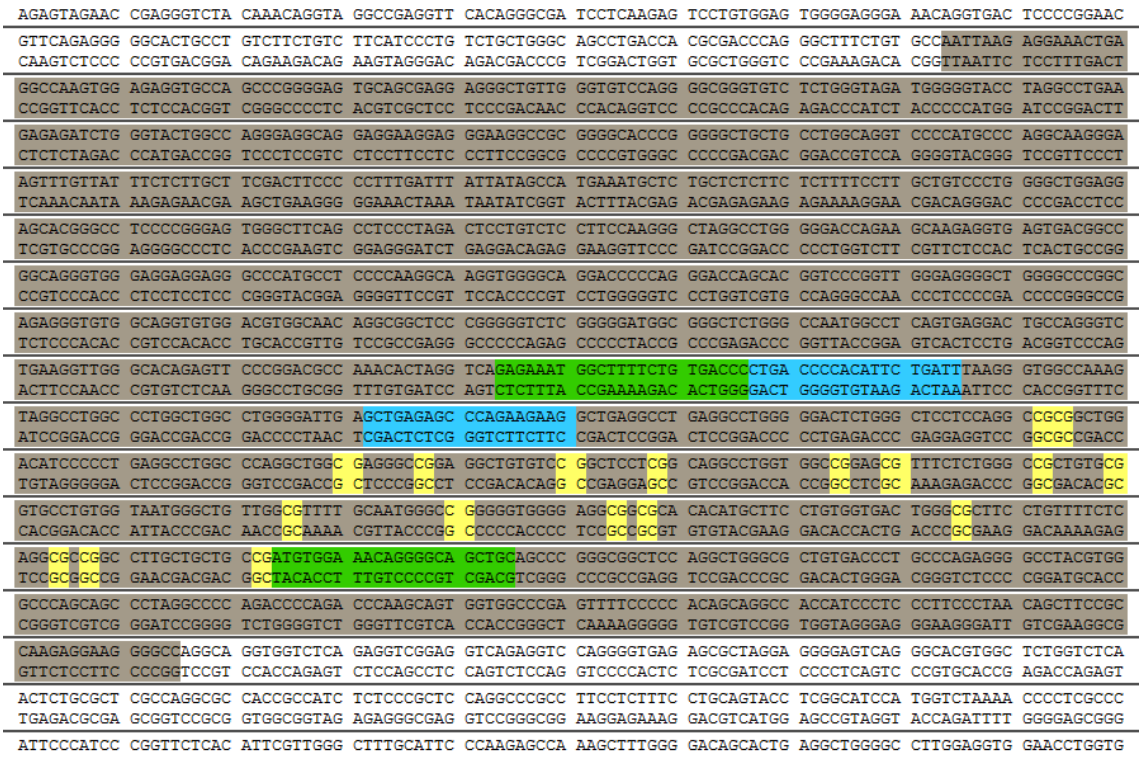


Figure 11: DNA sequence of URE site with primer positions: ChIP primers -16.6kb (blue), bisulfite conversion primers (green). CpG islands in yellow

4. Methods

4.1. Cultivation of OCI-M2 wild type cell line

The cells were cultivated in IMDM medium supplemented with 20% fetal bovine serum and 1% antibiotics (penicillin-streptomycin) at 37°C in a 4% CO_2 incubator

4.2. Selection and cultivation of AZA-resistant OCI-M2 cell line

Selection of AZA-resistant OCI-M2 cells was based on the fact, that sublethal AZA concentration for OCI-M2 cell line is 5 µM (Curik *et al.*, 2012)

The sensitive OCI-M2 cells were cultured in 150 µl of medium (IMDM + 20% fetal bovine serum + 1% penicillin-streptomycin) in three 96-well plates, in concentration 10.000 cells per well and maintained at 37°C in a 4% CO_2 incubator. Cells were grown for 2 days and then AZA was added as follows:

To the first plate, 0.1 µM AZA was added to each well.

To the second plate, 1 µM AZA was added to each well.

To the third plate, 10 µM AZA was added to each well.

Treatment with AZA continued for 5 days, AZA was added in appropriate concentration every second day (3 times in total). After the 5th day, no change in cell growth was observed in the first and second plate. On the contrary, in the third plate, no living cells were found.

Based on these results, experiment was repeated with the same conditions but different concentration of AZA - for three weeks, every second day 8 µM AZA was added to each well. After the three weeks, 5-8 living cells were detected in some wells. These cells were transferred to a new 96-well plate and separated, so each well contained only 1 cell. Cell clones were cultured under the same conditions, with 8 µM AZA added every second day, to confluence, when they were transferred to 48-well plate, then 6-well plate and finally into culture flasks. The whole process of selection of AZA resistant clones took approximately 1 month and 3 weeks. In total, only ten clones (N=10 from 96) survived and were able to proliferate with AZA treatment. These clones were considered as AZA-resistant and were used for experiments.

AZA solution was always freshly prepared by adding appropriate amount of AZA powder to sterile ddH_2O.

4.3. AZA treatment of OCI-M2 wild type cells

OCI-M2 wild type cells were cultured as described above. Then, 5 µM AZA was added. After 48 hours, cells were harvested and RNA and DNA were isolated.

4.4. Isolation of RNA

RNA was isolated from OCI-M2 cell lines by TRIzol reagent.

300,000 cells were harvested and centrifuged for 5 minutes at 1,500 rpm. Cell pellet was washed with 500 µl of PBS and centrifuged again for 5 minutes at 1,500 rpm. Cell pellet was lysed with 300 µl of TRIzol reagent and incubated for 5 minutes at room temperature (RT). Afterwards 60 µl of chloroform was added, sample was mixed and centrifuged for 7 minutes at 11,000 rpm. Upper aqueous phases was transferred into new tube and mixed with 150 µl of chloroform and subsequently centrifuged for 7 minutes at 11,000 rpm. Upper aqueous phase was transferred into fresh tube again and 1 µl of glycogen and 150 µl of isopropyl alcohol were added. Sample was then incubated at - 20°C overnight.

Next, the sample was centrifuged for 40 minutes at 13,000 rpm, 4°C. Pellet was washed by 200 µl of 80% ethanol, centrifuged for 5 minutes at maximum speed, 4°C. Supernatant was discarded and pellet was diluted in 30 µl of RNase-free water. Concentration of RNA was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

4.5. Reverse transcription polymerase chain reaction (RT-PCR)

Isolated RNA was transcribed to cDNA by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) following manufacturer´s protocol:

PCR reaction mix:

10x RT buffer	2.0 µL
10x dNTPs (100 mM)	2.0 µL
10x Random Primers	2.0 µL
Reverse Transcriptase	0.8 µL
RNA (800 ng)	variable
RNase-free water	variable
Total	20.0 µL

PCR program:

1.)	10 minutes	25°C	Repeated 40x
2.)	120 minutes	37°C	
3.)	5 minutes	85°C	
Hold		4.0°C	

4.6. Quantitative real-time PCR (qPCR) and analysis of gene expression

qPCR was performed in 384-well plates using 7900 HT SDS PCR machine (Applied Biosystems). Each well contained 4 µL of Sample mix and 4 µL of Primer mix (see below).

TaqMan technology was used for analysis of gene expression, SYBR Green protocol for quantifying DNA fragments in samples after chromatin immunoprecipitation. Each sample was performed as a technical duplicate or triplicate.

TaqMan sample mix: 1x

TaqMan Multiplex Master Mix	2.0 µL
ddH_20	1.0 µL
cDNA	1.0 µL
Total	4.0 µL

TaqMan primer mix: 1x

TaqMan Multiplex Master Mix	2.0 µL
ddH_20	1.7 µL
Roche Probe 0,1 M	0.1 µL
Primer 20 µM (Forward + Reverse)	0.2 µL
Total	4.0 µL

TaqMan PCR program:

1.)	15 seconds	95°C	Repeated 40x
2.)	1 minute	60°C	

SYBR Green sample mix: 1x

SYBR Green Master Mix	2.0 µL
ddH_20	1.0 µL
DNA	1.0 µL
Total	4.0 µL

SYBR Green primer mix: 1x

SYBR Green Master Mix	2.0 µL
ddH_20	1.8 µL
Primer 20 µM (Forward + Reverse)	0.2 µL
Total	4.0 µL

SYBR Green PCR program:

1.)	10 seconds	95°C	Repeated 40x
2.)	20 seconds	60°C	
3.)	30 seconds	72°C	

Evaluation of gene expression:

The data obtained from each sample PCR reaction were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative mRNA expression was then calculated using formula (Livak and Schmittgen, 2001):

$$2^{(C_{T(norm.gene)} - C_{T(sample)})}$$

The counted values from duplicated samples were averaged and standard deviation was calculated. Statistical analysis was performed using Students t-test (*p<0.05; **p<0.01; ***p<0.001).

4.7. Bisulfite sequencing

The level of DNA methylation can be determined by technique using sodium bisulfite. Under specific conditions (high temperature and low pH), is sodium bisulfite capable of converting unmethylated cytosines in single-stranded DNA to uracils, while methylated cytosines stay unchanged. Subsequent DNA sequencing can reveal which particular CpG island was methylated (Frommer *et al.*, 1992).

4.7.1. Conversion with sodium bisulfite

DNA was isolated from 2 million cells by DNeasy Blood and Tissue Kit (Qiagen) following instructions.

Isolated DNA was then converted with sodium bisulfite using EpiTect Bisulfite Kit (Qiagen) following manufacture´s instructions:

To the reaction, 1 μg of isolated DNA was added.

Procedure consists from 6 main steps:

1.) Sodium bisulfite treatment (conversion of unmethylated cytosines to uracils)

DNA is mixed with Bisulfite Mix and DNA Protect Buffer and converted using a thermal cycler (Mastercycler Gradient, Eppendorf). Before each incubation step, DNA has to be denaturated.

