T.C.
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HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

METHIONINE METABOLISM IN HUMAN FETAL, ADULT AND CANCER STEM CELLS

MSc. Özlem ALTUNDAĞ

Stem Cell Program

MASTER THESIS

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In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor Assoc. Prof. Dr. Betül ÇELEBİ SALTIK and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

Özlem ALTUNDAĞ

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ABSTRACT

Altundağ Ö., Methionine Metabolism in Human Fetal, Adult and Cancer Stem Cells, Hacettepe University Graduate School of Health Sciences Stem Cell Program, Master Thesis, Ankara, 2021. Methionine is an essential amino acid critical in protein synthesis and methylation reactions. In this thesis, it is aimed to determine the effect of methionine on different human stem cell groups. Mesenchymal stem cells (MSC) derived from human bone marrow (BM) and umbilical cord blood (UCB), breast cancer stem cells isolated from MDA-MB-231 cell line, were treated with different doses of L-methionine in culture. Cell surface marker and cell cycle assessment were performed by flow cytometry. WST-1 was applied for the cell viability determination. Changes in gene expressions *(OCT3/4, NANOG*, *DMNT1, DNMT3A* and *DNMT3B, MAT2A* and *MAT2B*) with methionine supplementation were examined by RT-qPCR, the changes in histone methylation (H3K4me3, H3K27me3) levels were demonstrated by western blot analysis and SAM/SAH levels by ELISA. As a result of treatment with 0, 10, 25, 50 and 100 µM methionine, cell viability was determined >80% and methionine application for five hours was determined as a fixed time in future studies. In all three cell groups, the cells were mostly arrested in the G0/G1 phase for each culture condition. It was evaluated that BM-MSCs increased all investigated gene expressions in the culture medium containing 100 µM methionine, in addition to SAM/SAH levels. On the other hand, UCB-MSCs were found to increase *OCT3/4, NANOG and DNMT1* gene expressions and decrease *MAT2A* and *MAT2B* expressions in culture medium containing 10 µM methionine. Moreover, an increase was observed in the He3K4me3 methylation profile. In addition, OCT3/4, NANOG, DNMT1 and MAT2B gene expressions in CSCs increased more at 50 and 100 µM starting from the addition of 25 µM methionine. An increase was determined in H3K4me3 protein expression at 50 and 100 µM methionine supplemented culture condition. This study demonstrates that methionine plays a critical role in metabolism and epigenetic regulation in different stem cell groups, and induces the maintenance of stem cells by regulating pluripotency-related gene expression and protein levels.

Keywords: : methionine, stem cell, pluripotency, methylation, metabolism

ÖZET

Altundag O., İnsan Fetal, Erişkin ve Kanser Kök Hücrelerinde Metiyonin Metabolizması , Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Kök Hücre Programı Yüksek Lisans Tezi, Ankara, 2021. Esansiyel bir amino asit olan metiyoninin, protein sentezi ve metilasyon reaksiyonlarında kritik rolü vardır. Bu tez çalışmasında, farklı tip insan kök hücre gruplarında metiyonin etkisinin belirlenmesi hedeflenmiştir. İnsan kemik iliği (Kİ) ve göbek kordon kanından (GKK) elde edilen mezenkimal kök hücreler (MKH) ile MDA-MB-231 hücre hattından izole edilen meme kanseri kök hücreleri, kültür ortamında farklı dozlarda L-metiyonin uygulamasına maruz bırakılmıştır. Yüzey belirteç ifadelerinin tayini ve hücre siklusu analizleri, akım sitometri ile yapılmıştır. WST-1 uygulaması ile hücre canlılığı tayin edilmiştir. Metiyonin takviyesi ile gen ifadelerindeki (*OCT3/4, NANOG, DMNT1, DNMT3A* ve *DNMT3B, MAT2A* ve *MAT2B)* değişiklikler RT-qPCR ile incelenmiş, histon metilasyon (H3K4me3, H3K27me3) seviyelerindeki değişiklikler western blot analizi ve SAM/ SAH seviyeleri ELISA ile gösterilmiştir. 0, 10, 25, 50 ve 100 µM metiyonin ile muamele sonucunda hücre canlılığı >80% olarak belirlenmiş olup, sonraki deneylerde sabit süre olarak beş saatlik metiyonin uygulaması belirlenmiştir. Her üç hücre grubunda ve her kültür koşulunda, hücreler çoğunlukla G0/G1 fazında asılı kalmıştır. Kİ-MKH' lerin 100 µM metiyonin içeren kültür ortamında araştırılan tüm gen ekspresyonlarını ve SAM/SAH seviyelerine ek olarak arttırdığı değerlendirilmiştir. Öte yandan, 10 µM metiyonin içeren kültür ortamında GKK-MKH' lerin *OCT3/4, NANOG ve DNMT1* gen ekspresyonlarını arttırdığı ve *MAT2A* ve *MAT2B* ekspresyonlarını azalttığı bulunmuştur. Ayrıca H3K4me3 metilasyon profilinde bir artış gözlemlenmiştir. KKH 'lerde *OCT3/4, NANOG, DNMT1* ve *MAT2B* gen ekspresyonları 25 µM metiyonin ilavesinden başlayarak 50 ve 100 µM'de daha fazla artmaktadır. 50 ve 100 uM metiyonin içeren kültür koşulunda H3K4me3 protein ifadesinde bir artış belirlenmiştir. Bu çalışma, metiyoninin farklı kök hücre gruplarında metabolizma ve epigenetik düzenlemede kritik bir rol oynadığını ve pluripotens ile ilgili gen ekspresyonunu ve protein seviyelerini düzenleyerek kök hücrelerin korunmasını indüklediğini göstermektedir.

Anahtar kelimeler: metiyonin, kök hücre, pluripotentlik, metilasyon, metabolizma

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ABBREVIATIONS

µl	mikroliter
µM	micromolar
µg	microgram
1-C metabolism	One Carbon Metabolism
5-mTHF	5-methyltetrahydrofolate
AECs	Amniotic Epithelial Cells
AF	Amniotic Fluid
Alpha-KG	Alpha Ketoglutarate
AMCs	Amniotic Mesenchymal Stem Cells
AMPK	AMP-activated protein kinase
BHMT	Betaine homocysteine methyltransferase
BM	Bone Marrow
CpG sites	Cytosine-phospho-Guanine sites
CSCs	Cancer Stem Cells
DHF	Dihydrofolate
DNMT1	DNA Methyltransferase 1
DNMT3A	DNA Methyltransferase 3A
DNMT3B	DNA Methyltransferase 3B
EAA	Essential Amino Acid
ESCs	Embryonic Stem Cells
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
Gly	Glycine
H3K4me3	Histone 3 Lysine 4 trimethylation
H3K27me3	Histone 3 Lysine 27 trimethylation
HAT	Histoneacetyltransferase
ICM	Inner Cell Mass
TCA	Tricarboxylic Acid
Hcy	Homocysteine
HMT	Histone Methyltransferase
HSCs	Hematopoietic Stem Cells

IGF-1	Insulin Growth Factor 1
ICM	Inner Cell Mass
IPSCs	Induced Pluripotent Stem Cells
IVF	In vitro Fertilization
JMDH	Jumonji-c Domain Histone Demethylase
miRNA	micro RNA
MAT	Methionine Adenosyltransferase
MAT2A	Methionine Adenosyltransferase 2A
MAT2B	Methionine Adenosyltransferase 2B
MEF cells	Mouse Embryonic Fibroblast Cells
MR	Methionine Restriction
MSCs	Mesenchymal Stem Cells
MTHFD	Methylenetetrahydrofolate dehydrogenases
MTHFR	Methylenetetrahydrofolate reductase
MTR	MethionineSynthase
NNMT	Nicotinamide N-methyltransferase
Nrf2	Nuclear factor erythroid 2-related factor 2
NSCs	Neural Stem Cells
NTD	Neural Tube Defect
OCT4	Octamer-binding transcription factor 4
OxPhos	Oxidative Phosphorylation
PBS	Phosphate Buffered Saline
PDSCs	Placenta Derived Stem Cells
PSCs	Pluripotent Stem Cells
SAH	S-Adenosyl-L-homocysteine
SAM	S-Adenosyl-L-methionine
SIRT1	Sirtuin 1
TEMED	Tetramethylethylendiamine
TET	Tet Methylcytosine Dioxygenase
TICs	Tumor-initiating Cells
TRAIL-R2	TRAIL receptor 2 (Deathreceptor 5,DR5)
UCB-MSCs	Umbilical Cord Blood Mesenchymal Stem Cells

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1. INTRODUCTION

Stem cells are distinguished from other cells by their capacity to form both differentiated cells and their identical twins. Both intrinsic and environmental regulatory factors promotes the potency and self-renewal characteristics of stem cells (1). Disturbances in this functioning during development from embryo to birth and beyond are associated with diseases. In this context, it is important to understand the metabolic regulation in different groups of stem cells. Due to the differences in their metabolic and epigenetic arrangements, the microenvironment they reside, stem cells at different stages display different characteristics (2). This leads to the formation of a defining metabolic and epigenetic signature in each stem cell group and requires shaping the microenvironment both *in vitro* and *in vivo* to adapt to changing metabolic activities.

As an essential amino acid in mammals, methionine is critical in protein synthesis and methylation reactions. Methionine metabolism is an intertwined network with the folate cycle and transsulfuration pathway, which is gathered under the name of single carbon (1-C) metabolism (3). Briefly, 1-C metabolism is responsible for the production of cofactors, metabolites, enzymes for the synthesis of nucleotides and maintaining amino acid levels (glycine, methionine, serine). In this way, the epigenetic identity and the redox defense system of the cell can be preserved (3). In particular, methionine metabolism is critical for stem cell proliferation, protection of pluripotency, embryo and fetus development by affecting gene expression profile and cellular functions. Any disruption in methionine and 1-C metabolism is associated with future diseases (4).

Activation and inhibition of gene expressions are involved in cell fate determination and are mediated by various epigenetic regulation. In fact, cell differentiation and specialization to form mature cells are regulated by the expression of specific gene sets and by epigenetic modifications (5). Hence, DNA methylation is especially important in early development, which is highly active during pregnancy and is essential for the maintenance of pluripotency (6). S-adenosyl methionine (SAM) synthesized from methionine by methionine adenosyltransferase (MAT) takes part in various reactions by providing the transmission of carbon units

throughout the methionine cycle (7). S-adenosyl homocysteine (SAH) is formed as a result of methyl group transfer from SAM to methyl transferases (8). Nutrition and *in vitro* culture medium composition provides epigenetic regulation in embryogenesis and during development as well as energy production and play a role in determining stem cell and cell identity (9).

Mesenchymal stem cells (MSCs) have the ability to differentiate into adipocyte, chondrocytes and osteocytes (10). MSCs isolated from different tissues have differences both epigenetically and metabolically. In this context, it is important to elucidate the differences in the metabolism of MSCs carrying lineage-specific markers and to reveal their relationship with stemness characteristics. It is known that cancer cells can arise from stem cells and more mature cells as a result of any disorder in regulation (11). The group of cells called cancer stem cells (CSC), which is a rare subpopulation in the tumor population, generally carries stem cell-specific markers, although they show different characteristics depending on tumor types (12). Similar metabolic and cellular properties of CSCs derived from triple negative breast tumor cells have been demonstrated with embriyonic stem cells (ESCs) (13). Recently, it has been reported that in the absence of methionine in ESC culture medium, intracellular SAM concentration, H3K4me3 protein levels and *NANOG* expression of cells were decreased. In addition, after prolonged methionine deprivation, cells expressed lineage-specific markers as well as increased p53 protein levels and apoptotic markers (14). Although studies have been conducted on the methionine dependence of CSCs, these studies have not been extensively studied on the basis of dose-dependent effects and stemness characteristics. In addition, although there are methionine related studies in MSCs, they are not intended to detail the metabolism. Therefore, this study focuses on methionine metabolism of CSC, UCB-MSC and BM-MSCs, which reflect different metabolic profiles and developmental stages of stem cells.

Within the scope of this master thesis, the expression of pluripotency-related genes, methionine metabolism-related protein levels as well as gene expressions and histone methylation levels of human UCB-MSC, BM-MSC and breast CSCs were evaluated after application of different doses of methionine in culture medium.

Hypothesis 1. Methionine-dependent culture medium conditions can affect the stemness properties of human BM-MSCs, UCB-MSCs and breast CSCs by changing their gene/protein expression profiles.

Objectives

- Culturing cells with 0, 10, 25, 50 and 100 µM methionine-dependent medium

- Investigation of expression of pluripotency-related genes (*OCT3/4, NANOG*)

-Identification of cells in G0/G1 phase by cell cycle analysis

-Determination of histone methylation levels (H3K27me3, H3K4me3).

Hypothesis 2. Methionine-dependent culture medium conditions of human BM-MSCs, UCB-MSCs and breast CSCs can change their levels of proteins and expression of genes involved in the methionine cycle.

Objectives

-Measurement of SAM and SAH levels

-Concentration-dependent assessment of *MAT2A, MAT2B, DNMT1, DNMT3A* and *DNMT3B* gene expression levels.

2. BACKGROUND

2.1 Stem Cells

The undifferentiated cells which have the ability to regenerate themselves and give rise to the formation of differentiated tissue cells are described as stem cells (15). Each stem cell makes a contribution to its own pool by asymmetric mitotic divisions and this way it maintains self renewal feature. In addition, thanks to their differentiation potential, they take part in tissue repair by replacing damaged and aged tissue cells (16). Both intracellular and extracellular factors, various signaling molecules contribute to the determination of cell fate decisions and maintain homeostasis by keeping the cell at lineage-specific differentiation potential (17).

2.1.1. Classification of Stem Cells

Stem cells are categorized according to their differentiation potential (unipotent, multipotent, pluripotent) or according to the source from which they are obtained (embryo, fetus, extra-embryonic tissues, adult tissues & organs) (18). The most potent cell that has the potential to generate all cells to form an organism, including extra-embryonic components, is the totipotent stem cell (19, 20). Specifically, the zygote as a single cell formed after fertilization can potentially be considered the most potent stem cell, capable of generating all tissue cells, including the extra-embryonic tissues of the organism (21). Cells capable of forming all cells of the three germ layers, except for extra-embryonic tissues, are pluripotent (Figure 2.1.). Multipotent stem cells, which often carry tissue-specific markers have more limited differentiation potential and are responsible for the regeneration of the tissue they are located (1, 22, 23). Adult stem cells can be multipotent to form lineage-specific cells in a tissue or lineage, or unipotent to form a single type of cell, with more limited differentiation potential (23).