Thermal cycler program:

Denaturation step	5 minutes	95°C
Incubation step	25 minutes	60°C
Denaturation step	5 minutes	95°C
Incubation step	85 minutes	60°C
Denaturation step	5 minutes	95°C
Incubation step	175 minutes	60°C
Hold		20°C

2.) Cleaning of converted DNA

Converted DNA is mixed with Buffer BL (binding buffer), which enables binding of converted DNA to membrane of EpiTec spin columns. Membrane is then washed using Buffer BW (washing buffer) to clean DNA from residual sodium bisulfite.

3.) Desulfonation

Membrane of EpiTec spin column is treated with Buffer BD (desulfonation buffer) and then again washed with Buffer BW.

4.) Elution of clean DNA

EpiTec spin column with bound DNA was placed into fresh micro-centrifuge tube and DNA was eluted using 30 µL of Buffer EB (elution buffer).

4.7.2. Polymerase chain reaction (PCR) of sodium bisulfite converted DNA

For further analysis of DNA methylation at URE site, URE sequence had to be amplified using PCR. Because of previous treatment of DNA with sodium bisulfite, primers had to be designed for converted DNA. For primer design, Bisulfite Primer Seeker (Zymo Research) online tool was used. Primers sequences are shown in table 5. Unconverted DNA sequence of URE site with primer positions is shown in Table 6. For PCR, thermal cycler Mastercycler Gradient (Eppendorf) was used.

PCR reaction mix:

10x PCR buffer JumpStart	2.0 µL
dNTPs 2.5 mM	2.0 µL
DMSO	1.0 µL
Betaine 2.5 M	8.0 µL
Primer 20 µM (reverse+forward)	2.0 µL
Taq polymerase JumpStart	0.4 µL
ddH_2O	3.6 µL
Converted DNA	1.0 µL
Total	20.0 µL

PCR program:

1.)	3 minutes	95°C	
2.)	30 seconds	94°C	
3.)	30 seconds	55.8°C	Repeated 35x
4.)	1 minute	72°C	
5.)	10 minutes	72°C	
Hold		4.0°C	

4.7.3. Agarose electrophoresis

For verification of PCR, amplified DNA was separated on 1.5% Agarose gel with Ethidium bromide (final concentration in gel was 0.5 µg/mL). To 20 µL of DNA sample, 3.3 µL of 6x Loading dye was added and mixture was transferred into wells in gel together with DNA size marker Gene Ruler 1 kb (10,000 – 250 kb). The gel was run in TBE buffer at 60 V for 1.5 hours.

TBE buffer 5x:

Tris base	54 g
Boric acid	27.5 g
EDTA 0.5 M (pH 8)	20 mL
ddH_2O	to 1 L

For gel electrophoresis, the 5x TBE buffer was diluted to 1x.

Amplified DNA sequence was then cut out from the gel and extracted using Gel/PCR Fragments Extraction Kit (Geneaid) following manufacture´s instructions.

The concentration of purified DNA was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

4.7.4. Ligation into the cloning vector and bacterial transformation

For sequencing of URE site, this amplified sequence had to be cloned into a vector. The cloning was performed using InsTAclone PCR Cloning Kit (Thermo Scientific), vector pTZ57R/T, following instructions. For the ligation reaction, 69 ng of purified DNA was used (according to manufacturer´s recommendation).

Ligation mix:

Vector pTZ57R/T	3.0 µL
5x Ligation Buffer	6.0 µL
PCR product (69 ng)	variable
Nuclease-free water	variable
T4 DNA Ligase	1.0 µL
Total	30.0 µL

Ligation mixture was incubated at RT for 1 hour and then at 4°C overnight.

Second day transformation of bacteria (DH5α Competent Cells – Invitrogen) by heat shock was performed:

Vial with bacteria was thawed on ice and then gently mixed by pipette tip with 2.5 µl of ligation mixture and incubated on ice for 30 minutes. After the incubation, heat shock was performed in 42°C water bath (NÜVE) for 40 seconds and immediately after that, the cells were cooled on ice for 2 minutes. Subsequently, 500 µL of S.O.C. medium was added and cells were incubated in Orbital Shaker incubator (Gallenkamp) for 1 hour at 37°C 225 rpm.

Bacteria were then spread on 2 agar plates, prepared as follows:

200 mL of dH_2O was mixed with 7 g of LB Broth with agar and sterilized in Sterimat Plus machine (BMT Medical Technology) for 20 minutes at 121°C. 200 µL of Ampicillin (100 mg/mL) was then added into the thawed and cooled agar in laminar flow cabinet (Telstar BioIIA) and mixed. The mixture was then transferred to Petri dishes. When agar solidified, 40 µL of X-Gal (20 mg/mL in DMSO) and 20 µL of IPTG (100mM) were added to each plate.

Plates were placed into incubator (TCH 100 – Labsystem Praha) overnight at 37°C.

4.7.5. Colony PCR

Next day, blue-white screening was performed. Insertion of PCR product disrupts LacZ gene, thus bacteria carrying plasmid with successfully cloned PCR product don´t express functional β-galactosidase and are not able to metabolize lactose analog X-Gal. Such bacterial colonies appear white. Non-recombinant colonies express functional β-galactosidase which cleaves X-Gal forming blue pigment 5,5'-dibromo-4,4'-dichloro-indigo resulting in blue color of non-recombinant colonies.

Ten white colonies were transferred with pipette tip into a new plate with Ampicillin. The same pipette tip was then dipped into PCR reaction mix (see below). For PCR reaction, M13 primers (Thermo Fisher) were used (Table 8).

The new plate was placed into incubator (TCH 100 – Labsystem Praha) overnight at 37°C.

PCR reaction mix:

10x PCR buffer JumpStart	2.0 µL
dNTPs 2.5 mM	2.0 µL
DMSO	1.0 µL
Betaine 2.5 M	8.0 µL
Primer 20 µM (reverse+forward)	1.0 µL
Taq polymerase JumpStart	0.2 µL
ddH_2O	5.8 µL
Total	20.0 µL

PCR program:

1.)	3 minutes	95°C	Repeated 35x
2.)	30 seconds	94°C	
3.)	30 seconds	55.8°C	
4.)	1 minute	72°C	
5.)	10 minutes	72°C	
Hold		4.0°C	

Agarose gel electrophoresis was performed after, to verify if DNA corresponding to URE site was cloned into the vector. Procedure of Agarose gel electrophoresis was described above.

4.7.6. Isolation of plasmid DNA

The next day, colonies were transferred by pipette tip from the new plate into tubes with 3 mL of bacterial growth medium LB Broth (prepared by adding 10 g of LB Broth into 400 mL of dH_2O). Before use, LB Broth medium was sterilized in Sterimat Plus machine (BMT Medical Technology) and 400 µL of Ampicillin was added. Tubes were incubated overnight in Orbital Shaker incubator (Gallenkamp) at 225 rpm, 37°C.

The next day, plasmid DNA was isolated from grown bacteria using High-Speed Plasmid Mini Kit (Geneaid) following instructions.

Isolated plasmid DNA was then cleaned up by ethanol precipitation. To 30 µL of plasmid DNA, 1.5 µL of glycogen, 3 µL of 3 M sodium acetate (pH 5.2) and 75 µL of 96% ethanol were added and mixture was precipitated overnight at -20°C. The second day, samples were centrifuged for 20 minutes at 14,000 rpm, 4°C. The pellet was washed with 700 µL of 75% ethanol, mixed and centrifuged for 5 minutes at 14,000 rpm, 4°C. Supernatant was discarded and precipitated plasmid DNA was diluted in 50 µl of elution buffer and incubated for 2 minutes at 42°C in thermomixer (Thermomixer comfort - Eppendorf). Concentration of plasmid DNA was measured by NanoDrop ND-1000 Spectrophotometer (Thermo Scientific).

4.7.7. Sequencing of plasmid DNA and analysis of DNA methylation

Samples with purified plasmid DNA from 10 white colonies were sequenced in Laboratory of DNA Sequencing at Faculty of Natural Science, Charles University in Prague. For the reaction, M13 forward primer (Thermo Fisher) was used (Table 8).

Reaction mix:

Primer 20 µM (forward)	2.5 µL
Plasmid DNA (200ng)	variable
ddH_2O	variable
Total	8.0 µL

DNA methylation was analysed at the 18 CpG islands (Fig. 8) using BiQ analyser software (Bock *et al.*, 2005), data from 10 white colonies for each CpG were statistically calculated.

4.8. Chromatin immunoprecipitation (ChIP) of methylated and hydroxymethylated DNA

$20x10^6$ cells were harvested, cross-linked by formaldehyde to final concentration 1% and incubated at RT for 10 minutes on shaker (Grant bio PMR-30 Mini Rocker-Shaker). Glycine was added to final concentration 0.125 M, sample was incubated at RT for 10 minutes on shaker and centrifuged for 5 minutes at 3,000 rpm, 4°C. Pellet was resuspended in 6 mL of PBS supplemented with freshly added protease inhibitors, incubated on ice for 10 minutes and centrifuged 5 minutes at 3,000 rpm, 4°C. Cells were lysed with 5 mL of ChIP lysis buffers 1, 2 (see below) supplemented with proteases inhibitors. After each lysis, sample was centrifuged for 10 minutes at 4,000 rpm, 4°C. Finally, pellet was diluted in 2 mL of ChIP lysis buffer 3 (see below).

Isolated chromatin in ChIP lysis buffer 3 was sonicated (Digital sonifier M500 - 2 seconds pulse on, 13 seconds pulse off, repeated 500 times, pulse intensity 20%). During procedure, sample was cooled in beaker filled with ethanol and ice. 20 µL of sheared DNA were removed and agarose electrophoresis was performed to verify DNA shearing. DNA fragments were checked on agarose gel to be 200-500 bp long.

Sheared DNA was centrifuged for 15 minutes at 13,000 rpm, 4°C, supernatant was transferred into fresh tubes and $\frac{1}{10}$ of volume was removed as 10% input. Input was stored at -20°C.

Sample was pre-cleared using 60 µL of protein A agarose beads and 50 µL of 10% BSA (BSA powder diluted in PBS) and incubated on shaker for 3 hours at 4°C. Afterwards, sample was centrifuged for 5 minutes at 3,000 rpm, 4°C and supernatant was divided into 4 fresh tubes. To 3 tubes, 5 µL of either 5-methylcytosine antibody, 5-hydroxymethylcytosine antibody or non-specific IgG was added. No antibody was added into the fourth tube. The samples were incubated on shaker for 3 hours at 4°C and then 60 µL of protein A and G agarose beads were added to each tube. The samples were shaking overnight at 4°C.

Next day, the samples were centrifuged for 5 minutes at 3000 rpm, 4°C. Supernatant was discarded and beads were washed with buffers TSE 1, 2, 3 and TE (see below) supplemented with fresh proteases inhibitors. Between each washing procedure, samples were let shaking for 10 minutes at 4°C and then were centrifuged for 1 minute at 1,000 rpm, 4°C. After the last washing step, samples were centrifuged for 1 minute at highest speed, 4°C. Precipitates were eluted by adding 200 µL of Elution buffer (see below) and centrifuged for 3 minutes at 13,000 rpm, RT. Supernatant was transferred into a fresh tube and elution step was repeated once more with 100 µL of Elution buffer. To 300 µL of eluted samples, 18 µL of 5 M NaCl were added and samples were incubated overnight at 65°C in thermomixer.

The next day, samples and also inputs were mixed with 75 µL of PK buffer (see below), 1.2 µL of proteinase K (10 mg/mL) and 1 µL of RNase and incubated for 2 hours at 55°C in thermomixer. Afterwards, phenol-chloroform extraction was performed. The samples were mixed in a ratio of 1:1 with phenol and centrifuged for 2 minutes at maximum speed. Upper aqueous phases was transferred into a fresh tube and mixed in a ratio of 1:1 with chloroform and centrifuged for 2 minutes at maximum speed. Upper aqueous phase was transferred into a fresh tube and ethanol precipitation was performed (as described previously).

Level of DNA methylation and hydroxymethylation was determined from quantity of DNA fragments present in samples using qPCR (SYBR Green protocol, primers in table 9). As a positive control of DNA hydroxymethylation, additional four DNA amplicons were used. Primers were designed according to data from Madzo, et al., 2014 to amplify sites with high levels of DNA hydroxymethylation (S1).

qPCR reaction was first performed with serially diluted DNA to obtain a standard curve for the primer pair. Using this equation, CT values of samples and 10% inputs from ChIP were converted into DNA copy numbers. The copy numbers of 10% inputs were divided by 10 to obtain copy numbers of 1% input. The DNA copy numbers for each

sample were then divided by the copy numbers of 1% inputs in order to calculate "percentage of the input". Because samples were performed as technical duplicates or triplicates, in the end "percentages of input" were averaged. Statistical analysis was performed using Students t-test (*p<0.05; **p<0.01; ***p<0.001).

ChIP Lysis Buffer 1

50 mM HEPES-KOH (pH 7.5)

140 mM NaCl

1 mM EDTA

10% glycerol

0.5% NP-40

0.25% Triton-X100

ddH_2O to final volume

ChIP Lysis Buffer 2

1 mM EDTA

0.5 mM EGTA

10 mM Tris-HCl (pH 8)

200 mM NaCl

ddH_2O to final volume

ChIP Lysis Buffer 3

1 mM EDTA

0.5 mM EGTA

10 mM Tris-HCl (pH 8)

ddH_2O to final volume

TSE 1

2 mM EDTA

1% Triton X-100

20 mM Tris-HCl (pH 8.1)

150 mM NaCl

0.1% SDS

ddH_2O to final volume

TSE 2

2 mM EDTA

1% Triton X-100

20 mM Tris-HCl (pH 8.1)

500 mM NaCl

0.1% SDS

ddH_2O to final volume

TSE 3

1 mM EDTA

0.5 M LiCl

10 mM Tris-HCl (pH 8.1)

1% NP40

1% DOC

ddH_2O to final volume

TE Buffer

10 mM Tris-HCl (pH 8)

1 mM EDTA

ddH_2O to final volume

PK Buffer

50 mM Tris-HCl (pH 7.5)

25 mM EDTA

1.25% SDS

ddH_2O to final volume

Elution Buffer

0.1 M $NaHCO_3$

1% SDS

ddH_2O to final volume

4.9. Methylated and hydroxymethylated DNA immunoprecipitation (MeDIP and hMeDIP) analysis

DNA was isolated from $2x10^6$ cells by DNeasy Blood and Tissue Kit (Qiagen) following instructions and sonicated (Digital sonifier M500 - 1 second pulse on, 12 seconds pulse off, repeated 50 times, pulse intensity 20%). DNA fragments were approximately 300 bp long (checked on agarose gel).

Sheared DNA was used for MeDIP and hMeDIP performed by commercial kits Methylated DNA Immunoprecipitation Kit - MeDIP and Hydroxymethylated DNA Immunoprecipitation Kit - hMeDIP (Abcam) following manufacturer´s instructions. Both kits are based on specific binding of methylated or hydroxymethylated DNA fragments to antibody coated wells, which are in next step washed and DNA fragments are eluted.

Quantity of DNA fragments was analysed using qPCR (SYBR Green protocol, primers in table 9), qPCR was evaluated as described in chapter 4.8.

4.10. siRNA mediated gene knockdown

For each siRNA reaction $2x10^6$ cells were harvested, centrifuged for 5 minutes at 1,500 rpm and supernatant was discarded. Pellets were washed with 1 mL of PBS and centrifuged again for 5 minutes at 1,500 rpm. Specific TET1, TET2 or TET3 siRNA and non-specific control siRNA were added (the most efficient siRNA concentration was previously found out – S2). 0.2 µL of GFP control vector was cotransfected. Afterwards 90 µL of nucleofector solution with supplement (provided by the Nucleofector Kit) was added to each sample. The Samples were subsequently electroporated in nucleofector cuvettes using Amaxa Nucleofector II (Lonza) - programme Y-001. Transfected cells were transferred in to 12-well plate containing 1 mL of warm growth medium and incubated in 4% CO_2 for 48 hours at 37°C.

Cells were harvested 48 hours later, RNA and DNA were isolated.

TET1, TET2 and TET3 siRNAs (ON-TARGETplus siRNA, Dharmacon) were kindly provided by Kateřina Trejbalová, M.Sc., Ph.D. from the Institute of Molecular Genetics of the ASCR.

5. Results

5.1. AZA-resistant OCI-M2 display different levels of PU.1 expression

Previous publication from our laboratory revealed that subset of high-risk MDS patients displayed significantly lower levels of PU.1 mRNA. PU.1 downregulation correlated with the levels of DNA methylation at the URE and treatment with AZA in OCI-M2 cell line led to significant URE demethylation and upregulation of PU.1 expression resulting in restarted myeloid differentiation. The group of patients underexpressing PU.1 displayed even significantly shorter median survival after treatment with AZA (Curik *et al.*, 2012). Based on these results we further asked, whether the dynamics of epigenetic modifications at the URE of the *PU.1* gene are associated with AZA resistance.

In order to reveal a role of epigenetic modifications at *PU.1* gene in AZA resistance, we treated OCI-M2 cells with appropriate dose of AZA (see methods 4.2.) for 1 month to select AZA resistant clones. After the selection procedure we got ten AZA-resistant clones of OCI-M2. Initially, we wanted to know, whether these clones express lower levels of PU.1 mRNA, similarly to the subgroup of MDS patients with shorter median survival. Interestingly we found AZA-resistant clones to considerably differ in PU.1 expression. In comparison with AZA sensitive OCI-M2 (wild type OCI-M2), seven clones expressed significantly higher PU.1 levels, two clones expressed lower levels and one clone expressed approximately the same level of PU.1 mRNA (Fig. 12). Two clones (marked by numbers “3” and “21”) expressing significantly higher levels of PU.1 mRNA (we set the limit to “high expression” to more than 60 % of wild type OCI-M2 PU.1 expression) were termed “high expressors”, while two clones (clones “34” and “30”), expressing significantly lower levels of PU.1 (less than 60 % of wild type OCI-M2) were named “low expressors”. PU.1 mRNA levels of the other six clones were comparable with mRNA levels of wild type OCI-M2, (intermediate expressors). We hypothesize that AZA resistance is accompanied by inefficient DNA demethylation at the URE. The different PU.1 expression in OCI-M2 clones also led us to think about different mechanism of AZA resistance establishment.