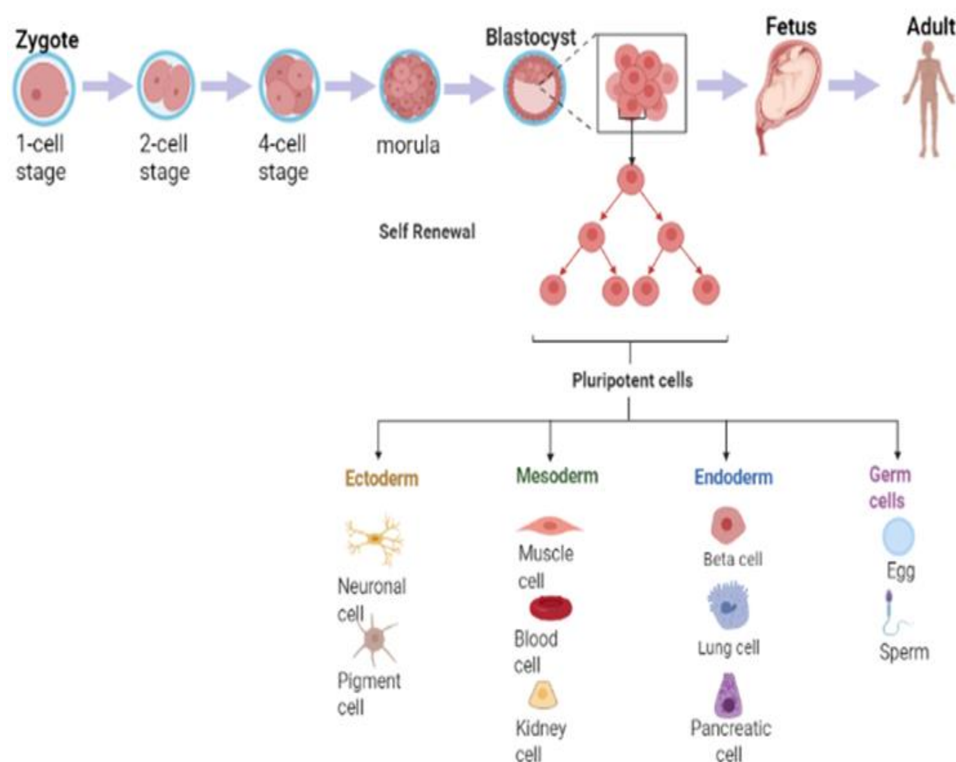


Figure 2.1. Human embryonic development from the zygote.

After fertilization, a diploid zygote is formed from two haploid cells. This cell will form the new organism as a result of many mitotic divisions. Each blastomere formed by the first cleavage of the zygote is totipotent. After the genome activation, the cells become polarized and form two separate cell groups, and the outer layer of the trophectoderm and the inner layer, which is defined as the inner cell mass (ICM), are separated from each other. ICM cells show pluripotent characteristics.

At the 4-5 days of embryonic development, the inner cell mass (ICM) cells isolated from blactocysts stage embryos are **embryonic stem cells** (**ESC**s) with pluripotent characteristics (24). There exist many differences for mouse ESCs and human ESCs in gene expression profiles, cellular morphologies, growth factors required to maintain their pluripotency *in vitro* (25).

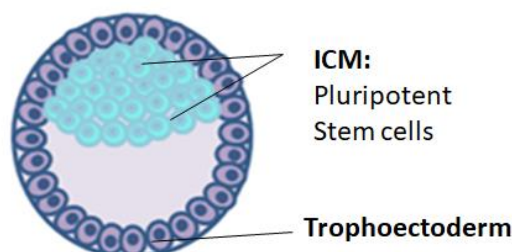


Figure 2.2. Early blastocyst stage

Stem cells isolated either from the appropriate tissue cells that make up the fetus itself or from the supporting, extra-embryonic tissue cells surrounding it are defined as fetal stem cells. Fetal stem cells originated from extra-embryonic tissues attract the attention of researchers due to their clinical availability (Figure 2.3.) (26-29). The circulating cells isolated of umbilical cord blood (UCB) were the first fetal stem cells isolated and cultured in hematopoietic culture conditions (30, 31). Pluripotent primordial germ cells, first isolated by John Gearhart and colleague and developed *in vitro* (1998), are fetal stem cells, known as precursors of sperm and egg cells, found in the gonadal ridge and mesentery of fetuses 5-9 weeks after therapeutic abortion (32, 33).

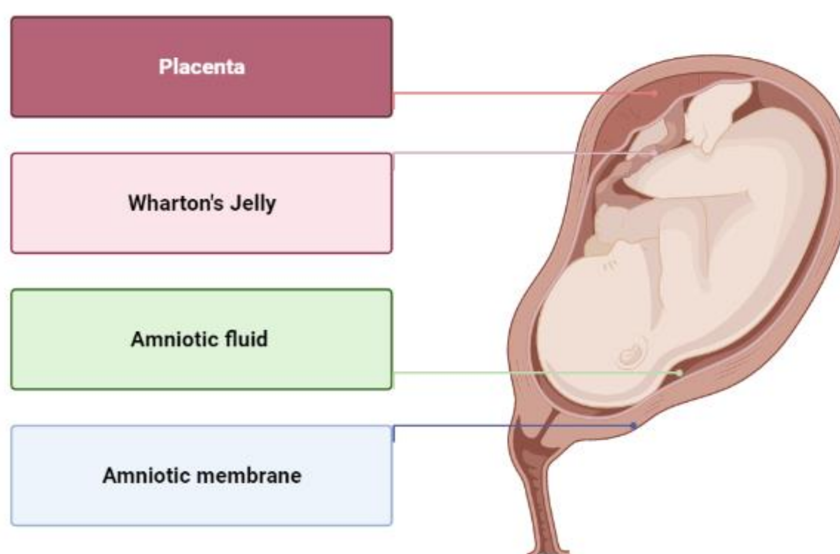


Figure 2.3. The supportive extra-embryonic sources for stem cells.
Placenta, Wharton's Jelly, Amniotic fluid, Amniotic membrane (from extra-embryonic structures) are fetal sources rich in stem cells.

Cells in fetal tissues consist of heterogeneous subpopulations and some of these cells can be characterized by the pluripotency markers such as NANOG, OCT3/4, SSEA-3, TRA-1-60, SSEA-4, TRA-1-81 (34). They have the expression of pluripotent-like cell surface markers but they display multipotent properties. Instead of ESCs which cause problems in terms of ethical consideration, they often preferred in studies (27). There are multiple (heterogeneous) stem cell populations resident in the amniotic fluid (AF) and these cells can be obtained before birth (26, 35).

Although heterogeneous subsets of AF stem cells differ in origin, morphology, and potency, they generally predominate in epithelial form and have a low proliferation rate *in vitro* (36). These stem cells can give rise to the formation of *OCT3/4*-expressing pluripotent cells and differentiate into three germ layer cells (27, 37, 38). Wharton's Jelly, which is the connective tissue structure in the umbilical cord, surrounds one vein and two arteries and is known to be rich in proteoglycan. Mesenchymal stem cells (UC-MSCs) located in this connective tissue can be obtained with an average yield of 400,000 cells in human (39, 40). UC-MSCs with positive expression of NANOG, SOX2, CD29 / CD54, c-Kit, OCT4 can differentiate into many mesodermal lineages cells such as adipocyte, osteocyte and skeletal muscle *in vitro* (41, 42). They have positive for mesenchymal (CD29, CD44), pluripotent (OCT4, REX1) stem cell surface markers and also the expression of various genes specific to ectoderm, endoderm, and mesoderm origin (43). The amniotic membrane is a structure formed by three separate layers. The basement membrane, which is the outermost of the amniotic membrane, contains stem cells with epithelial appearance and multipotent properties (44). These Amniotic MSCs (AMSCs) are positive for the stem cell-expressed markers SOX2, NANOG, REX1, OCT4, FGF4, SSEA-4, TRA-1–60, TRA-1–81, and SSEA-3 (27, 34). AMSCs, which also carry markers expressed by neural stem cells such as Musashi 1 and Nestin, are able to differentiate into cardiomyocyte originating from the mesoderm, neural cell types originating from the ectoderm, pancreatic and hepatocyte cells originating from the endoderm (34, 45).

Tissue (adult) stem cells support the regeneration of somatic cells and homeostasis of tissue by forming specific progenitor cells with their multipotent or unipotent characteristics. Stem cells, which are resident in every tissue/organ (fat, skin, brain, bone marrow,liver) and contain different subpopulations, differ in their embryonic origins by their surface markers, their differentiation capacity and the lineages what they will form (46). Among adult stem cells, MSCs show multipotent characteristics with spindle-shaped cell morphologies very similar to fibroblasts (47). MSCs are defined by their plastic adherence, high replication and proliferation capacities. They can be isolated from adult (adipose tissue bone-marrow, peripheral blood etc.) and fetal (umbilical cord and cord blood, placenta, amniotic fluid etc.)

tissues (48). MSCs are of clinical importance due to their ability to form all mesodermal cells (osteoblast, chondrocyte, adipocyte, fibroblast) *in vitro* and *in vivo* (49). While these cells do not have the expression of CD14, CD45, CD34 which are the markers of HSC lineage, they have the expression of CD73, CD90 and CD105, which are determined in the guidelines proposed by the International Society for Cellular Therapy.

Apart from all stem cell groups, there are subgroup cells that are rarely found among tumor cells. They are less abundant, enter the cell cycle less frequently, are in the G0/G1 resting phase, and are characterized by stem cell-like properties (50). Like stem cells, they show self-renewal and differentiation properties and cause tumorigenesis as a result of transplantation. CD44, CD133, CD166, EpCAM, CD24, and ALDH1 are commonly accepted cell surface proteins often used to identify CSCs of lung, colorectal, renal, pancreas, ovarian and breast cancers (51). Recent findings demonstrate that there exist several similarities between CSCs and ESCs in surface markers, gene and protein expressions, metabolisms, and requirements in their *in vitro* culture conditions (52). Among the features determined to be present in both CSCs and mouse/human ESCs, expression of SSEA-1, TRA-1-60, NANOG, SSEA-3, TRA-1-80, SOX2, SSEA-4, OCT4 as well as c-MYC, crypto and EPCAM surface marker positivity indicate that they both possess markers of pluripotency (53). While CSCs continue their symmetrical mitotic divisions to self-renew and maintain their reserves, they also perform asymmetric divisions to form progenitor cells as in stem cells (54). Considering the fact that oncogenic genes are associated with self-renewal and the genes that help suppression of self-renewal act as tumor suppressors, the mechanism behind the link between cancer and stemness arouses curiosity (54). Based on this, the self-renewal and proliferation ability of the cell and the findings of studies on suppressing these mechanisms are essential for providing therapeutic approaches focused on metabolism in cellular therapy and in order to understand oncogenesis (12). The tumor cell population, which has heterogeneity in different tumor types and even within the same tumor tissue, leads to secondary tumor development after chemotherapy and complicates the accurate targeting of treatment-resistant CSCs (55). Therefore, detection of their metabolic activities and biomarkers is essential to ensure accurate targeting. In particular, it has been shown

that breast cancer stem cells with $CD44^{+}$ and $CD24^{-/low}$ cell surface protein expression signature use the 1-C metabolism very actively, express the genes and proteins in this metabolic network at a high rate and are dependent on the amino acid methionine (56, 57).

2.2 Amino Acids

Molecules that play a role as building blocks in the synthesis of various molecules such as vitamins, minerals, fatty acids and amino acids must be taken from the diet in necessary amounts. In this way, many metabolic, neuronal and physiological diseases are prevented by providing homeostasis (58-60). Balanced dietary intake of amino acids, which are not only the building blocks for protein synthesis, but also play critical roles in metabolic pathways, is essential for a healthy immune system, growth and cell development (60, 61). There are 20 different types of amino acids, which are involved in the structure of proteins and mostly cannot be synthesized by mammals and these essential amino acids (EAAs) must be obtained from external sources through diet (62). Among these 20 amino acids; histidine (His), methionine (Met), valine (Val), threonine (Thr), leucine (Leu), isoleucine (Ile), lysine (Lys), tryptophan (Trp) and phenylalanine (Phe) are EAAs for human (63). The importance of amino acids in mammalian cells culture are also highlighted for the proper functioning of cells. The intracellular concentrations of amino acids and their transporters within cells are affected by the culture media ingredients (63). Considering the differences in metabolism and functioning of different stem cell types, it is important to carry out studies that examine the cellular and extracellular interactions caused by amino acids in the culture medium, and the interaction differences with the medium components.

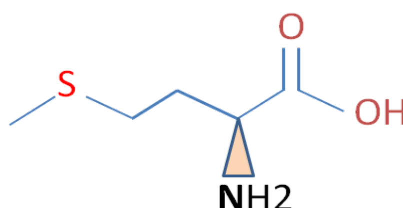
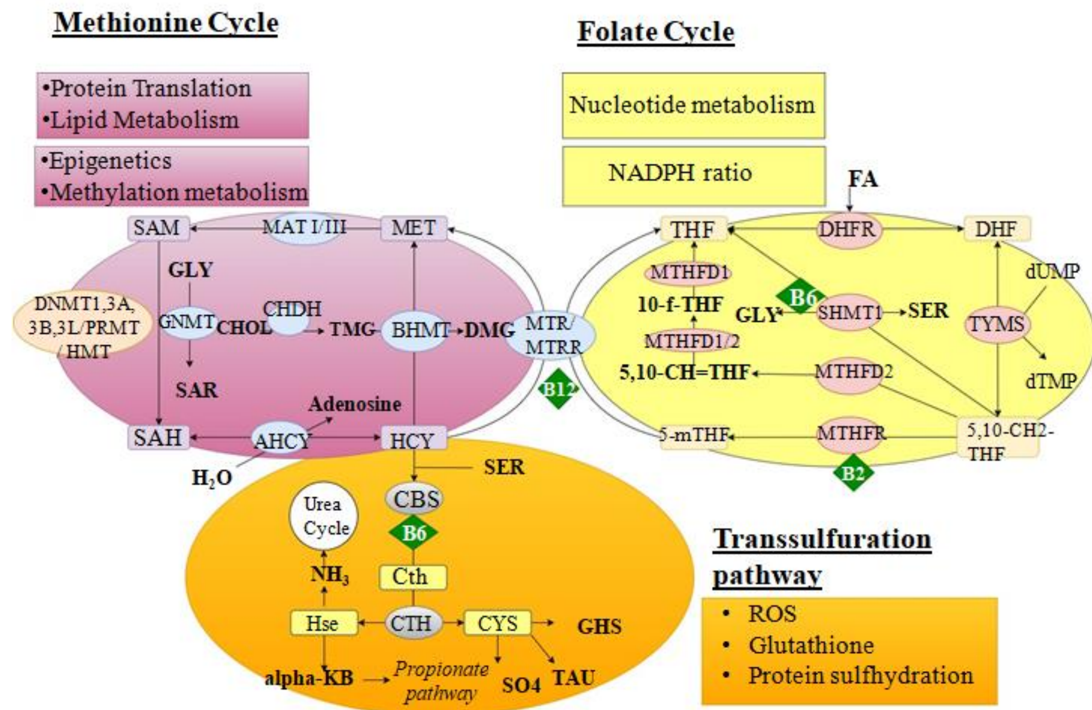


Figure 2.4. The molecular structure of methionine.

AUG codon acts as the initiator sequence of protein synthesis in eukaryotes and encodes the amino acid methionine. It is among the an aliphatic, sulfur-containing amino acids and acts as a precursor for SAM, succinyl-CoA, Homocysteine (Hcy), Cystein (Cys), creatin and carnitine biosynthesis reactions (Figure 2.4.) (64). In addition, the SAM molecule is an intermediary to provide the S atom required for the production of biotin and lipoic acid (65). In mammals, SAM is mostly involved as a methyl unit donor in methyltransferase reactions and formation of the methylated DNA, histone, protein, polysaccharides and several other molecules (66).

2.3 One Carbon Metabolism

Metabolism, to be defined in a broad summary, is the combination of all the biochemical processes performed by a living organism. Methionine metabolism, folic acid metabolism and transsulfuration pathways are three metabolic pathways that operate in conjunction with each other through metabolites, precursors and enzymes, and all three are collectively called 1-C metabolism (Figure 2.5.) (3). Cellular redox state of the cells, the epigenetic marks of chromatin structures, methylation reactions, polyamine synthesis, folate metabolism are all responsible for survival and also proper amino acid intake is essential for the management of these systems (67, 68). Thus, the investigation of metabolic requirements/regulations of different types of stem cells is vital for stem cell maintenance.



DNA and histone methylation are dependent on **SAM**

Embryonic stem cell must keep **SAM/SAH** ratio high

Among the DNMT enzymes, **DNMT1**, **DNMT3A** and **DNMT3B** has the methyl transferase activity.