PU.1

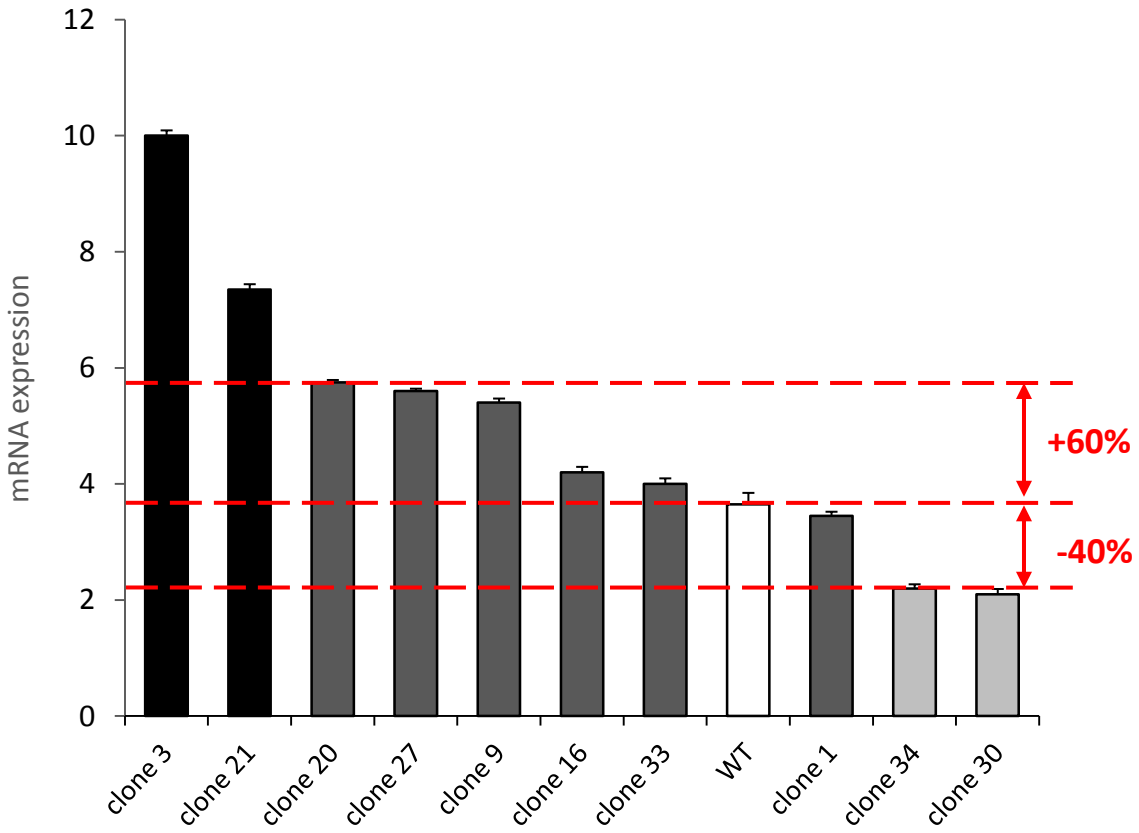


Figure 12: **Relative mRNA expression of PU.1 in AZA-resistant clones (black and grey bars) and wild-type OCI-M2 (white bar).** Clones 3 and 21 (black bars) expressing more than 60% of wild type OCI-M2 PU.1 expression represent "high expressors", clones 34 and 30 (light grey bars) expressing less than 60 % of wild type OCI-M2 PU.1 expression represent "low expressors". Clones 20, 27, 9, 16, 33 and 1 displaying comparable PU.1 mRNA levels to wild type OCI-M2 represent "intermediate expressors". The expression was measured at 48 hrs, data were normalized to GAPDH expression, error bars indicate SE of two independent experiments.

5.2. AZA-resistant OCI-M2 with low expression of PU.1 display higher rate of DNA methylation at URE

Gene expression can be regulated by multiple mechanisms, including epigenetic phenomenon - methylation of DNA. To resolve, why AZA-resistant clones differ in PU.1 expression, we initially decided to analyse epigenetic modifications at 18

CpGs within URE in high and low PU.1 expressing OCI-M2 AZA resistant clones. As controls we used the following cell cultures: i) wild type AZA sensitive OCI-M2 either non treated or harvested 48 hours after treatment with sublethal dose (5 µM) of AZA, ii) healthy human bone marrow CD34+ cells (Curik *et al.*, 2012; Burda *et al.*, 2016). The analysis was initially performed by bisulfite sequencing technique to determine DNA methylation of each individual CpG within URE.

Indeed we observed a significant difference in DNA methylation levels between low and high PU.1 expressors (Fig. 13). High expressors displayed lower DNA methylation rate at URE CpGs in comparison to low PU.1 expressors (at least four times), wild type OCI-M2 (approximately five times), and even wild type OCI-M2 after treatment with AZA (approximately two times). Conversely, low expressors showed higher average DNA methylation at the URE in comparison not only to high expressors, but also to wild type OCI-M2 after treatment with AZA, and comparable DNA methylation levels to wild type OCI-M2 (Fig. 13).

These results showed relationship between epigenetic status of URE and the expression of PU.1 in AZA-resistant clones. However, bisulfite sequencing has a limitation, it is unable to distinguish between 5mC and 5hmC (Huang *et al.*, 2010), more precise analysis was performed.

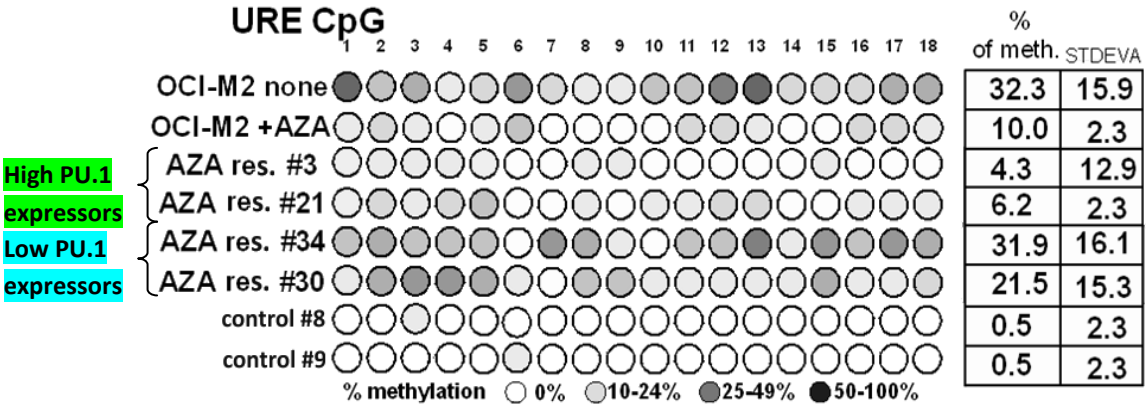


Figure 13: **DNA methylation analysis at 18 CpGs within URE of *PU.1* gene in OCI-M2 performed by bisulfite sequencing**. Comparison of DNA methylation rate in wild type OCI-M2 not treated with AZA (OCI-M2 none), wild type OCI-M2 following treatment with 5 µM AZA – cells harvested 48 hours after treatment (OCI-M2 + AZA), high PU.1 expressors (clones 3 and 21), low PU.1 expressors (clones 34 and 30) and healthy bone marrow CD34+ cells (clones 8 and 9). Each circle represents one CpG. Color shading represents % of DNA methylation. Box on the right shows average relative percentage of DNA methylation at all CpGs, STDEVA was calculated from two independent experiments.

5.3. Expression of PU.1 correlates with DNA methylation and hydroxymethylation at URE

Unlike DNA methylation at promoter regions, which have a role in gene repression, DNA hydroxymethylation appears to be associated with activation of gene transcription (Stroud, *et al.*, 2011; Madzo *et al*., 2014). Therefore it's crucial to distinguish between these two epigenetic marks. To test, whether DNA at URE in high and low expressors display methylated or hydroxymethylated state, we decided to perform ChIP using 5mC and 5hmC antibodies followed by qPCR using primers covering the URE.

ChIP data revealed hydroxymethylated DNA at URE in both high PU.1 expressing clones, however only in high expressor clone 3 we observed significant levels of the DNA hydroxymethylation. DNA methylation was not detected in high expressors. According to our expectation low expressors had a trend toward high DNA methylation rate, DNA hydroxymethylation at URE was not detected at all. In wild type OCI-M2 we detected both marks 5mC and 5hmC (Fig. 14)

These results suggest that the epigenetic modifications detected by bisulfite sequencing referred primarily to DNA hydroxymethylation in high expressors, and to DNA methylation in low expressors. Both epigenetic marks appear to be involved in regulation of PU.1 expression and their proportion apparently contribute to PU.1 expression regulation in OCI-M2.

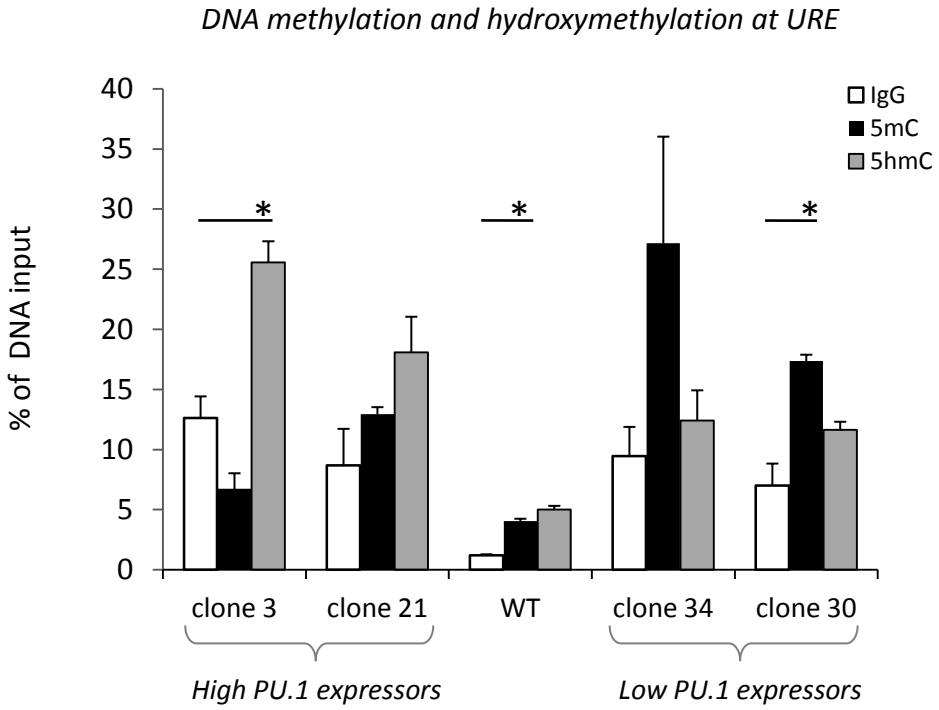


Figure 14: ChIP of methylated and hydroxymethylated DNA in OCI-M2 analysed by qPCR using primers covering URE (-16.6 kb). Comparison of DNA methylation and hydroxymethylation rate in high (clones 3 and 21) and low (clones 34 and 30) PU.1 expressors and in wild type OCI-M2 (WT). White bars indicate nonspecific signal, black bars DNA methylation, grey bars DNA hydroxymethylation. Specific signals represent % of DNA input. Error bars indicate SE of two independent experiments. (*$p<0.05$)

5.4. AZA-resistant clones express significantly lower levels of TET2 and DNMT1

5hmC is a product of activity of three TET enzymes (TET1, TET2, TET3), which catalyze oxidation of methyl group of 5mC generated by DNMT enzymes (Ito *et al.*, 2010). The determination of TET and DNMT1 levels in OCI-M2 cells could bring an insight into 5hmC conversion rate. Because *TET2* is frequently mutated gene (19 - 26%) in MDS patients, we supposed that in hematopoietic cells, TET2 is the main enzyme responsible for production of 5hmC (Delhommeau et al., 2009; Langemeijer et al., 2009). Based on this assumption and our results, that DNA at URE is in OCI-M2 cells hydroxymethylated, we decided to measure TET2 and DNMT1 expression in high and low expressors and wild type OCI-M2 by RT-qPCR.