DNMT1: Mitotic DNA methylation
DNMT3A & DNMT3B: *de novo* methylation

Figure 2.5. One carbon metabolism.

One carbon metabolism as an interconnected metabolic network: Transsulfuration pathway, methionine and folate cycles. This metabolic network is involved in SAM production in stem cells, SAM/SAH (methylation index) protection, regulation of DNA and histone methyltransferases activity, etc. It plays a critical role in cell fate by being responsible for many processes.

2.3.1 Methionine Metabolism

After methionine is taken with the diet, free methionine is obtained as a result of its degradation in the small intestine and is either used for protein synthesis or conversion to SAM (69). In the first stage of methionine metabolism, SAM synthesis takes place from methionine and ATP mediated by MAT enzymes, which are evolutionary preserved from *E.coli* to humans (70). MAT enzymes are of three main multimeric types: MAT1, MAT2 and MAT3 (71). SAM is the cofactor that takes the most active role in enzymatic reactions after ATP molecule in the body acts as an aminopropyl group donor for polyamine synthesis and supplier of a carbon unit (methyl) for the formation of methylated DNA and histone structure (72, 73). Based on this, SAM can be considered as a gene expression regulator by methyl donating to methyltransferases, turning target gene regions on and off by histone/DNA methylation (74). After methylation of the substrate, SAH molecule is formed from SAM and this molecule is further is irreversibly hydrolized to Adenosine and Hcy by SAH Hydrolase (SAHH/AHCY). The SAM/SAH ratio is also known as the 'methylation index' of the cell, and a decrease in this ratio leads undermethylation of DNA since DNMTs has higher binding affinity for SAH than SAM (8). At the same time, high SAH levels also increase the HCy level of the cell, leading to the hyperhomocysteinemia associated with most diseases (75). By the remethylation reactions, resultant Hcy regenerates and the methionine cycle is completed. When the methionine levels are low, methionine synthetase (MTR/MS) enzyme catalyzes the remethylation of Hcy for methionine regeneration. This reaction takes place by the vitamin B-12 dependent 5-methyltetrahydrofolate as a one-carbon donor. This step is the intersection point of the folate and methionine cycles. The second way of Hcy remethylation is mediated by Betaine Hcy methyl transferase (76, 77). BHMT functions specifically in the liver and kidney, although conversion via the MTR/MS enzyme also occurs in nonhepatic cells (78). Apart from its remethylation, Hcy can also be used to produce Glutathione (GSH) via the transsulfuration pathway (Figure 2.5.).

2.3.2 Transsulfuration Pathway

When both intracellular folate and methionine levels are sufficient, high SAM production triggers Hcy degradation and directs it to the transsulfuration pathway (79). SAM inhibits the activation of cystathionine ß-synthase, which is the transsulfuration initiating step, by allosteric inhibition of methyl-THF reductase (MTHFR). Cystathionine is produced by this enzyme as a result of the serine and Hcy condensation reaction (8). In the next step, cystathionine is γ-lyase-catalyzed to form cysteine, a precursor of GSH which is the major regulator of cellular redox metabolism (80).

2.3.3 Folate Cycle

Folate carriers in reduced form, proton-coupled carrier molecules and receptors are involved in the transport of dietary folate to the target cells (81). Once taken into the cell, folate monoglutamates provide the formation of 5-mTHF molecule as a result of polyglutamylation reaction, and 5-mTHF is a critical coenzyme in 1-C metabolism. Folic acid is not coenzyme as the fully oxidized form of folate, so it can be reduced to participate in the folic acid cycle as either DHF or THF (82). THF, the reduced form, first contributes to the formation of 5.10-methylenetetrahydrofolate (5,10-CH2-THF) and then the irreversible formation of 5-mTHF. These reactions take place via B6 and B12 dependent enzymes, as an alternative route for 5-mTHF conversion, a series of reactions resulted in 10-formly tetrahdrofolate (10-f-THF) by methylenetetrahydrofolate dehydrogenases (MTHFD) (83). Finally, 5-mTHF intersects with the methionine cycle by donating a methyl group for Hcy remethylation via B-12 dependent MTR/MS, and the folate cycle is completed (76).

2.4 Metabolic Regulation of Stem Cells

Intracellular/extracellular, physiological and biochemical factors, signaling molecules, growth factors are stimulants for cell to reply, adapt and survive in their microenvironment. As a result of the modification of these dynamic parameters, the cell also adapts to the situation by changing its metabolism and response, by reviewing its energy need, the molecules it uses as fuel, biomass production, gene,

protein, post-translational and epigenetic programs (84, 85). Discovered by Schofield in 1978 in HSCs, stem cells located in specialized regions called "niche" can fulfill their metabolic functions. HSCs show different metabolic activities in osteoblastic and endosteal regions from two different microenvironments in the BM, and this is regulated by the gradiently decreasing oxygen concentration. Similarly, during embryonic development, ICM cells are referred to as naïve and primed stages in pre- and post-implantation stages, respectively, and the epigenetic signatures of cells, the genes they express, their differentiation potential and metabolic activity vary in these two stages (86). In addition, glycolysis and oxidative phosphorylation (Ox-Phos) transitions in energy metabolism also affect stem cell maintenance, self-renewal, differentiation capacity, metabolite production and epigenetic regulation changes accordingly (Figure 2.6.) (87).

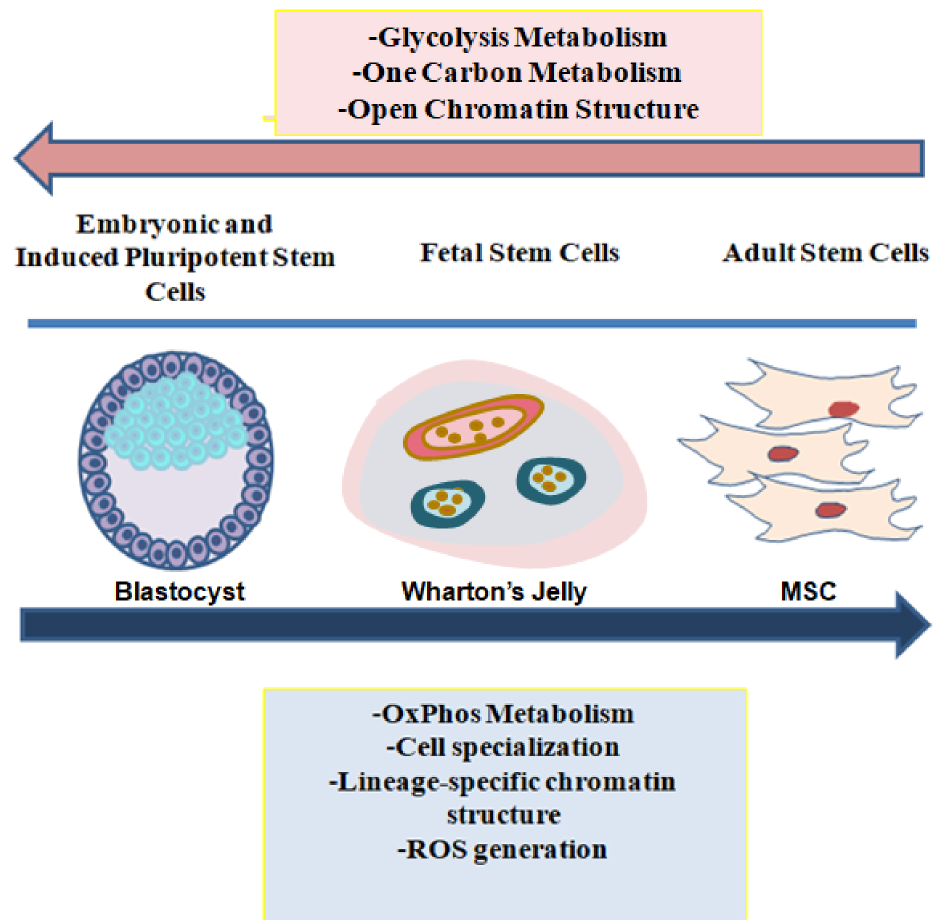


Figure 2. 6. Metabolic adaptations through developmental stages.

2.5 Methionine Metabolism in Stem Cells

The effects of the microenvironment and the reorganization of the genomic structure are observed in embryogenesis, organogenesis, fetal development periods and in the formation of differentiated tissue-specific cells (88-90). In all these stages, 1-C metabolism plays an essential part in SAM methyl donation, protein translation via DNMT and histone methyl transferases, lipid metabolism, polyamine synthesis, maintaining biosynthesis reactions by preserving NADPH ratio, and providing cellular redox balance that plays a decisive role in stem cells (4) (Figure 2.7.).

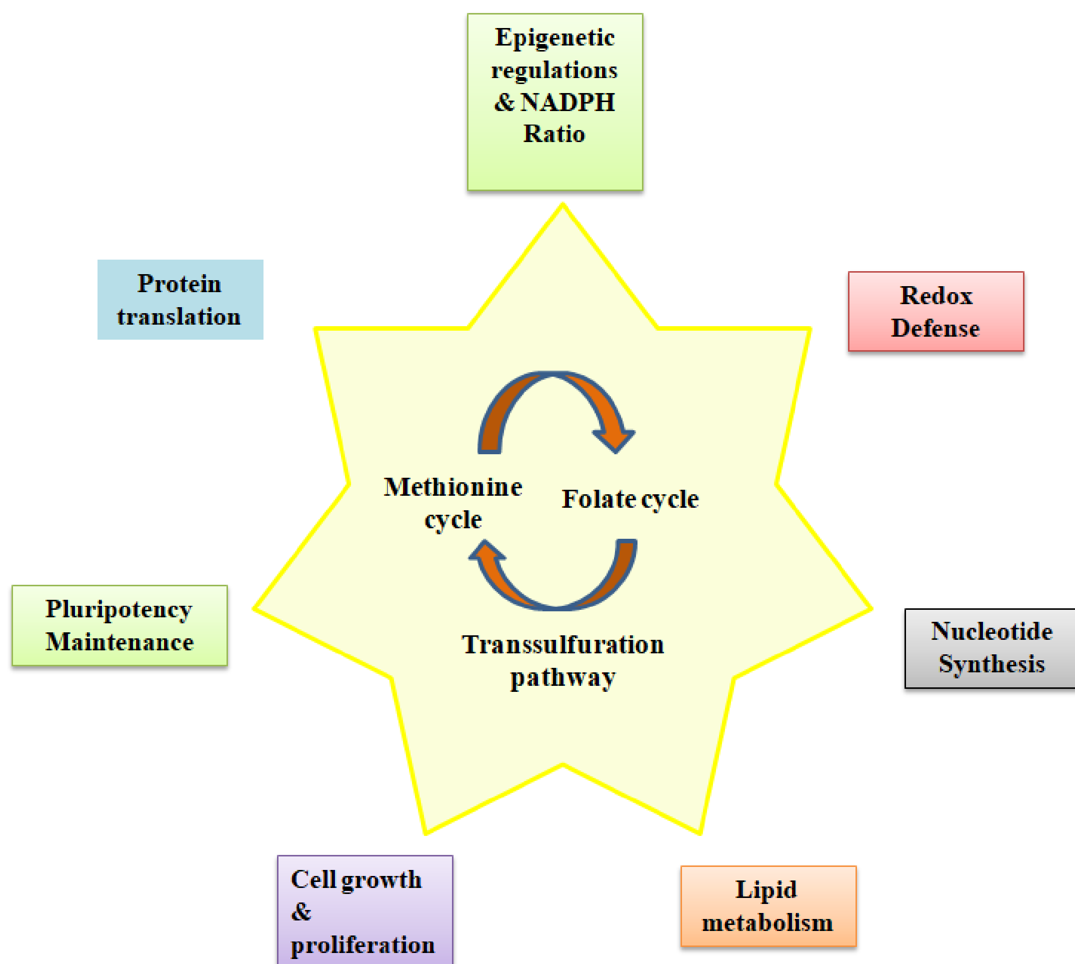


Figure 2. 7. Methionine metabolism related processes in stem cells.

2.5.1 Methionine Metabolism in Pluripotent Stem Cells

Studies in both mouse ESCs (mESCs) and human ESCs (hESCs) have shown that both groups of cells need amino acids for healthy development, proliferation, maintenance of pluripotency and their undifferentiated states. Although pathway analysis studies demonstrated the metabolic profiling of both cells similarly, mESCs are mostly dependent on threonine AA to protect their undifferentiated pluripotent characteristics in culture (91). Unlike hESCs, mESCs provide glycine amino acid and the Acetyl-CoA pool required for acetylation reactions, as a result of Threonine dehydrogenase (Tdh) activity that can catabolize threonine (Thr) (91).

It has been demonstrated quite recently by Shiraki et al. that supplementation of the amino acid methionine externally to the culture medium in hESCs is vital for these cells (14). There are differences between ESCs and somatic cells in terms of amino acid metabolism and maintenance of pluripotency, that glycine-methionine metabolism is more actively regulated and Glycine decarboxylase enzyme (Gldc) is highly expressed in PSC. In this way, folate intermediates are produced and participates in the cycle (92, 93). PSCs maintain their rapid proliferation ability and depend on folate metabolism for the necessary nucleotide synthesis and for the production of molecules required for biosynthesis reactions, and Gly is very important with its critical role in this cycle (90). In early developmental stages, regulation Acetyl-CoA and alpha-ketoglutarate (α-KG) as epigenetic regulators, maintenance of their pools, regulation of euchromatin and heterochromatin structures, preservation of methlyation/demethylation/acetylation levels in DNA/histone regions and the activation of stemness-determining gene regions should be critically managed in PSCs (92). To maintain SAM levels and epigenetic status, mESCs have been shown to be sensitive to the amino acid Thr *in vitro* and *in vivo* (91). Through the Thr-Gly-Met amino acid triad and the pathways in which they participate, processes such as preservation of pluripotent stem cell (PSC) self-renewal feature, maintenance of folate metabolism, protection of SAM and Acetyl-CoA pools, nucleotide synthesis, epigenomic and metabolic regulation of pluripotency demonstrates the importance of external amino acid levels in PSCs (14).

Cytosine methylation, which occurs at CpG sites on DNA and often leads to suppression of gene expression, is a central regulatory mechanism in tissue-specific expression. Contrary to the suppressive role of CpG methylation, studies show that DNA methylation that occurs in non-CpG regions is found in highly transcribed gene regions in ESCs (94). With the lineage commitment, a decrease in non-CpG methylation in DNA is observed (94). Until they begin to differentiate during development, there are bivalent marks (H3K27me3 and H3K4me3) in pluripotency-related gene and chromatin regions of PSCs/ ESCs (95). The presence of these markers in specific development-related gene regions and the fact that they carry the H3K4me3 and H3K27me3 markers together indicate that the cells are open to interaction with the external environment and the decision mechanism is more active. After differentiation, these markers are transferred lineage-specifically, with H3K27me3 or H3K4me3 remaining more dominant (96). As a result of the immunoblotting analysis performed by Steele et al. with the induced pluripotent stem cell (IPSC) cell line BGN01, it was determined that the enzymes in 1-C metabolism were highly expressed (97). Fernandez-Arroyo et al. found that SAM/SAH ratio, 5-mTHF and Hcy levels were significantly different in IPSC cells (approximately 10-fold increase, approximately 10-fold increase, 3.7-fold decrease, respectively) compared to mouse embryonic fibroblasts (98). PSCs (IPSCs and ESCs) have to keep the SAM/SAH ratio high, SAM synthesis under control, and pluripotency/development-related gene regions active through H3K4me3 modification (14). In their study on hESC cell line khES3 and hIPSC 201B7 cells, Shiraki et al. found that cells tested for amino acid requirements by culturing them in CSTI medium without amino acids, were sensitive to methionine. They also highlighted the optimum concentration value of methionine to be 25 μM in terms of proper morphology, gene expression levels and maintenance of pluripotency of these cells. Since folate receptors are highly expressed in human oocytes, this indicates that folate uptake into the cell and regular functioning of 1C- metabolism and methyl transfers are essential in the transition from maternal oocyte structure to zygote (99). Although studies have shown that PSCs are methionine-sensitive and dependent, there are no studies on the relationship between folate deficiency/addition or dose/time-dependent study that may affect pluripotency, cell plasticity, stemness, and

healthy physiological functioning. In a study with rhesus monkeys, it was determined that the embryoid body and rosette formation (developmental signature of neuro-progenitors in cultures of differentiating ESCs) of cells were decreased with folic acid deprivation, along with a decrease in the expression of *NESTIN, SOX-1*, *PAX-6* neuronal markers (100).

2.5.2 Methionine Metabolism in Fetal Stem Cells

Besides genetic factors, extracellular and environmental factors play a regulatory role on gene expressions of cells with the effect of organization of the genetic structure during embryogenesis, organogenesis and fetal development stages (101). In addition, studies have supported that dietary foods and their amino acids contents as well as the pathways in which these amino acids take place, play a critical role in ESCs and during embryonic development. In this context, when studies reflecting the fetal period were examined, *in vivo* measurements were mostly made on folate metabolism.

During pregnancy, daily 0.4–0.5 mg folic acid intake is recommended for women until the 10th week in terms of healthy development of the fetus, in line with the criteria determined worldwide (102). If maternal folic acid levels decrease during pregnancy, there is a strong correlation between pregnancy complaints, intrauterine growth restriction, diseases caused by developmental disorders, learning disorders, and mental problems (103-106). Folate level is also important in terms of transformation of methyl groups in one-carbon metabolism, epigenetic arrangements, and preservation of DNA and methylation pattern, and the changes that will occur result in disease and disorder in the child (107, 108). Similarly, the increase in the level of Hcy in methionine metabolism has a toxic effect on the cells, and in case of an increase during pregnancy (hyperhomocysteinemia), it leads to various psychiatric and metabolic disorders (109).

Disturbances in 1-C metabolism, in particular in the folate cycle, are associated with neural tube defects which then lead to spontaneous infant aportions and premature abruption of the placenta (109-112). In addition, the most common of the polymorphisms for 1-C metabolism is C677T in the MTHFR and occurs in folic

acid deficiency(113). Van mil, N. H et al. who are investigating metabolites that may be precursors of disorders of 1-C metabolism in early pregnancy, showed that maternal folic acid deficiency in the newborn epigenome was associated with DNA hypomethylation (114). It has been shown by Steegers-Theunissen that after folic acid supplementation, insulin-like growth factor 2 expression and epigenetic regulatory mechanisms have been altered in umbilical cord-derived white blood cells and demethylated regions in DNA become highly methylated (115). In a more recent study, changes in cell metabolism and transcriptional activity were observed during the first 5 months of life in human UC-MSCs from infants born to normal-weight and obese mothers. Amino acid concentrations and gene expression pattern specific to these cells that obtained from obese mothers were found to be lower than those of normal-weight mothers (116). According to these studies, folic acid is necessary during pregnancy and associated with fetal development disorders. However, the methionine cycle intersecting with folate metabolism and its effect on fetal stem cells should be clarified with other studies. *In vitro* proliferation capacity was found to be higher in neural stem cells (NSCs) that received methyl donors maternally with diet than those fed a lower diet (117). Another study, as a result of examining labeled metabolites in newborns through the 1-13C methionine methionine cycle, indicated an increase in csyteine synthesis was observed for the increased need for glutathione synthesis in the transsulfuration pathway (118).

2.5.3 Methionine Metabolism in Adult Stem Cells

Mostly, tissue adult stem cells can continue their metabolic activities by being in a quiescent state for a long time in a suitable niche environment. For the generation of a specific genotype and phenotype for the cell, and for the lineage commitment, cells must adapt their metabolism by making decisions based on their interaction with their environment. As a result, they carry some metabolic biomarkers. Mass spectrometry and metabolic analyzes were performed by Sinclair et al. to examine methionine cycle, methyl donors, protein and nucleotide methylations in murine T cells (67). Expression of methionine transporters, which determined as rate-limiting in protein synthesis, is found to be responsible for the regular functioning of the methionine cycle. In addition, T cells provided their

epigenetic regulation by increasing methionine transport during their maintenance, proliferation and differentiation (67). It has been suggested that increasing reactive oxygen species levels could be inhibited by the effect of SAM in rat BM-MSCs under 1 mM hydrogen peroxide exposure. Based on this study, it has been deduced that the SAM molecule has an anti-apoptotic effect (119). MTHFR polymorphisms, which are known to be common mutations in 1-C metabolism, are shown as risk factors for diseases such as psychiatric disorders, schizophrenia, bipolar disorder, and depression (120). In addition, there is a decrease in Hcy remethylation, which completes the methionine cycle, in Alzheimer's patients and neurodegenerative diseases (121). When NSCs were isolated from 8-weeks old C57BL/6 mice and examined after 4 weeks of diet of various combinations devoid of methionine/ choline, normal crow, methionine/choline supplementation, it has been found that methionine supplementation regulates the hippocampus function and balances NSCs ability of differentiation, maintenance and proliferation. When Hcy was added into the culture medium, a decrease in the proliferation rate of NSCs and DNA hypomethylation were also noted (122).

2.5.4 Methionine Metabolism in Cancer Stem Cells

Cancer cells with high proliferation capacity are strongly dependent on 1-C metabolism to maintain their proliferation abilities such as nucleotide synthesis, methylation, redox system, etc. In this context, the use of anti-folates and the use of drugs targeting single carbon metabolism through the folate cycle are studied in cancer treatment (123). Sydney Farber targeted folate metabolism for the first time and revealed the decrease in the number of leukemic cells in the event of a dietary deficiency of folate (123). As a result of this invention, drugs have been produced to target anti-folates in cancer, and the most widely known of these is methotrexate (124). The serine-glycine pathway, the mechanism that enables methyl groups to participate in the metabolic cycle as a result of SHMT1/2 activity by using 5-mTHF, is used extensively in cancer (72, 125, 126). Nishimura et al. identified 139 EGF-related gene expressions and found them to be associated with lung adenocarcinoma. In addition, they showed that MTFHD2 can also be targeted in drug resistance studies for tumor-initiating CSCs (127). Presence of Hcy and deprivation of

methionine in culture medium suppressed the growth of CSCs (128). Methionine dependence of CSCs is a striking feature among the heterogeneous tumor population.Various CSCs from different tumor tissues are sensitive and dependent on the amino acid methionine, similar to PSCs, and must maintain high levels of SAM within the cell (129). Recently, Wang et al. demonstrated that the MAT2A enzyme and the methionine cycle were shown to be highly active in lung CSCs (129). As a result of therapeutic targeting of MAT2A, SAM formation from methionine can be prevented and DNA, protein, lipid, nucleotide synthesis, development and epigenetic regulation of the cell can be targeted (129). As a result, the tumor initiating capacity of CSCs was reduced and their stemness was lost (55). Similarly, in a study on triple negative (ER- / PR- / HER2-) breast cancer cells with variable methionine doses in culture medium, the mammosphere-forming capacity, histone methylation markers, primarily H3K4me3, CD44+/CD24-/low CSCs were damaged (56). In a different study conducted by the same researchers, it was noted that after lexatumab treatment of breast cancer cells where grown in culture medium without methionine, increased expression of TRAIL-R2 in cells was reported. In a study examining the in vitro effects of 10 different essential amino acids on Hs578t and MDA-MB-231 cell lines, it was stated that methionine was the primary amino acid that affects migration and metastasis (57). In Table 2.1., the comparison of CSCs and ESCs/IPSCs in terms of their methionine metabolism is demonstrated.

Table 2.1. Metabolic and epigenetic effects of methionine in Cancer Stem Cells and Pluripotent Stem Cells (ESCs and IPSCs) (14, 56)

ESCs / IPSCs	CSCs
They are marked by increased expression of genes and proteins involved in the methionine cycle.	They are marked by increased expression of genes and proteins involved in the methionine cycle.
Optimum methionine concentration was determined to be 25 µm for maintenance and healthy growth of the cells and prevention of cell cycle arrest.	These cells are dependent on the amino acid methionine for growth.
Homocysteine levels should be kept low due to its pro-inflammatory and oxidative effects.	Result of **increase in** SAM/SAH: Hypermethylation of tumor suppressor genes Result of **decrease in** SAM/SAH: -Oncogene expression increase
It was found that the SAM/SAH ratio was kept high.	The MAT2A enzyme, which catalyzes the formation of SAM from methionine, is of therapeutic importance.
Methionine deprivation results in: - Decrease in intracellular SAM concentration, -Decrease in H3K4me3 profile, -Decrease in Nanog expression, -Marker expression towards ectoderm, endoderm, mesoderm lineages -It increased the expression of p53 proteins	**Methionine deprivation** results in**:** -Reduction in mammosphere formation, -Reduction of stem cell feature, -Ensuring cancer stem cell elimination without reducing viability in MCF7 cells, -MDA-MB-231 cells lead to apoptosis by increasing the gene expression level of TRAIL-R2.

3. MATERIAL-METHOD

3.1 Experimental Design

At the beginning, MACS method was used for breast CSC isolation based on their CD44+ and CD24-/low expression profile. Cells were characterized both morphologically and by flow cytometry. CSC cells were cultured according to the mammosphere culture conditions for five days based on the literature (130). BM-MSC and UCB-MSC cells were characterized morphologically and by flow cytometry according to CD73, CD90 and CD105 surface markers. After the methionine dose range to be applied to the cells literature (0, 10, 25, 50 and 100 µM), cell viability was determined by WST-1 analysis for 0, 2, 5, 12, 24 and 48 hours (14). All following experiments were applied to cells cultured in methionine-supplemented and control medium for 5h after WST-1 analysis (Figure 3.1.). The effect of methionine on the cell cycle of cells cultured under methionine-dependent culture conditions for 5h was determined using flow cytometry. In this way, the frequency of the cells in the GO/G1, S and G2/M phases was determined. After RNA isolation and cDNA synthesis of cells cultured under the same conditions, gene expressions were determined by RT-qPCR method. Primers *OCT3/4, NANOG, DNMT1, DNMT3A, DNMT3B, MAT2A and MAT2B* were used for gene expression analysis. Protein isolation of cells cultured with different doses of methionine for 5h was performed in accordance with the Abcam histone extraction protocol. Pierce BCA Protein Assay Kit was used for protein measurement. For Western Blot Analysis, H3K4me3, H3K27me3 and H3 total antibodies were used to determine histone protein levels of human BM-MSCs/UCB-MSCs and breast CSCs by the gel imaging FluorChem FC3 System (Protein Simple, USA). SAM/SAH determination was performed using SAM and SAH ELISA Combo Kit according to the according to manufacturer recommendations. The measurement of absorbance values was measured with a spectrophotometer at the 450 nm primary wave length.

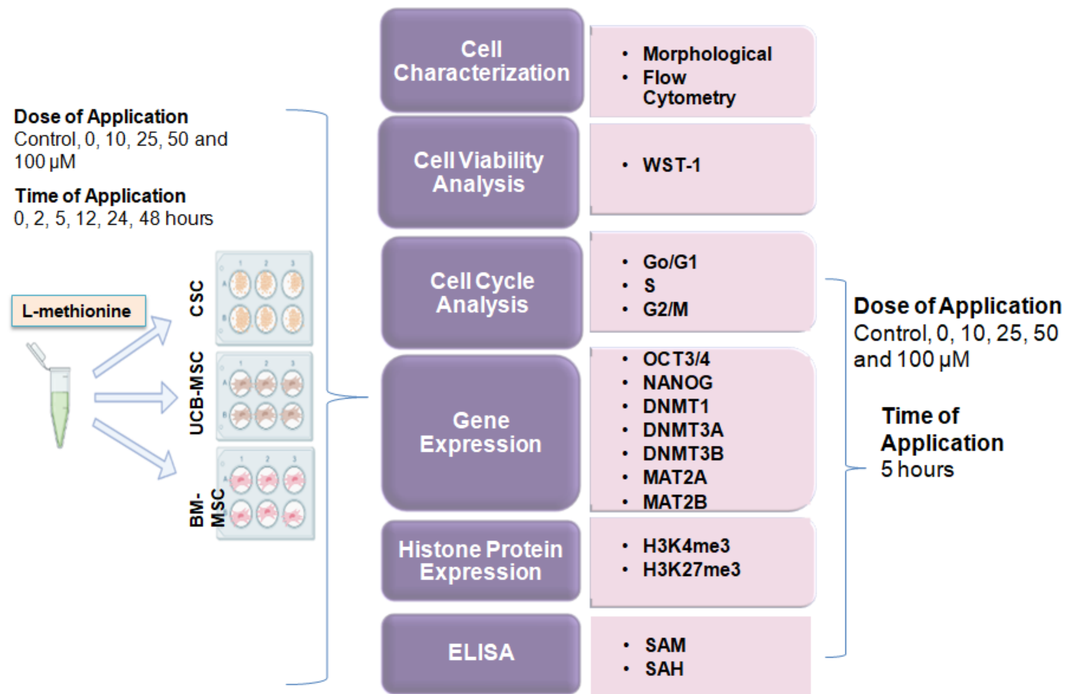


Figure 3.1. The experimental design of the study.

3.2 Cell Culture and Characterization of Fetal and Adult Mesenchymal Stem Cells (BM-MSCs and UCB-MSCs)

Within the scope of this thesis, human umbilical cord blood mesenchymal stem cells (UCB-MSCs) were used with the permission of Ethics Committee of Hacettepe University Health Sciences Institute (GO19/794). Human UCB-MSCs which isolated by our research group were used in this thesis research (n=3). Human BM-MSCs were obtained from Lonza (n=3). After being removed from the nitrogen tank, the cell solution in the cryo vial was quickly processed by keeping it in a water bath set at 37 ^{0}C. Then, cells were transferred into cell culture medium with a total volume of 10 mL and centrifuged at 1500 rpm for 5 minutes in 15 mL centrifuge tubes. The supernatant was discarded after centrifugation. By adding 1 mL of cell culture medium onto the pellet, the pellet was dissolved and trypan blue (Invitrogen) was used to count viable cells by ideal dilution. UCB-MSCs and BM-MSCs were cultured by adding L-glutamine (Sigma), Penicillin-streptomycin (Sigma) and 20% FBS (Invitrogen) into minimum essential medium with alpha (α-MEM, Biological Industries). The cells cultured in this standart culture medium were considered as control group. Cells were kept in an incubator set at 37 ^{0}C and 5% CO_2. Flow cytometry (Becton Dickinson Biosciences (BD)) analysis was performed for the characterization of cell surface proteins of passage 3 MSCs. Anti-human CD90-phycoerythrin (PE, BD), anti-human CD105- allophycocyanin (APC, BD), anti-human CD73- fluorescein isothiocyanate (FITC, BD) were used for phenotypic characterization of MSCs. After seven days of culture, cells were examined using phase contrast microscopy (Olympus CKX-41).