Data in Figure 15 describe the levels of TET2 mRNA, high expressors expressed significantly higher levels of TET2 mRNA than low expressors, which demonstrates, that TET2 expression corresponds to levels of 5hmC in URE in AZA-resistant clones. However, the highest levels of TET2 mRNA were observed in wild type OCI-M2. Concerning the expression of DNMT1, we detected low expressors to differ in DNMT1 mRNA levels. While clone 34 expressed the lowest levels of DNMT1 mRNA from all OCI-M2 cells, clone 30 displayed trend toward high DNMT1 expression. However, in comparison with wild type OCI-M2, all AZA-resistant clones displayed lower DNMT1 expression (Fig. 16). Nevertheless we have to admit, that DNA at the URE could be methylated and hydroxymethylated despite lower DNMT1 and TET2 levels.

Our previous result from analysis of DNA methylation and hydroxymethylation revealed, that URE in wild type OCI-M2 contains equal levels of 5mC and 5hmC. Now we observed, that DNMT1 and TET2 mRNA levels are higher in wild type OCI-M2 than in AZA-resistant clones. Higher rates of both enzymes may be one of possible explanations, why wild type OCI-M2 displays both DNA methylation and DNA hydroxymethylation in URE. High levels of DNMT1 can increase the efficiency of DNA methylation process, while high levels of TET2 can convert more of these 5mC to 5hmC.

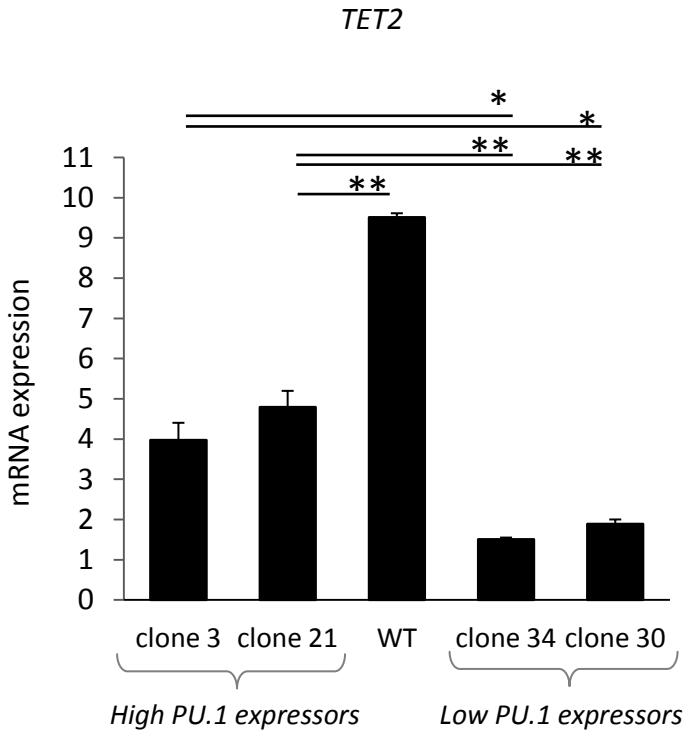


Figure 15: Relative TET2 expression in OCI-M2. The X axis shows high (clones 3 and 21) and low (clones 34 and 30) PU.1 expressors and wild type OCI-M2 (WT). The Y axis indicates relative TET2 mRNA expression. The expression was measured at 48 hours, data were normalized to GAPDH expression, error bars indicate SE of two independent experiments. (*p<0.05; **p<0.01)

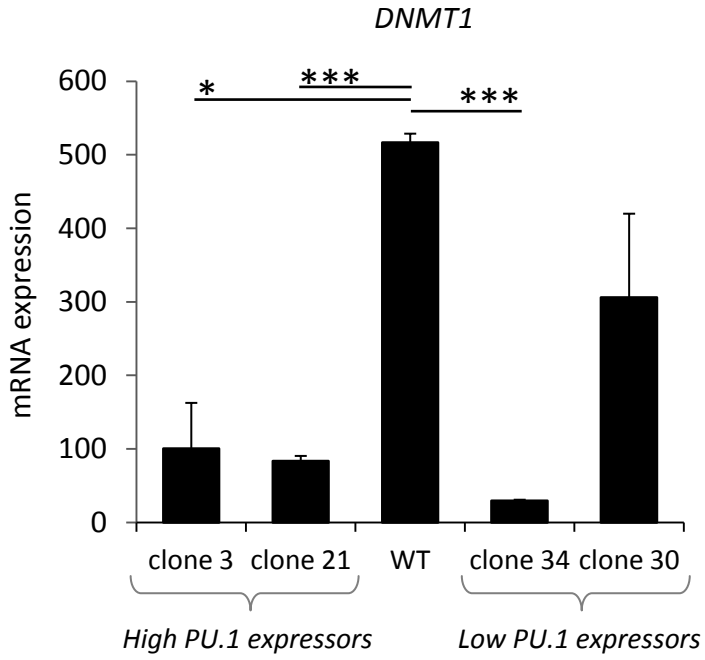


Figure 16: Relative DNMT1 expression in OCI-M2. The X axis shows high (clones 3 and 21) and low (clones 34 and 30) PU.1 expressors and wild type OCI-M2 (WT). The Y axis indicates relative DNMT1 mRNA expression. The expression was measured at 48 hours, data were normalized to GAPDH expression, error bars indicate SE of two independent experiments. (*p<0.05; ***p<0.001)

5.5. TET1 is the most expressed enzyme among all TET proteins in OCI-M2

As mentioned in introduction chapters, it is not clear, whether are TET proteins fully interchangeable concerning their biological functions (Putiri *et al.*, 2014; An *et al.*, 2015). Initially we hypothesized that the dominant role in production of 5hmC in hematopoietic cells belongs to TET2, due to frequent mutations observed in MDS and AML patients (Delhommeau et al., 2009; Langemeijer et al., 2009). However, all TET proteins are capable of 5hmC generation as was revealed in experiment with human HEK293T and U2OS cell lines (Ito *et al.*, 2010). Thus we measured mRNA levels of all TET proteins in high and low expressors and wild type OCI-M2. Unexpectedly, among all TET proteins, TET2 was absolutely the least expressed TET enzyme (Fig. 17). Expression of TET1 and TET3 was considerably higher than expression of TET2 in all OCI-M2 clones. In wild type OCI-M2, where the diference in expression between TET proteins was most obvious, we detected almost twenty times higher levels of TET1

mRNA and ten times higher levels of TET3 mRNA than TET2. We did not observe substantial difference in expression between TET1 and TET3 in AZA-resistant clones, but in wild type OCI-M2, TET1 was the most expressed enzyme. These results led us to test if DNA hydroxymethylation in OCI-M2 cells is processed rather by TET1 and/or TET3, and not by TET2.

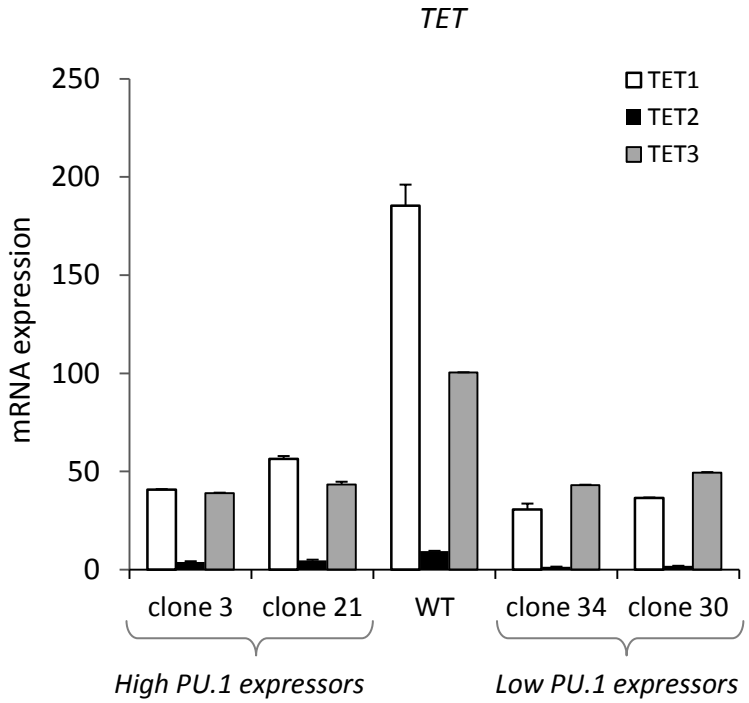


Figure 17: Relative TET proteins expression in OCI-M2. Comparison of mRNA expression of TET1 (white bars), TET2 (dark) and TET3 (grey) in high (clones 3 and 21) and low PU.1 expressors (clones 34 and 30) and wild type OCI-M2 (WT). The expression was measured at 48 hours, data were normalized to GAPDH expression, error bars indicate SE of two independent experiments.