3.3 Cell Culture and Characterization of Cancer Stem Cells ($CD44^{+}$ / $CD24^{-/low}$) from MDA-MB-231 Cells

MDA-MD-231 breast cancer cell lines that were obtained from ATCC (n=3), were cultured in DMEM-F12 medium (Invitrogen) containing 10% FBS, L-glutamine, Penicillin-streptomycin and in an incubator at 37 ^{0}C and 5% CO_2. The media of the cells were removed twice a week and freshly from the culture flask where the medium was removed and washed with phosphate salined buffer (PBS, Sigma). Cells were removed by applying TrypLe (Gibco) solution. Then, cell

detachment from the flask surface was checked under the light microscope. For each flask, DMEM-F12 culture medium was added as the same amount of TrypLe and cells were transferred to a 50 mL falcon tube. After centrifugation at 1500 rpm for 5 minutes, the supernatant was removed from the falcon tube. MACs method was used for CSC isolation. Magnetic-activated cell sorting (MACS) buffer solution containing the ordered components was prepared and kept cold (2−8 ^{0}C): PBS (pH 7.2,), 2 mM EDTA (Sigma) and 0.5% bovine serum albumin (BSA, AppliChem). Firstly, CD24- negative cell selection protocol was applied, then CD44+ labeled cells were collected. After centrifugation, cell pellet was resuspended in MACS Buffer and cell number was determined. The centrifugation was repeated, and the supernatant was completely removed. Then, MACS buffer was added to the cell pellet, calculated as 40 µL for each 10^7 cells. Then, 10 µL of Biotin-labeled CD24 kit component (Miltenyi Biotech) was added for each 10^7 cells, and after mixing by finger tapping on the tube, it was left in the refrigerator for 15 minutes in the dark. When the incubation was over, 1 mL of MACS buffer was added to each 10^7 cells and the centrifuge step was repeated. After the liquid part on the pellet was completely discarded, this time 80 µL of MACS buffer was added for each 10^7 cells. Anti-Biotin labeled MicroBeads in the form of 20 µL for each 10^7 cells were also added to the solution and kept in the refrigerator as indicated in the previous step. In this step, LS column (Miltenyi Biotech) was prepared with 3 mL buffer. Then, 500 µL of buffer added onto cell suspension and filtered through column. The flow-through cells were $CD24^{-/\ low}$ cells and they were used for $CD44^{+}$ selection as the same. Surface protein expression characterization of CSCs was performed by flow cytometry and Anti-human CD44- FITC (BD), Anti-human CD24- PE (BD) were used for phenotypic characterization of human breast CSCs. In order for CSCs to maintain their *in vitro* stemness properties, they should be cultured with Sphere Formation Assay. Thus, the isolated human breast CSCs were cultured in Ultra-Low Attachment Multiple Well Plate (Sigma) in RPMI 1640 medium containing 10 ng/ml bFGF, 20 ng/ml EGF, 1xB27 (Sigma). The cells cultured in this standart culture condition were considered as control group. The mammosphere assay images were taken under light microscope on the first five days of the cell culture. After five days of CSC culture, cells were used for further experiments.

3.4 Cell Viability and Cell Cycle Analysis

For methionine deprivated culture, RPMI 1640 medium with no methionine (Gibco) and for dose determination; 0 µM, 10 µM, 25 µM, 50 µM and 100 µM methionine-dependent medium was applied for the following experiments. The cells cultured in standart culture condition were considered as control groups. Cell viability test (WST-1) for human UCB and BM-MSCs and CSCs seeded with 2200 cells per well of 96-well cell culture plates was applied to cells kept in culture for 48 hours. Three replicates were measured for each indicated dose (control, 0 µM, 10 µM, 25 µM, 50 µM and 100 µM). Following 0, 2, 5, 12, 24 and 48h of incubations, water-soluble tetrazolium based analysis (10% WST-1) was applied for the measurement of metabolic activities of cells. 100 µL of cell culture medium transferred to ELISA microplates (96-wells plates, Corning Life Sciences, USA) spectrophotometric measurements of microplate reader (Tecan) at 450 nm wavelength were recorded with 600 nm reference wavelength. The data was calculated and presented as indicated in the following equation.

$$\frac{(O.D\ value)sample - (O.D\ value)blank}{(O.D\ value)sample} \text{x}100$$

Cell cycle analysis was performed based on the determination of the number of cells in G0, G1 and S phase by applying flow cytometry method. Cells washed after trypsinization were centrifuged and cell counts were made in a hemocytometer by applying trypan blue. $1.5\text{x}10^6$ cells/mL were used in this experiment after fixation with 96% alcohol (Merck). The tube was vortexed to mix well 70 µl of RNAse (Sigma, St. Louis, MO, USA) and 100 µl of propidium iodide (Sigma) added to the cells. Cells kept for 20 minutes at room temperature and away from light (covered with foil) were then analyzed using a flow cytometry device (Cytoflex; Beckman Coulter Inc., Brea, CA, USA). The proportional values indicating the phase of the cell cycle of the cells and the ratio of apoptotic cells were analyzed by using McCycle software (Phoenix Flow System, San Diego, CA, USA) and obtaining dichotomous variable DNA histograms data.

3.5 Total RNA isolation, cDNA synthesis and RT-qPCR

In order to determine changes in gene expression of the *MAT2A, MAT2B, OCT3/4, NANOG, DNMT1, DNMT3A and DNTM3B* genes (Table 3.1.) by fetal, adult and cancer stem cells in response to methionine, total RNA was isolated. For the isolation of total RNA from the cell, the Nucleospin RNA Mini kit (Machery-Nagel) was used and the A260/A280 purity and extraction quality as well as its concentration were determined using the NanoDrop ND-1000 spectrophotometer (Thermo Ficher Scientific Inc., USA). Subsequently, deoxyribonucleic acid (cDNA) synthesis was performed from these RNAs using the iScript cDNA Synthesis Kit (Bio Rad, USA). LightCycler® 480 II (Roche, Germany) device was used for real-time qPCR (RT-qPCR). PCR samples set up to reach 10 µL final volume contained the following components: cDNA, nuclease-free water, master mix and 1X Primer Probe (Taqman Gene expression assays, Thermo Fisher Scientific Inc., USA). Quantitative PCR condition: denaturation step at 95 °C for 10 minutes; The PCR step was set as 10 seconds at 95° C, 30 seconds at 60 °C and 1 second at 72 °C, and a cooling step at 40 °C for a total of 45 cycles. For each sample and experimental setup, three replicates were studied. Gene expression analysis was performed on treshhold Cycle (Ct) values relative to the reference gene (GAPDH) using sing the LightCycler® 480 II software. For the calculation of the changes in gene expression, relative ($2^{-\Delta\Delta Ct}$) quantitation was used.

Table 3.1. PCR gene sequences

Gene Name	Forward	Reverse
GAPDH	CGAGATCCCTCCAAAATCAA	CATGAGTCCTTCCACGATACCAA
MAT2A	CCACGAGGCGTTCATCGAGG	AAGTCTTGTAGTCAAAACCT
MAT2B	TGGGGAGCACTTGAAAGAG	CTTAGCGGCAACATGGG
NANOG	AAAGAATCTTCACCTATGCC	GAAGGAAGAGGAGAGACAGT
OCT3/4	GTATTCAGCCAAACGACCATC	CTGGTTCGCTTTCTCTTTCG
DNMT3A	GGGGACGTC CGCAGCGTC ACA	CAGGGTTGGACTCGA GAAATCGC
DNMT3B	ACC GC TGAATT ACT CAC GCC	ATGTCCCTCTTGTCG CCAAC
DNMT1	AGGTGGAGAGTTATGACGAGGC	GGTAGAATGCCTGATGGTCTGC

3.6 Histone Protein Extraction and Western Blot Analysis

Abcam histone extraction protocol was applied for protein isolation and Triton Extraction Buffer (TEB) prepared according to the following recipe: 0.5% Triton (Sigma), 1 mM EDTA (Gibco), 0.02% NaN3 (Sigma) in PBS. Briefly, cold PBS was added twice on the counted cells and centrifuged. Then, 1 ml of TEB was added to each 10^7 cells and the cell pellet was pipetted well. From this stage onwards, the cells and each working solution were kept on ice. Pipetting was done for 10 minutes to mix well and obtain cell lysate. Post-treatment supernatant was completely removed from cells, which were centrifuged to precipitate nuclei in a centrifuge set at 6,500 xg for 10 minutes and at 4°C. Next, the centrifugation step was repeated by adding half the amount of TEB added in the previous step onto the nebula-appearing nuclei. This time the pellet was treated at 4°C overnight by adding 1 mL of 0.2 N HCl per $4x10^7$ cells. The next day, the centrifugation step was repeated and the supernatant was transferred to new tubes and neutralized by adding

2M NaOH. Protein concentration was determined using Pierce BCA Protein Assay Kit (Thermofisher Scientific, USA) and aliquots were stored at -80°C until sample preparation for western blot experiment. 15 µg protein samples prepared in 4X laemmli buffer and diluent were denatured at 100 ºC for 5 minutes. Samples were then run on a previously prepared resolving gel containing 15% Acrylamide and stacking gel containing 6% acrylamide. A semi-dry transfer system device (BioRad) was used for the transfer of proteins from the gel to the membrane (Advansta). For this, the 1A-15V for 30 min program in the device was preferred and the transfer was done at room temperature. A 1% solution of Tris-Buffered saline-tween 20 (TBS-T) containing 5% non-fat milk powder was prepared and after transfer the membrane was blocked in this solution for 1 hour at room temperature. Manufacturer's recommended incubation times were followed for the incubation of all histone antibodies, and all primary antibodies (1:500 diluted H3K4me3,Stj Lab ; 1:500 diluted H3-Total, Biolegend ; 1:500 diluted H3K27me3, Biolegend) were incubated at 4 °C overnight and secondary antibodies (1:10000 diluted HRP-Anti-Rabbit, Santa Cruz ; 1:20000 diluted HRP-anti-mouse, Biolegend) were incubated for 1 hour at room temperature. After each incubation steps, the membrane was washed at least 3-5 times for 5-10 minutes by TBS-T. Then, the bands were visualized by Chemiluminescence Reagent for Horseradish Peroxidase Reagent (Serva, Germany). Gel imaging FluorChem FC3 System (Protein Simple, USA) was used to visualize and examine the protein bands.

3.7 S-Adenosyl-Methionine (SAM) / S-Adenosyl Homocysteine (SAH) Determination

SAM / SAH determination was performed using SAM and SAH ELISA Combo Kit (Cell Biolabs). In addition to the materials included in the kit, the necessary solutions were prepared according to the manufacturer's recommendation. The SAM and SAH standards required for the preparation of standard curves were prepared according to the manufacturer's recommendation. For sample preparation (n=2), the cells collected after methionine treatment were centrifuged at 2000 xg for 10 minutes at 4 ° C and the supernatant was discarded and 500 µl PBS was added to the cell pellet cell. Then, the cells were sonicated at 4 ° C for 8 minutes, the supernatant was taken on ice and the protein was measured, and the samples to be

studied were kept at -80 ° C. First, SAM and SAH conjugates diluted 1: 100 and 1: 1000 were used for the preparation of SAM - SAH conjugate coated plates, respectively. Protein binding plate was made ready for experiment by storing at 4ºC overnight. Then, the liquid on the plate surface was removed, the wells were washed 3 times with 200 µL PBS, then 200 µL of Assay Diluent was added and left on the shaker at room temperature for 1 hour. Assay diluent was removed, samples and standards were added to the wells in a volume of 50 µL, and after incubation on an orbital shaker for 10 minutes, 50 µL of diluted Anti-SAM (1: 500) and Anti-SAH (1: 500) antibodies were added. After 1h incubation at room temperature, the wells were washed 3 times with 250 µL of 1X Wash Buffer and the excess liquid was completely removed and 100 µL of diluted Secondary Antibody HRP (1: 1000) was added. After 1h incubation at room temperature, the washing step with 1 X Wash Buffer was repeated, and 100 µL of Substrate solution was added to each well and left for 10 minutes. After observing the color change, 100 µL of Stop Solution was added to each well to terminate the enzyme reaction, and the measurement of absorbance values was measured with a microplate reader (Tecan) at 450 nm wavelength.

3.8 Statistical Analysis

Statistical analyzes were made with the help of SPSS Inc by making comparisons between different experimental groups and using t-test, ANOVA, parametric and non-parametric analysis methods. Data with a p value less than 0.05 were considered statistically significant.

4. RESULTS

4.1 Characterization of Bone Marrow Mesenchymal Stem Cells, Umblical Cord Blood Mesenchymal Stem Cells and Cancer Stem Cells

First, we confirmed the phenotype and morphology of the stem cells from different sources and independent donors. The morphological characterization of both human BM-MSCs and UCB-MSCs were done on first, third and seventh day of the cell culture by phase contrast microscope. On the other hand, human breast CSCs isolated from MDA-MB-231 cell line were observed for first five days of cell culture and characterized.

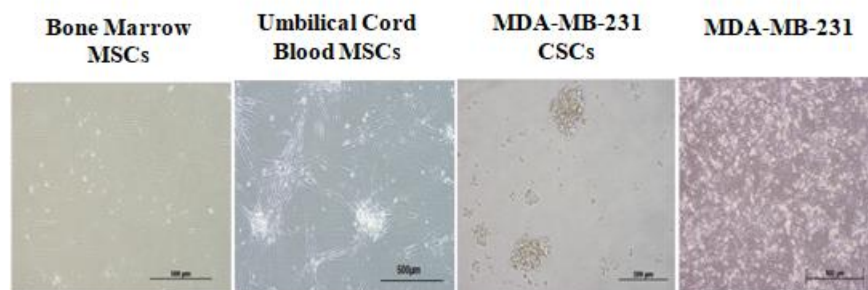


Figure 4.1. Morphological characterization of stem cells. A- Human Bone Marrow MSCs B- Human Umbilical Cord Blood MSCs C- MDA-MB-231 derived human breast CSCs D- MDA-MB-231 cell line.

UCB- and BM-MSCs had spindle-shaped morphology and were adhesive to the culture flask. Unlike these cells, CSCs displayed partial-adhesive properties to the culture plate. Both BM-MSCs and UCB-MSCs were analyzed for CD73, CD90 and CD105 cell surface protein expression (Figures 4.2 and 4.3). BM-MSCs were positive for for CD73, CD90 and CD105 for 98.6±0.1%, 94.0±0.0% and 95.6±0.4% respectively (Figure 4.2.). UCB-MSCs were positive for CD73, CD90 and CD105 as 98.6±0.1%, 85.9±2.3% and 82.8±1.8%, respectively (Figure 4.3.). CSCs isolated from MDA-MB-231 triple negative (ER-, HER2-, PR-) breast cancer cell line displayed $CD44^{+}/CD24^{-/low}$ expression (Figure 4.4.).

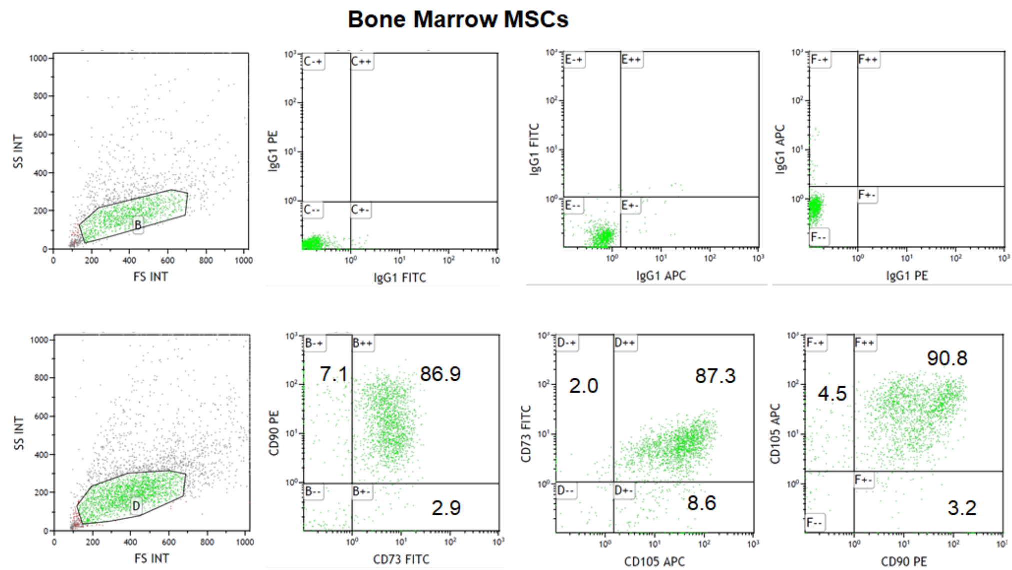


Figure 4.2 Cell surface marker expressions of bone marrow mesenchymal stem cells.

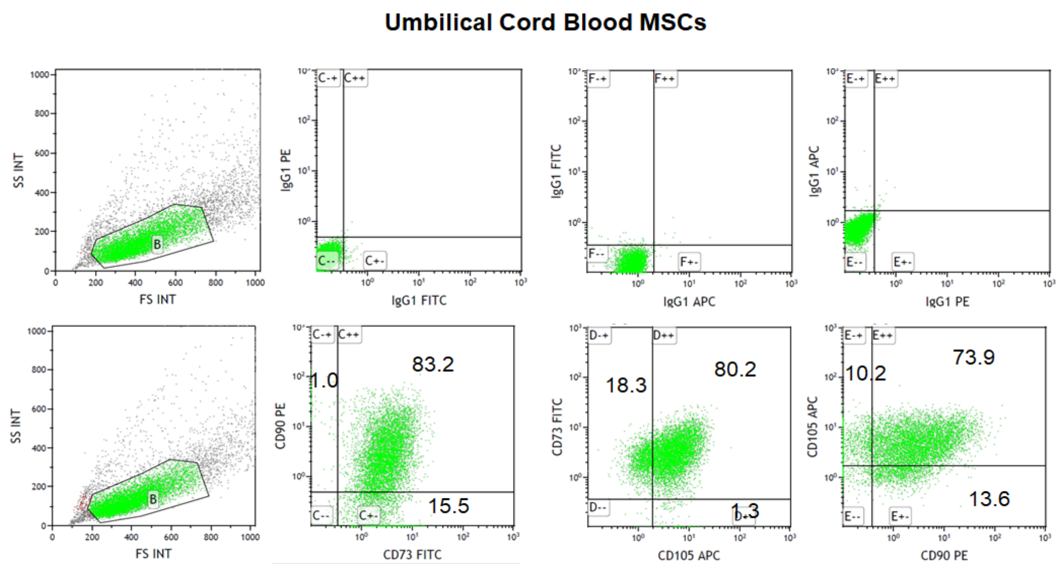


Figure 4.3. Cell surface marker expressions of umbilical cord blood mesenchymal stem cells

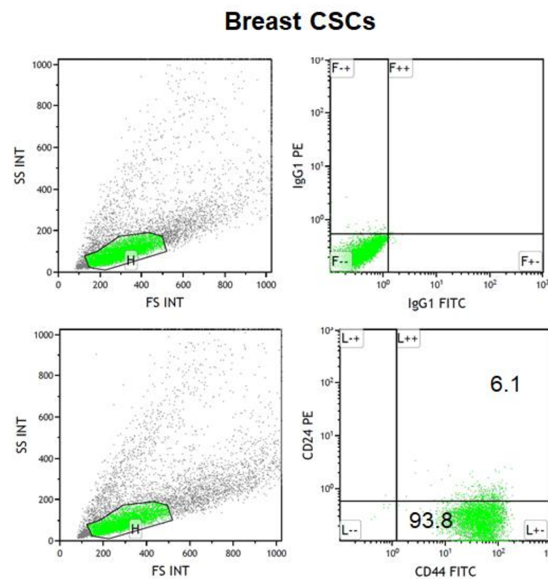


Figure 4.4. Cell surface marker expressions of breast cancer stem cells.

4.2 Cell Viability, Dosage and Time Interval with WST-1

The first objective of this work was to identify the non-toxic dose of L-Methionine condition for stem cells culture. For this concept, MSCs and CSCs on the fifth day of the culture were exposed to methionine application with sufficient confluent levels. First, the culture media were removed and treated for 48 hours in RPMI 1640 medium with no methionine, 10 µM, 25 µM, 50 µM and 100 µM methionine-dependent medium. Standard culture conditions for MSCs and CSCs were used as control. WST-1 analysis was performed for determination of effective dose and time. Among these, it was determined that methionine was not toxic for all three cell groups in any dose range and had no lethal effect in the chosen application range of 0-100 µM and control group (Figure 4.5. and - 4.6).

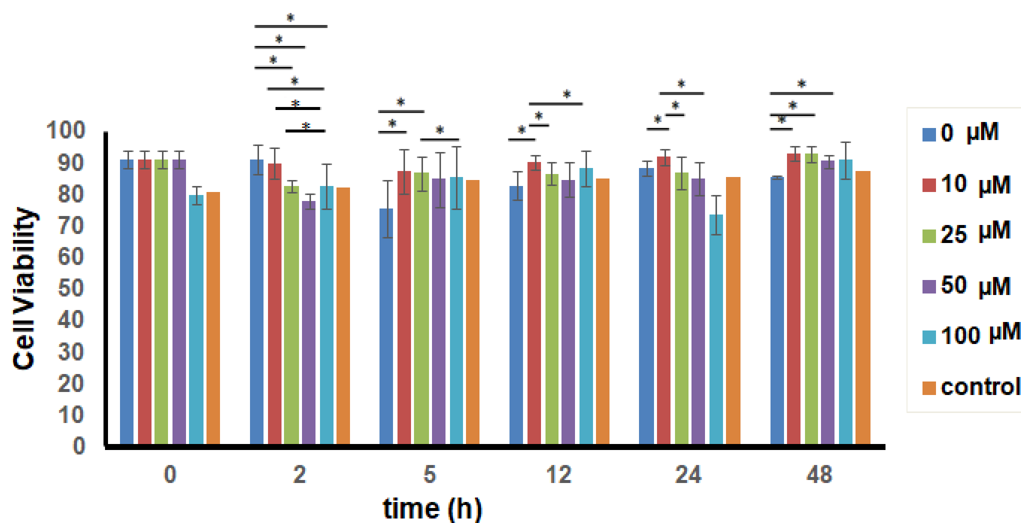


Figure 4.5. Viability assay of bone marrow mesenchymal stem cells.

Mean ± Std of at least three independent experiments is shown. *Significant differences (p<0.05) were analyzed with t-test.

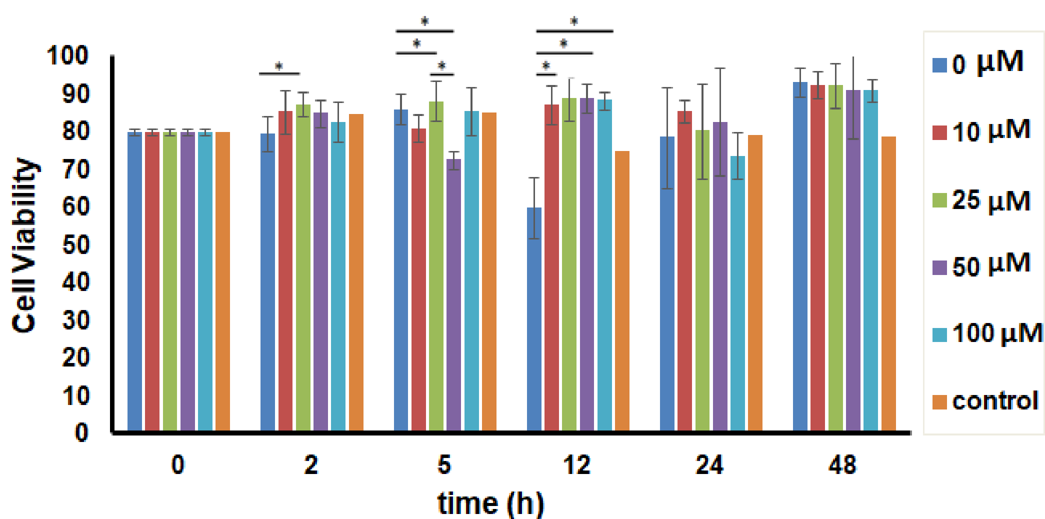


Figure 4.6. Viabiliy assay of umbilical cord blood mesenchymal stem cells.

Mean ± Std of at least three independent experiments is shown. *Significant differences (p< 0.05) were analyzed with t-test.

According to WST-1 analysis, cell viability was found to be approximately 80-90% in cells. However, it has been determined that this method is not suitable for CSCs due to the semi-suspended culture conditions. Dose determination is

emphasized by examining the BM-MSC and UCB-MSC groups, and determining the significant differentiation in cell viability at the fifth hour in the article referenced in the literature. Supporting this, BM-MSC in the methionine-free culture condition and UCB-MSC almost every dose has a fluctuating viability profile at fifth hour (5h). For this reason, the application of methionine was limited by focusing on the 5h in order to determine the significant effect as soon as possible and the experiments were continued.

4.3 Cell Cycle Analysis

MSCs and CSCs on the fifth day of the culture were exposed to different concentrations of methionine. First, the culture media were removed and cells were cultured for 5h in control medium and RPMI 1640 medium with no methionine (0 µM), 10 µM, 25 µM, 50 µM and 100 µM methionine concentrations. Then, cells in G0 / G1, S and G2 / M phases were evaluated for all three cell types by flow cytometry. The results of the cell cycle analysis were shown in Figure 4.7.-4.9. and Table 4.1.-4.3.

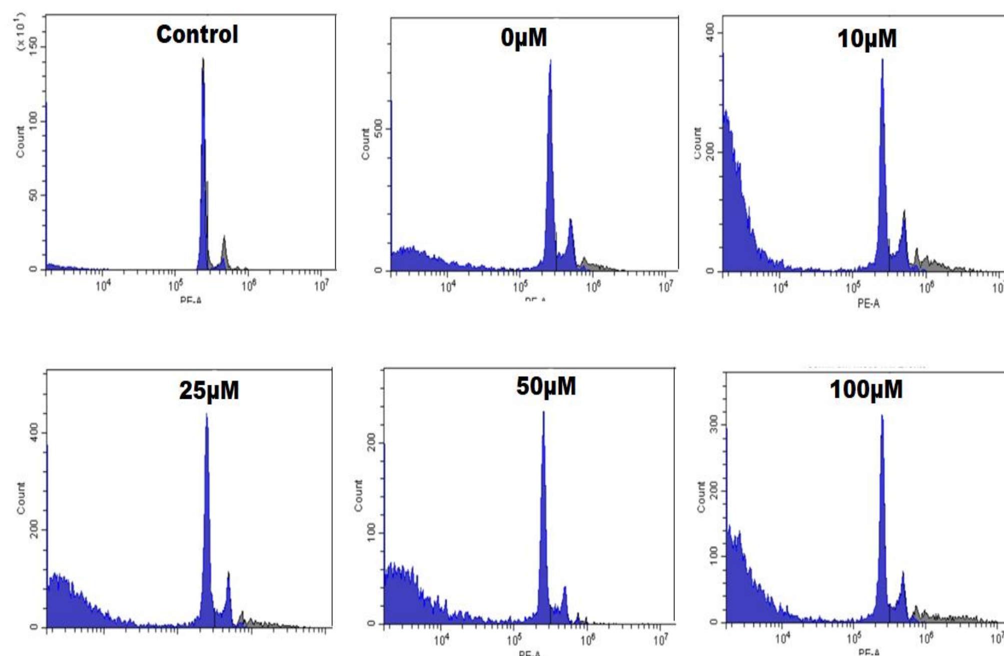


Figure 4.7. Representative data of cell cycle analysis of bone marrow mesenchymal stem cells.

The concentration of L-methionine in the culture medium between 0 µM-100 µM vs. control.

Table 4.1. Cell cycle analysis of bone marrow mesenchymal stem cells.

BM-MSC			
Condition	G0/G1	S	G2/M
Control	94.5±2.1	5.0±1.4	0.3±0.6
0 µM Met	75.6±24.0	24.4±12.0	0.3±0.6
10 µM Met	81.3±5.8*	21.7±0.6	0.3±0.6
25 µM Met	92.0±5.3★	4.5±5.0.7	0.3±0.6
50 µM Met	94.5±4.9	5.5±4.9	0.0±0.0
100 µM Met	92.5±7.8	7.5±7.8	0.0±0.0

* statistically significant compared to the control. ★ statistically significant compared to 10 µM Met. Mean ± std, n=3, p<0.05.

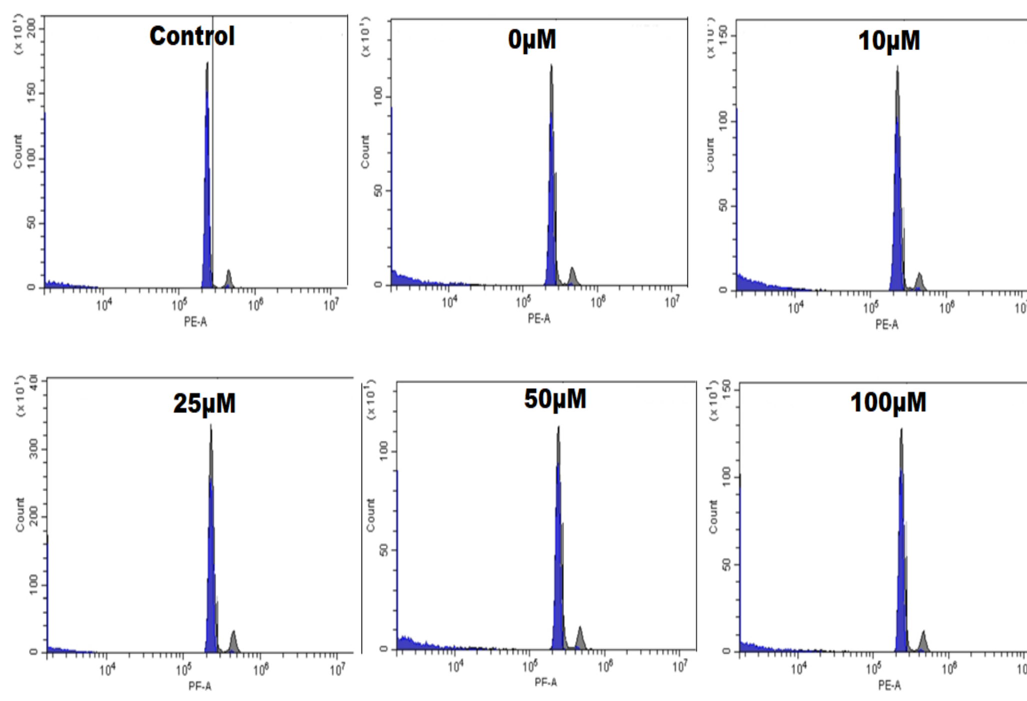


Figure 4.8. Reprentative data of cell cyle analysis of umbilical cord blood mesenchymal stem cells.

Representative data of cell cycle analysis of UCB-MSCs, The concentration of L-methionine in the culture medium between 0 µM-100 µM vs. control.

Table 4. 2. Cell cycle analysis of umbilical cord blood mesenchymal stem cells.

UCB-MSC			
Condition	**G0/G1**	**S**	**G2/M**
Control	92.5±0.7	7.5±0.7	0.0±0.0
0 μM Met	91.5±0.7	8.5±0.7	0.0±0.0
10 μM Met	90.5±3.4	9.5±3.5	0.0±0.0
25 μM Met	89.0±1.1*	11.0±1.1	0.0±0.0
50 μM Met	90.0±1.1	10.0±1.1	0.0±0.0
100 μM Met	92.0±1.14	8.0±1.1	0.0±0.0

* statistically significant compared to the control. Mean ± std, n=3, p<0.05.