5.6. TET3 siRNA mediated knockdown leads to decreased levels of DNA hydroxymethylation and increased levels of DNA methylation at URE

TET knockdown experiment performed by Putiri *et al.*, 2014 revealed that individual TET family members may act on distinct sites depending on chromatin

modification or CpG density. In order to resolve which TET protein is responsible for DNA hydroxymethylation at URE, we depleted each TET protein using sequence specific siRNA and analysed DNA methylation and hydroxymethylation at URE.

Firstly, we transfected wild type OCI-M2 cells with each TET protein specific siRNA. The most efficient siRNA concentration for each TET protein was first optimalized (S2 A, B). After 48 hours, RNA and DNA were isolated. RT-qPCR revealed, that TET2 and TET3 siRNA were sufficient to decrease TET2 and TET3 mRNA levels to about 47% and 39% respectively (Fig.18 A, B). TET1 siRNA was capable of decreasing TET1 mRNA levels to about 24 % (Fig. 18 C).

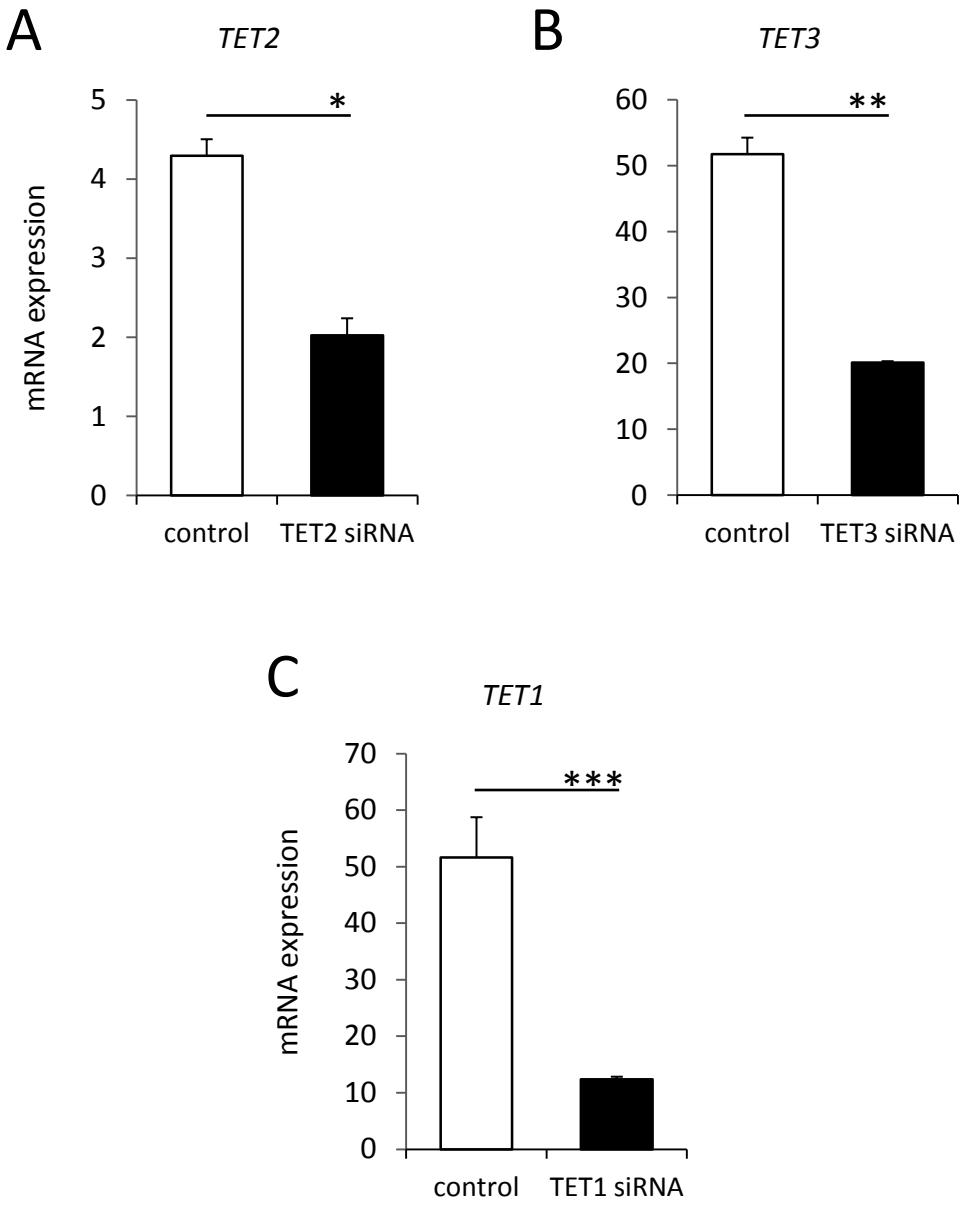


Figure 18: Relative TET proteins expression in wild type OCI-M2 after transfection with TET specific or control siRNA. A) TET2 expression after transfection with TET2 and control siRNA (250 nM). B) TET3 expression after transfection with TET3 and control siRNA (250 nM). C) TET1 expression after transfection with TET1 and control siRNA (100 nM). The expression was measured 48 hours after the transfection, data were normalized to GAPDH expression, error bars indicate SE of two independent experiments. (*p<0.05; **p<0.01; ***p<0.001)

For analysis of DNA methylation and hydroxymethylation levels, we used commercial kits MeDIP and hMeDIP (Abcam). The immunoprecipitated DNA was then analysed by qPCR using primers covering URE (Fig.19, 20).

As shown in Fig. 19, only TET3 siRNA mediated knockdown led to a significant decrease in DNA hydroxymethylation at URE (to about 23%). The reduction of 5hmC was simultaneously associated with increased DNA methylation (Fig. 20). Surprisingly, treatment with TET1 siRNA resulted in increase in DNA hydroxymethylation (Fig. 19). In contrast with TET1 and TET3, TET2 knockdown led to decrease in DNA methylation (to about 39%) (Fig. 20).

These results demonstrate, that TET3 significantly contributes to DNA hydroxymethylation establishment in URE in OCI-M2 cells and its downregulation leads to accumulation of DNA methylation at this site.

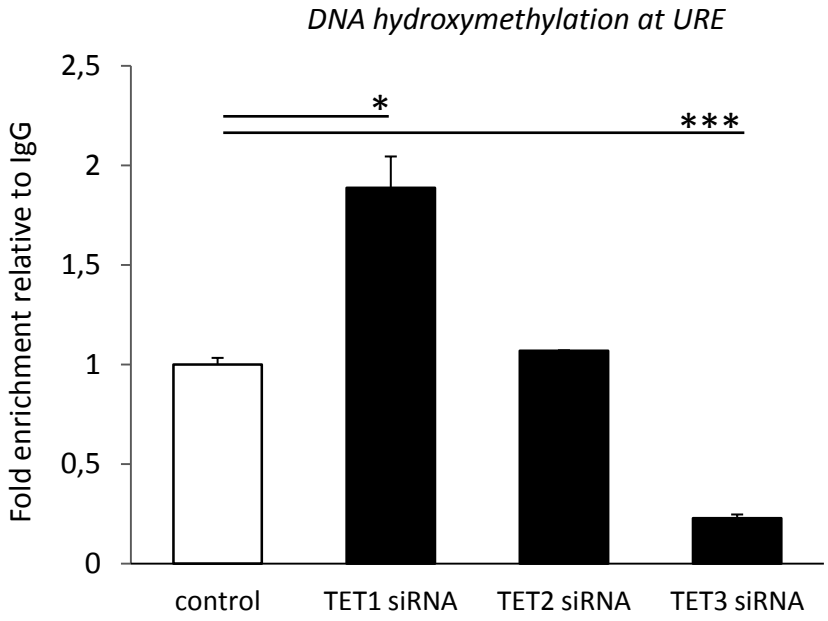


Figure 19: hMeDIP in wild type OCI-M2 after transfection with TET specific or control siRNA analysed by qPCR using primers covering URE (-16.6 kb). The Y axis shows fold enrichment relative to IgG, specific signals were divided by nonspecific signal. Error bars indicate SE of two independent experiments. (*p<0.05; ***p<0.001)

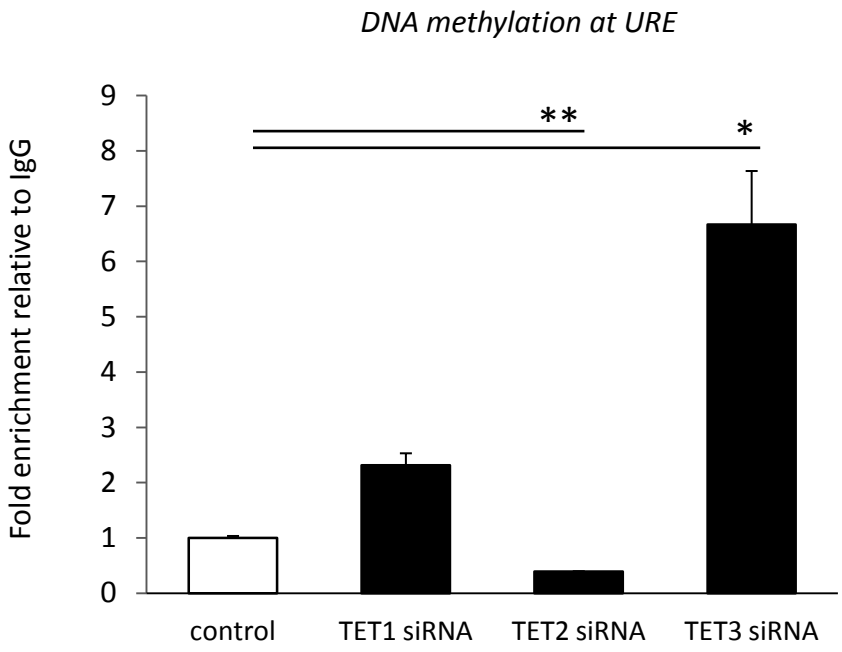


Figure 20: MeDIP in wild type OCI-M2 after transfection with TET specific or control siRNA analysed by qPCR using primers covering URE (-16.6 kb). The Y axis shows fold enrichment relative to IgG, specific signals were divided by nonspecific signal. Error bars indicate SE of two independent experiments. (*p<0.05; **p<0.01)

5.7. Inhibition of TET3 results in downregulation of PU.1

DNA methylation at URE negatively correlates with PU.1 expression and as our results suggest, correlation between DNA hydroxymethylation and PU.1 expression may also exist (Curik *et al.*, 2012). Because knockdown of TET proteins changed DNA methylation and hydroxymethylation patterns at URE, we asked whether also PU.1 expression was changed.