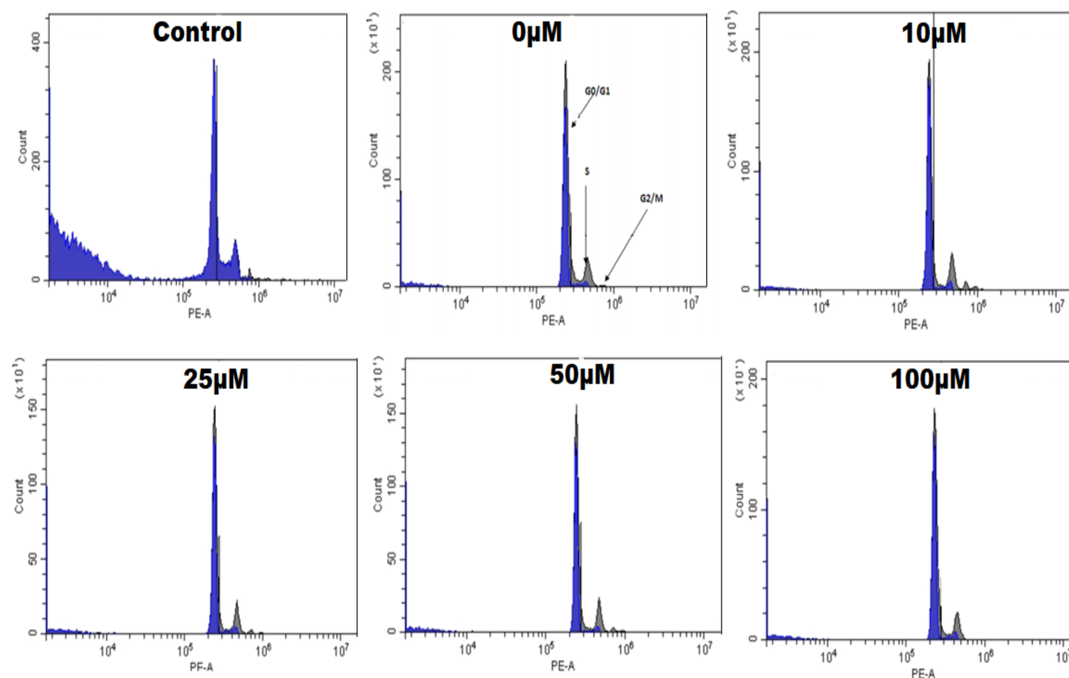


Figure 4.9. Representative data of cell cycle analysis of CSCs.

The concentration of L-methionine in the culture medium between 0 μM-100 μM vs. control.

Table 4.3. Cell cycle analysis of breast cancer stem cells.

CSC			
Condition	G0/G1	S	G2/M
Control	85.7±5.5	14.3±5.5	0.0±0.0
0 µM Met	82.5±0.7	17.5±0.7	0.3±0.6
10 µM Met	84.5±7.8	15.50±7.8	0.0±0.0
25 µM Met	86.0±2.83	14.0±2.83	0.0±0.0
50 µM Met	78.5±4.9	21.5±4.9	0.0±0.0
100 µM Met	91.5±7.8	8.5±7.8	0.0±0.0

* statistically significant compared to the control. Mean ± std, n=3, $p<0.05$.

When the cell cycle data were examined, it was observed that cells remain arrested in S phase with low concentrations of methionine (0 and 10 µM) in the culture medium of BM-MSCs. This trend was different from control and other tested doses. In terms of the frequency of cells in S phase, there was a 4-fold ($p<0.05$) increase in the BM-MSCs that cultured in the medium without methionine compared to the control), and a 5-fold ($p>0.05$) increase in 10 µM application (Table 4.1.). While the percentages of G0/G1 and S phase in BM-MSCs changed sharply between doses, a slight variation was observed in UCB-MSCs. Contrary to the application of 25 µM in BM-MSC culture, with the supplementation of 25 µM methionine in UCB-MSC culture, the frequency of cells in the S phase reaches its highest (11.0±1.1, $p<0.05$) and G0-G1 phase reached its lowest percentage (89.0±1.1 , $p<0.05$) among other doses. This decrease was statistically significant compared to the control (Table 4.2.). For CSC, the distribution of cell frequency in the S phase showed a profile between UCB-MSC and BM-MSC. Again, although the critical dose value was 25 µM for methionine supplementation, it was observed that at this dose, the tendency to S phase in CSCs was the least (14.0±2.8) as control (14.3 ± 5.5). CSCs in 50 and 100 µM methionine supplemented culture medium displayed 1.5- and 2- fold increase to arrest in S phase compared to control condition, but these differences were not statistically significant (Table 4.3.).

4.4 Gene Expression Analysis

The effect of methionine supplementation in culture medium at different doses (control, 0, 10, 25, 50 and 100 µM) during 5h period on BM-MSCs, UCB-MSCs and CSCs was investigated by RT-qPCR in terms of their gene expressions (*OCT3/4, NANOG, MAT2A, MAT2B, DNMT1, DNMT3A, DNMT3B*). The gene expression of cells cultured in different amount (0, 10, 25, 50 and 100 µM) of L-methionine was normalized to the gene expression of cells in control condition. According to gene expression analysis, *OCT3/4* expression level of BM-MSCs in depleted medium has significantly increased 1.5, 2 and 2.5-fold ($p<0.05$) at 10 µM, 25 µM and 100 µM methionine concentrations, respectively, compared to depleted medium. At 25 µM and 50 µM concentration, *OCT3/4* expression was decreased compared to control ($p< 0.05$). *NANOG* gene expression has significantly increased in BM-MSCs at 10, 25 and 50 and 100 µM methionine concentration (2-fold, 2-fold, 2.6-fold and 3-fold, $p< 0.05$), compared to depleted medium. *NANOG* expression of cells cultured in depleted medium was significantly reduced compared to the cells cultured in control condition ($p<0.05$). *MAT2B* expression level of BM-MSCs at 10, 25, and 100 µM L-methionine containing medium was increased (1.7-fold, 1.6-fold, 3.5 fold, $p<0.05$) compared to methionine-depleted medium (Figure 4.10.). Oppositely, at 50 µM methionine condition, BM-MSCs have decreased (0.7-fold, $p<0.05$) *MAT2B* gene expression. On the other, as shown in Figure 4.1, *MAT2A* expression of cells in 25 µM and 100 µM methionine concentration was 1.3-fold and 3-fold ($p<0.05$) vs. to the cells in depleted and control medium. Besides, there was a significant increase in *MAT2A* expression level of cells in 0 µM (2-fold, $p< 0.05$), 10 µM (3.5-fold, $p< 0.05$) ,25 µM (3-fold, $p<0.05$), 50 µM (2-fold, $p<0.05$) and 100 µM (8-fold, $p<0.05$) L-methionine containing medium compared to control medium. *DNMT1* expression has significantly increased in BM-MSCs cultured in 10, 25 and 50 µM L-methionine compared to depleted medium (1.6-fold, 1.5-fold and 4-fold, respectively, $p< 0.05$). At 10 µM and 25 µM, *DNMT1* expression has significantly increased compared to cells in control medium ($p< 0.05$). Increased *DNMT3B* expression of cells was indeed achieved at 100 µM (2-fold vs. depleted medium and 4-fold vs. control condition, $p<0.05$) and 100 µM (4-fold vs. depleted medium and 8-fold vs. control condition $p<0.05$ methionine concentration). *DNMT3A* expression of

cells in 10, 25, 50 and 100 µM methionine concentration has increased significantly vs. depleted medium ($p < 0.05$). Similar trends were observed both cells in 10 and 25 µM methionine concentration vs. control medium ($p < 0.05$) (Figure 4.10.).

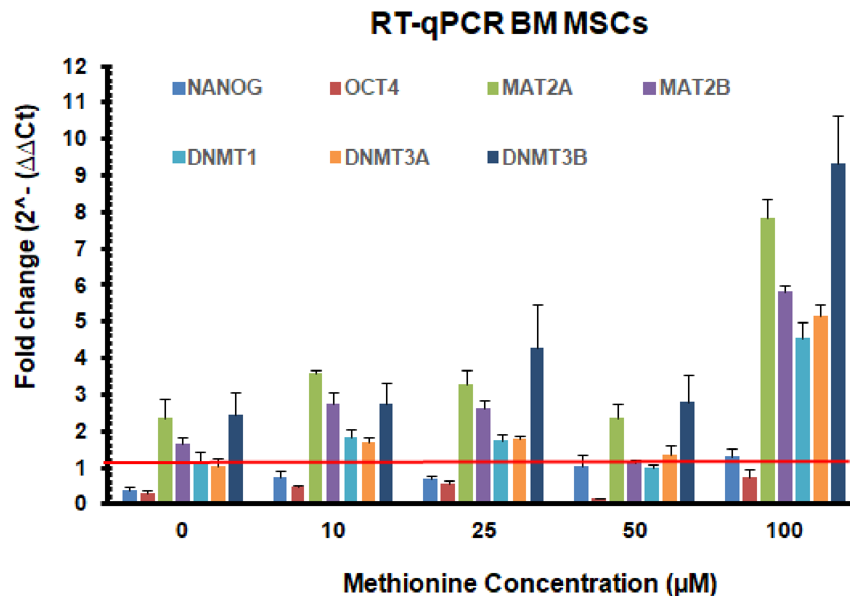


Figure 4.10. The gene expression profile of bone marrow mesenchymal stem cells.

The expression of pluripotency and methionine metabolism specific genes increased when BM-MSCs incubated with different concentration of L-methionine (mean ± std of six independent experiments shown). (OCT3/4: 10 , 25 µM Methionine vs depleted condition, $p<0.05$. NANOG: 10,25,50 µM Met Methionine vs depleted condition, $p <0.05$. DNMT1: 10, 25, 50 µM Met Methionine vs depleted condition, $p <0.05$. DNMT3A: 10, 25, 50 and 100 µM Methionine vs depleted condition, $p <0.05$. DNMT3B: 100 µM Methionine vs depleted condition, $p <0.05$ MAT2A: 25 and 100 µM Methionine vs depleted condition, $p <0.05$. MAT2B: 10, 25, 50 and 100 µM Met Methionine vs depleted condition, $p <0.05$). Data given are the fold change according to expression of cells in control culture medium.

According to gene expression profile analysis, ***OCT3/4*** expression of UCB-MSCs significantly increased at 10 µM methionine concentration compared to the cells in depleted medium ($p<0.05$). Similarly, ***NANOG*** expression of cells significantly increased 1.5 and 2-fold ($p<0.05$) at 10 and 50 µM methionine concentration compared to cells in depleted medium. There was a significant increase (3-fold, $p<0.05$) of ***NANOG*** expression of UCB-MSCs in control medium vs. depleted medium. ***MAT2A*** expression of UCB-MSCs significantly decreased at

10, 25, 50 and 100 µM methionine concentration ($p<0.05$) compared to cells in depleted medium (Figure 4.11.). ***DNMT1*** expression of cells at 10 and 50 µM methionine concentration increased significantly (2- and 1.5-fold, $p<0.05$, respectively) compared to cells in depleted medium. On the other hand, *MAT2B*, ***DNMT3A*** and ***DNMT3B*** expressions of UCB-MSCs showed no significant differences compared to both control condition and depleted medium.

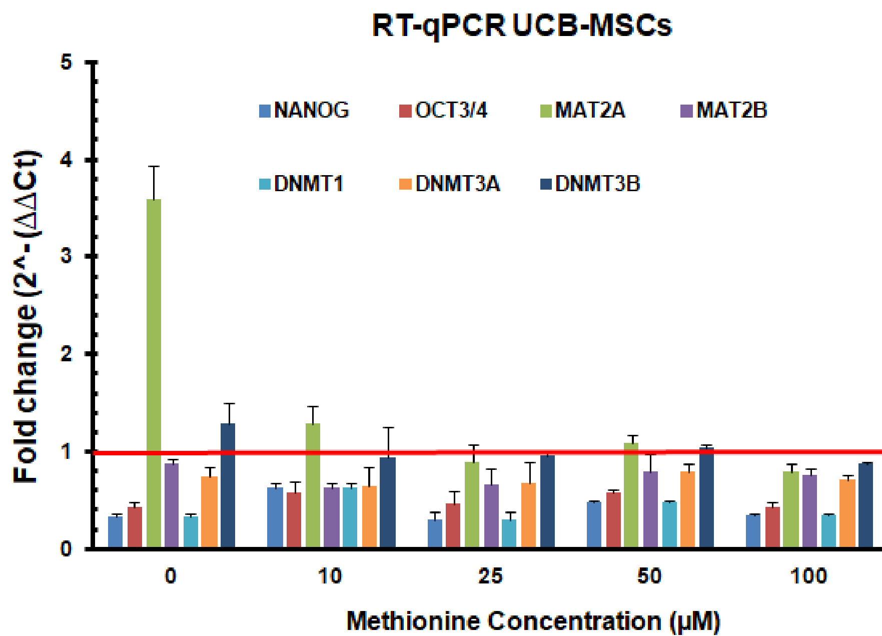


Figure 4.11. The gene expression profile of umbilical cord blood mesenchymal stem cells.

The expression of pluripotency and methionine metabolism specific genes increased when UCB-MSCs incubated with different concentration of L-methionine. (mean ± std of six independent experiments shown). (OCT3/4: 10 µM Methionine vs depleted condition, $p<0.05$. NANOG: 10, 50 µM Met Methionine vs depleted condition, $p<0.05$. DNMT1: 10, 50 µM Met Methionine vs depleted condition, $p<0.05$. MAT2A: 10, 25, 50, 100 µM Met Methionine vs depleted condition, $p<0.05$. MAT2B: 10 µM Met Methionine vs depleted condition, $p<0.05$.). Data given are the fold change according to expression of cells in control culture medium. Data given are the fold change according to expression of cells in control culture medium

Cancer stem cells and MSCs differed in terms of gene expression profiles (Figure 4.12.). *OCT3/4* expression of CSCs in 10, 25, 50, and 100 µM methionine concentration showed significant increase compared to both depleted medium and control condition ($p<0.05$). *NANOG* expression of CSCs in 25, 50 and 100 µM methionine concentration significantly increased compared to depleted medium

(p<0.05). Similar tendency was observed among cells in control medium, cells in 100 µM methionine containing medium vs. depleted medium, but the diffrence was not significant (p>0.05). *MAT2A* expression of CSCs in depleted medium was increased 9-fold compared to control condition. Also, MAT2A expression of cells in depleted medium was increased at least 2-fold among all other doses, though the differences were not significant. At 10 µM, 25 µM and 100 µM methionine concentrations, ***MAT2B*** expressions of CSCs decreased (p<0.05) compared to cells in the methionine depleted condition. In all doses of methionine dependent medium has increased MAT2B expression of CSCs than control condition. ***DNMT1*** expression of cells at 10, 25 and 50 µM methionine concentration was increased significantly compared to cells in depleted medium (1.2- 2- and 2-fold, respectively, p<0.05). Figure 4.. showed a 1.5-fold increase of ***DNMT3A*** expression of cells in 25 and 50µM (p>0.05) methionine concentration vs. cells in depleted medium. Besides, ***DNMT3B*** expression of cells in 50 µM methionine condition significantly increased compared to cells in depleted medium (3-fold, p<0.05).