Analysis of PU.1 mRNA levels revealed that TET3 siRNA mediated knockdown leads also to decrease in PU.1 expression to about 26%. The decrease was observed also after inhibition of TET2, however the results were not significant (Fig. 21 A) In contrast with TET3, inhibition of TET1 does not changed PU.1 expression at all (Fig. 21 B).

These results suggest that expression of PU.1 is dependent on DNA methylation/hydroxymethylation ratio at the URE. TET3 seems to be the most suspected DNA methylation/hydroxymethylation converter in OCI-M2. The cooperative role of other TET proteins we cannot exclude.

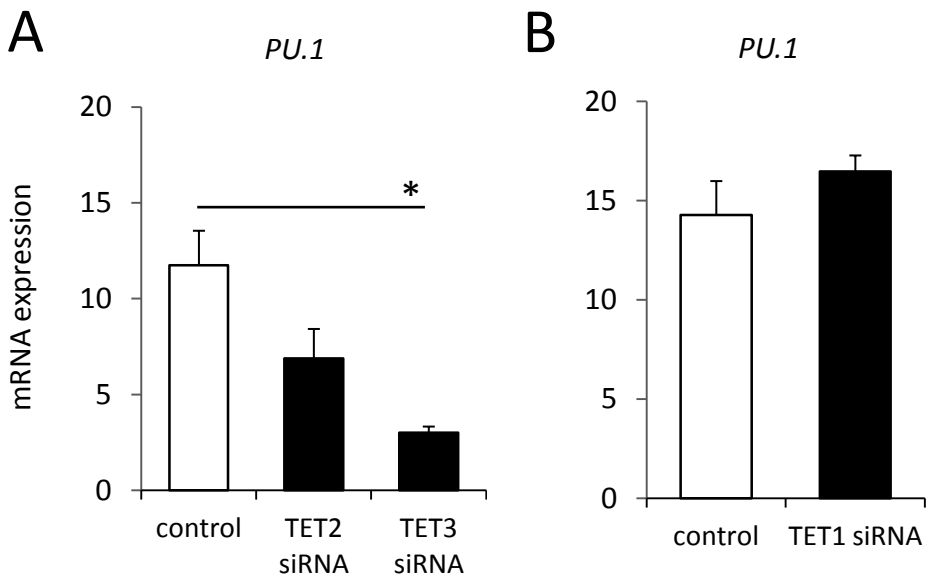


Figure 21: Relative PU.1 expression in wild type OCI-M2 after transfection with TET specific or control siRNA. A) PU.1 expression after transfection with TET2, TET3 and control siRNA (250 nM). B) PU.1 expression after transfection with TET1 and control siRNA (100 nM). The expression was measured 48 hours after the transfection, data were normalized to GAPDH expression, error bars indicate SE of two independent experiments. (*$p<0.05$)

6. Discussion

The only curative option for MDS and AML patients is HSCT, however many patients, due to advanced age, rely on chemotherapy only. AZA is the most used drug in treatment of high-risk MDS patients and subset of patients with AML. Accordingly, AZA resistance represent a considerable problem. Few possible mechanisms of AZA resistance were proposed in last five years, including upregulation of ribosomal genes and antiapoptotic protein BCL2L10, or downregulation of uridine-cytidine kinase and tumor-supresor miRNAs (Belickova et al., 2016; Cluzeau et al., 2012; Valencia et al., 2014; Solly et al., 2016). Purpose of this thesis was to analyse epigenetic modifications at PU.1 gene enhancer URE in AZA resistant OCI-M2 in order to find a potential mechanism at least contributing to AZA resistance.

We observed a large heterogeneity in epigenetic status and expression profile of AZA-resistant clones. While two AZA-resistant clones displayed higher rate of DNA methylation at URE associated with low expression of PU.1, other two clones exhibited opposite features. Our results suggest, that in PU.1 low expressors, AZA is not sufficient to demethylate URE as in wild type OCI-M2 (Curik et al., 2012). We propose that this may be one of mechanisms of AZA-resistance. Low levels of PU.1 caused by ineffective DNA demethylation may contribute to leukemic differentiation block (reviewed in Tenen, 2003). We don´t know, which mechanisms could be responsible for AZA-resistance in clones expressing high levels of PU.1. However, we found, that AZA-resistance in low and high PU.1 expressors is not a result of DNMT1 upregulation as described in SKM1 acute myeloid leukemia cell line (Solly et al., 2016). The expression of DNMT1 in AZA-resistant clones was lower compare to wild-type OCI-M2 cells.

In agreement with publications Madzo et al., 2014 and Stroud et al., 2011 were our results from analysis of DNA hydroxymethylation. Both publications observed association of 5hmC with active histone marks. We detected higher rates of 5hmC at URE in clones expressing high levels of PU.1. Whether these marks represent just intermediates of DNA demethylation pathway, or function as stable marks is not known. Madzo et al., 2014 also observed, that patients with chronic myelomonocytic leukemia with TET2 mutation display globally lower levels of 5hmC. We also found a correlation between TET2 and 5hmC, high PU.1 expressors with high rate of 5hmC at URE displayed also higher levels of TET2 mRNA. However, TET2 was the least expressed enzyme among all TET proteins and its siRNA mediated knockdown did not result in decrease of DNA hydroxymethylation at URE.

In wild type OCI-M2 we detected higher levels of TET and DNMT1 mRNAs than in AZA-resistant clones. Furthermore, URE contained approximately equal levels of

5mC and 5hmC. We assume that occurrence of both epigenetic marks at URE is a result of efficient DNA methylation process supported by high levels of DNMT1 and efficient conversion of these 5mC to 5hmC caused by high levels of TET proteins. Alternative explanation can be a difference in these epigenetic modifications at the alleles of wild type OCI-M2 cells. The cells may contain one allele with methylated URE, while URE in the second allele is hydroxymethylated, thus we detect both epigenetic marks simultaneously. We cannot exclude that population of wild type OCI-M2 cells was heterogeneous and contained more clones varying in DNA methylation and DNA hydroxymethylation patterns at URE.

We found that only inhibition of TET3 leads to decrease in DNA hydroxymethylation at URE. Moreover TET3 knockdown resulted in dramatic increase in DNA methylation with consequent downregulation of PU.1. TET3 siRNA inhibition lead also to decrease in expression of TET2 to about 57% (S3 C). However, TET2 siRNA decreased TET2 levels more efficiently (to 47%) and DNA hydroxymethylation at URE remained unchanged. Thus we concluded, that decrease in DNA hydroxymethylation observed after TET3 siRNA transfection was caused by inhibition of TET3 and not TET2. Based on these results, TET3 appears to be the most important among TET proteins for 5hmC generation at URE. However AZA-resistant clones, which significantly differed in DNA methylation and hydroxymethylation at URE, did not differ in TET3 expression. The only observed difference was in expression of TET2.

According to Curik et al., 2012, DNA methylation at URE inhibits PU.1 expression. We observed the same correlation in AZA-resistant clones. However, our results from TET2 siRNA transfection in wild type OCI-M2 were in contrast with these previous observations. Unexpectedly, TET2 downregulation caused more than two fold decrease in DNA methylation at URE followed by decrease in PU.1 mRNA levels. However the PU.1 decrease was not significant. Because in AZA-resistant clones we observed correlation not only between DNA methylation and PU.1 expression, but also between DNA hydroxymethylation, we suppose that decrease in DNA methylation did not result in PU.1 upregulation, because for its upregulation may be important not only to reduce DNA methylation at URE, but also to considerably increase levels of 5hmC.

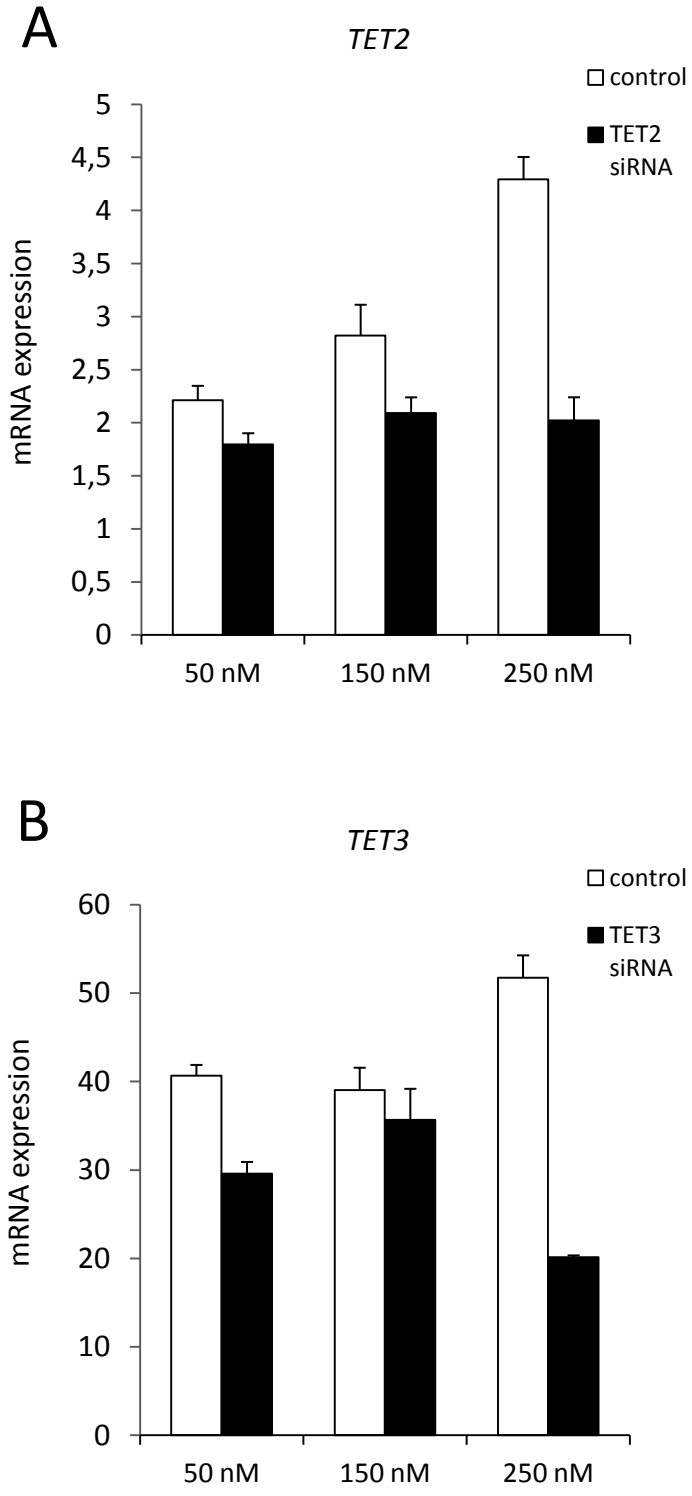
The elevation of DNA hydroxymethylation at URE after TET1 inhibition can be explained by future identification of other involved mechanisms/factors. We cannot exclude that TET1 role is primarily in DNA demethylation pathway. Thus its inhibition results in increase of 5mC, which can be subsequently converted by other TET proteins into 5hmC. However this explanation is in conflict with assumption from Putiri et al, 2014, who identified only TET2 and TET3 and not TET1 to be involved mainly in DNA demethylation pathway.

Collectively this work brings new insight into mechanisms of PU.1 gene regulation and AZA-resistance establishment in AML/MDS. We found an association between AZA-resistance and epigenetic status of URE. Indeed further experiments should be made to resolve, why AZA is not capable to demethylate DNA at the URE sufficiently. It is possible that this event may be associated with disrupted processing of AZA in the cells, for example with downregulation of uridine cytidine kinase or ribonucleotide reductase (Valencia et al., 2014). Because link between AZA-resistance and 5mC and 5hmC at URE was found only in limited number of clones, we have to take into account the existence of other mechanism of AZA resistance which don't have to be associated with PU.1 function at all or are associated with PU.1 in another context.

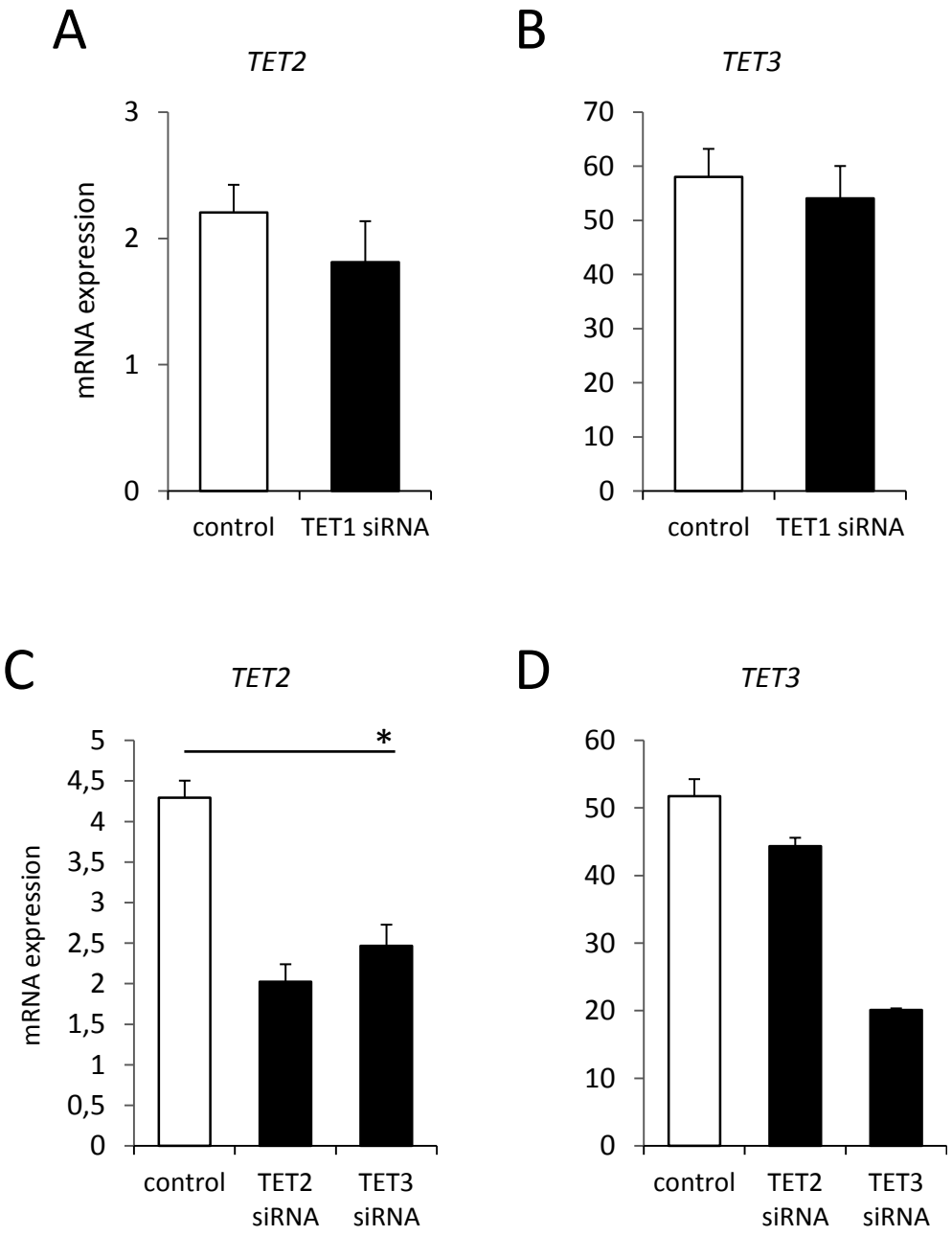
7. Supplementary data

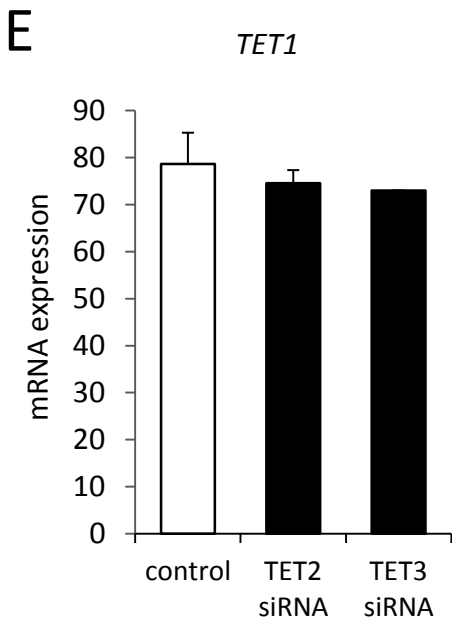
Primer´s name	Primer´s sequence	Primer´s location	Amplicon length
Control 1 - F	ACAGGGAACTCAGTGGTCTTGTGG	chr11:5263341+5263490	150 bp
Control 1 - R	ACTCCCTCCCCAGCTCTTAGGCA		
Control 2 - F	TCTCACAGTGCTGGTCTGTTTCTCA	chr11:5268841+5269002	162 bp
Control 2 - R	CCCATGCCCTCAAGTGTGCAG		
Control 3 - F	CTCCTTCTCTCACCCTCCACCC	chr11:5295750+5295899	150 bp
Control 3 - R	GCTCAAACTCACCTGCAAACTTCGT		
Control 4 - F	TGTGAAGTGTCCAGGTGGTTCCAT	chrX:48652904+48653066	163 bp
Control 4 - R	GCGTCATGCCCAGCCACAAG		

S1: Primers used for qPCR after ChIP as positive controls of DNA hydroxymethylation. F - forward primer, R – reverse primer.



S2: Relative TET2 and TET3 expression in wild type OCI-M2 after transfection with TET specific or control siRNA in different concentrations. A) TET2 expression after transfection with either 50 nM, 150 nM or 250 nM TET2 (black bars) and control siRNA (white bars). B) TET3 expression after transfection with either 50 nM, 150 nM or 250 nM TET3 and control siRNA. The expression was measured 48 hours after the transfection, data were normalized to GAPDH expression, error bars indicate SE of two independent experiments


A
TET2
mRNA expression
3
2
1
0
control
TET1 siRNA
B
TET3
70
60
50
40
30
20
10
0
control
TET1 siRNA
C
TET2
*
mRNA expression
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
control
TET2 siRNA
TET3 siRNA
D
TET3
60
50
40
30
20
10
0
control
TET2 siRNA
TET3 siRNA



S3: Relative TET proteins expression in wild type OCI-M2 after transfection with TET specific or control siRNA. A) TET2 expression after transfection with TET1 and control siRNA (100 nM). B) TET3 expression after transfection with TET1 and control siRNA (100 nM). C) TET2 expression after transfection with TET2, TET3 and control siRNA (250 nM). D) TET3 expression after transfection with TET2, TET3 and control siRNA (250 nM). E) TET1 expression after transfection with TET2, TET3 and control siRNA (250 nM). The expression was measured 48 hours after the transfection, data were normalized to GAPDH expression, error bars indicate SE of two independent experiments. (*$p<0.05$)

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