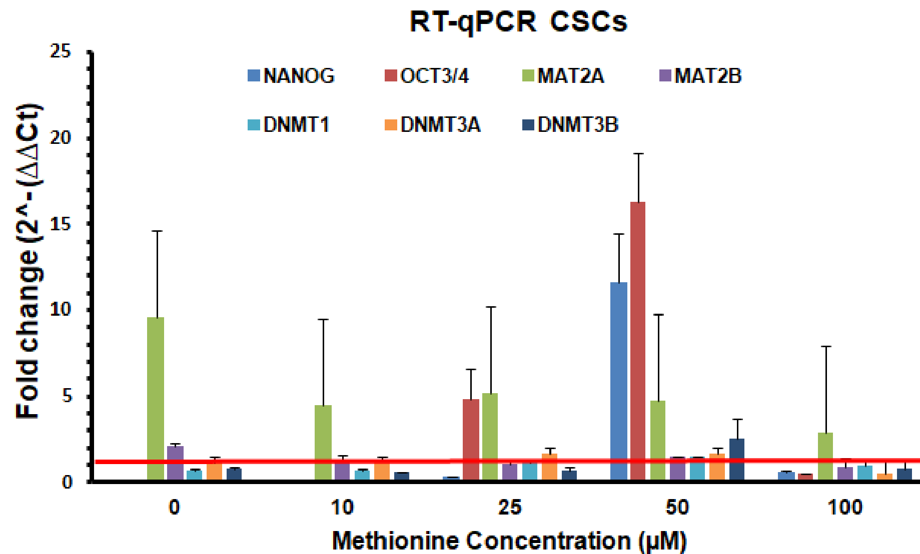


Figure 4.12. The gene expression profile of breast cancer stem cells.

The expression of pluripotency and methionine metabolism specific genes increased when CSCs incubated with different concentration of L-methionine. (mean ± std of six independent experiments shown). (OCT3/4: 10, 25, 50, 100 µM Methionine vs depleted condition, p<0.05 NANOG: 25, 50, 100 µM Met Methionine vs depleted condition, p <0.05 DNMT1: 10, 50 µM Met Methionine vs depleted condition, p <0.05 MAT2B: 10, 25, 100 µM Met Methionine vs depleted condition, p <0.05). Data given are the fold change according to expression of cells in control culture medium

4.5 Histone Protein Expression Analysis

Western blot analysis was performed to examine the methylation expression of three cell groups exposed to culture media of varying methionine concentrations. In this context, H3K4me3, H3K27me3 expressions were examined by normalizing to the H3 total expression. MSCs and CSCs were exposed to the different methionine concentrations. The expression level of H3K27me3 and H3K4me3 of BM-MSCs in control group was decreased as similar as cells in 25 µM methionine compared to other depleted and methionine supplemented conditions (Figure 4.13). The decrease in H3K27me3 expression levels compared to the cells in depleted medium was significant ($p<0.05$). In the methionine-free culture medium, the H3K27me3 expression of BM-MSCs showed a significant 1.6-fold increase compared to the cells in control condition ($p<0.05$). Similarly, after 100 µM methionine induction, a 1.2-fold ($p<0.05$) increase in H3K4me3 expression was observed in BM-MSCs compared to the cells in 50 µM methionine supplemented culture medium (Figure 4.13).

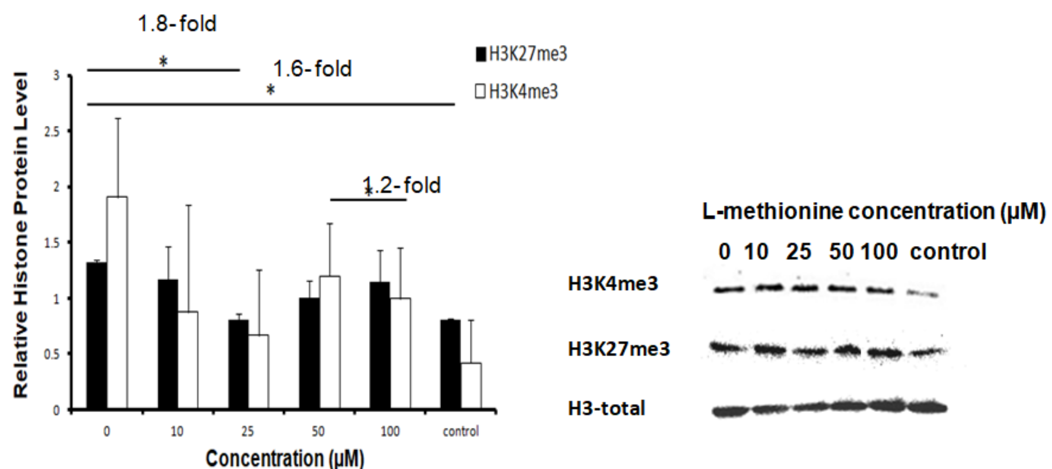


Figure 4.13. Histone protein levels of bone marrow mesenchymal stem cells. Mean ± std, n=3, *p<0.05.

UCB-MSCs cultured in control medium had the lowest H3K4me3 expression levels. The 25 µM methionine supplemented culture medium stimulated the expression of H3K4me3 in UCB-MSCs compared to the cells in control (1.2-fold, $p<0.05$, Figure 4.14.). In addition, compared to 10 µM methionine-supplemented

medium, UCB-MSCs in 50 µM methionine condition displayed an increased H3K3me3 expression level (1.3-fold, p<0.05).

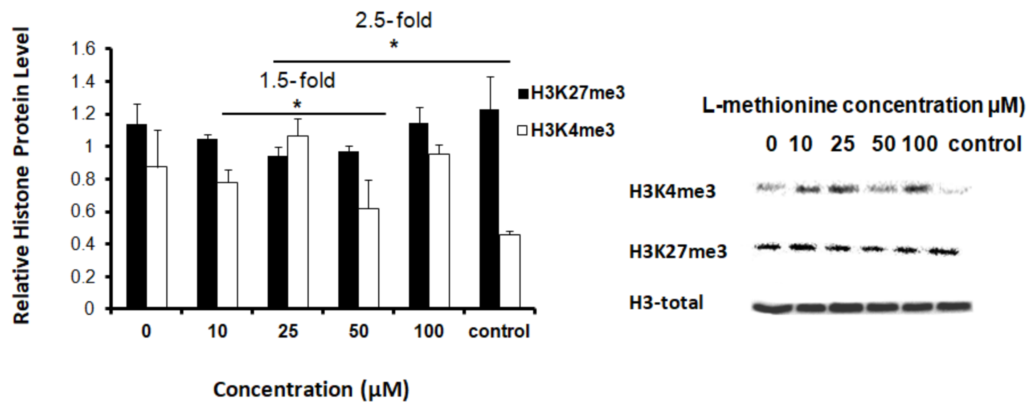


Figure 4.14. Histone protein levels of umbilical cord blood mesenchymal stem cells. Mean ± std, n=3, *p<0.05.

CSCs in 50 and 100 µM methionine supplemented culture medium increased their H3K27me3 expression levels compared to the cells in 10 µM methionine supplemented condition (1.3- and 1.4-fold, respectively, p<0.05). There was a significant decrease in H3K27me3 expressions of CSCs in the culture medium without methionine vs. the control (1.4-fold, p<0.05, Figure 4.15.). The H3K4me4 expression level was elevated in CSCs in methionine depleted medium compared to the cells in 25 µM methionine containing medium. In addıtion, H3K4me3 expression level of CSCs increased (1.3-fold, p<0.05) at 50 µM methionine concentration compared to the cells in control condition.

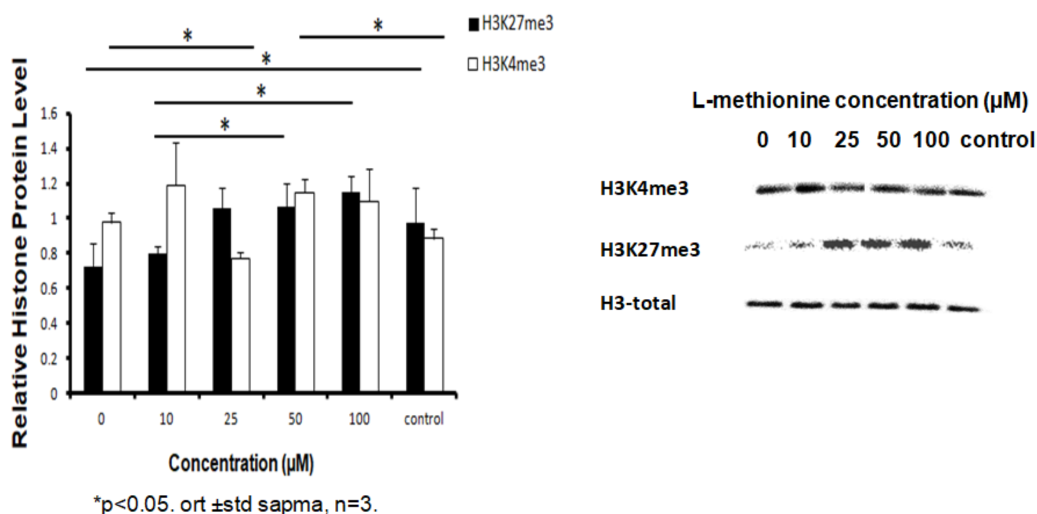


Figure 4.15. Histone protein levels of breast cancer stem cells. Mean ± std, n=3, *p<0.05.

4.6 SAM/SAH Analysis

The SAM and SAH standards curves were determined and the R^2 levels for both SAM and SAH were accepted as 0.92 for efficiency (Table 4.4. and -4.5.). SAM amount in BM-MSCs were found to be similar as 18.8± 5.4 µg/ml and 18.8± 2.5 µg/ml in methionine-depleted and 100 µM methionine supplemented medium. The lowest SAM/SAH ratio in BM-MSCs was observed in the control group. However, similar tendency was observed in CSCs with 25 µM methionine (10.7±4.4) supplemented condition. For UCB-MSCs, SAM and SAH levels were very low to detect by ELISA (Table 4.4. and - 4.5.).

Table 4. 4. Evaluation of SAM analysis by ELISA for different sources of stem cells.

SAM			
methionine concentration	BM-MSCs mean ±std (µg/ml)	UCB-MSCs mean ±std (µg/ml)	CSCs mean ±std (µg/ml)
0 µM	18.8±5.2	0.0±0.0	19.2±3.7
10 µM	13.1±3.1	0.0±0.0	19.9±0.2
25 µM	15.7±0.8	0.0±0.0	10.7±4.4
50 µM	15.7±0.7	0.0±0.0	21.5±1.0
100 µM	18.8±2.5	0.0±0.0	17.9±1.0
control	3.7±3.4	0.0±0.0	20.3±1.3

Mean± std, n=2.

Table 4. 5. Evaluation of SAH analysis by ELISA for different sources of stem cells.

SAH			
methionine concentration	BM-MSCs mean ±std (µg/ml)	UCB-MSCs mean ±std (µg/ml)	CSCs mean ±std (µg/ml)
0 µM	99x10-5±0.0	0.0±0.0	99x10-5±0.0
10 µM	0.0±0.0	0.0±0.0	0.0±0.0
25 µM	99x10-5±0.0	0.0±0.0	75x10-4±0.0
50 µM	99x10-5±0.0	99x10-5±0.0	0.0±0.0
100 µM	99x10-5±0.0	14x10-4±0.0	0.0±0.0
control	0.0±0.0	99x10-5±0.0	99x10-5±0.0

Mean± std, n=2.

5. DISCUSSION

The research question of this thesis is to analyze whether the response of stem cells at different developmental stages to methionine is different, based on the differences in the metabolism of embryonic, fetal, and adulthood development in humans. The reason for choosing different stem cell groups in this study is that it is aimed to reveal the change of metabolism and epigenetic regulation of stem cells referring to the existing studies in the literature on the importance of methionine and folate metabolism in especially CSCs and ESC/IPSCs (Table. 2.1). In this thesis study, ESCs could not be used due to ethical concerns, and although it is ethically permissible to work with mouse ESCs, they could not be used in this thesis concept because of differences in human methionine metabolism and threonine metabolism in the mouse (91). For this reason, tumor initiating cells (CSCs) isolated from MDA-MB-231 used in this thesis, due to their similarities to ESCs in terms of their stemness characteristics. As a result of studies in the literature on dietary folate and amino acid levels that revealed their importance during pregnancy, it is known that regulation of 1-C metabolism is important for embryo development and is associated with diseases in adulthood in case of deficiencies (112, 116, 156). In the light of the knowledge that amino acid regulation and 1-C metabolism are responsible for regulating methylation profile in pregnancy and as a result of our literature research, we aimed to observe the effect of external methionine application in cell culture medium on UCB-MSCs, based on the fact that these studies are not detailed *in vitro* on fetal stem cells. BM-MSCs with tissue-specific methylation pattern were selected as the cell group reflecting adult stem cells within the scope of this thesis study. Collectively in this thesis research, the effect of methionine on different human stem cell groups (BM-MSCs, UCB-MSCs and breast CSCs) was investigated.

BM- and UCB-MSCs used in this thesis showed a spindled-shaped morphology and adherence to the culture plastic, as well as expressing MSC markers. In addition, MDA-MB-231 had $CD44^{+}$, $CD24^{-/low}$ expression, similar to the literature (131). Similar to other research groups, the CSCs we studied also developed in a partial adherent form in the culture dish, unlike the adhesion ability of MSCs. (130, 132-134). In order to preserve the stemness characteristics of CSCs,

they were developed with appropriate culture medium and mammosphere medium components specified in the literature in an ultra-low attachment plate for culturing in ESC-like suspended form (135).

WST-1 cell viability analysis provided choosing the suitable condition for methionine treatment which is non-toxic for cells by 85-90% viability for all doses (0, 10, 25, 50 and 100 μM) during 48 hours and all three cell groups. As CSCs cultured in mammosphere culture conditions, during WST-1 analysis, after each centrifugation and washing steps, a serious amount of cells were lost. For this reason, WST-1 data of CSCs could not be properly detected. 5h of methionine-supplementation, in which the difference between groups in both UCB- and BM-MSCs was statistically significant, was chosen for the next experimental setup. In addition, as Shiraki et al. defined the optimum concentration for methionine in the culture medium as 25 μm and they observed cell cycle arrest and expression of apoptotic markers in the time interval of 5-24 hours of methionine depletion in ESCs/ IPSCs (14). Collectively, we have tested the effect of 0-100 μM methionine treatment in CSCs, BM and UCB-MSCs for 5h.

Cell cycle analysis of all three cell groups were investigated by the percentage of cells in G0/G1, S and G2/M phases after 5h of methionine treatment. Our results revealed that, the cells mostly tend to stay in G0/G1 phase and at the same time, they tend to stay S phase dependent on the dose applied. Methionine might tend to keep BM-MSCs, UCB-MSCs and CSCs in G0/G1 phase in cell cycle by application for 5h, and the cell cycle profile of cells would change if the application time of methionine would exceed their doubling times, which have been postulated by Zhan et al. for BM-MSCs and UCB-MSCs (26.2± 0.242h, 35±0.558h) (136). Similar to our results, Lin et al. observed the effect of MAT knockdown and methionine depletion for 16h, and they found that lymphoblast precursor cells remained G1/S arrested and largely remained in the G1 phase (137). As Shiraki et al reported, cell cycle disruptions related to methionine deprivation in ESCs started at 5h (14). The reason of the tendency of most cells to be arrested in G0/G1 might be that SAM is a supplier of methyl unit necessary for nucleotides and folate cycle takes part in the biosynthesis of DNA.

For gene expression analysis in cells kept in methionine-supplemented medium for 5h, pluripotency-related genes *(OCT3/4, NANOG),* genes that play an active role in methionine metabolism *(MAT2A, MAT2B)* and DNA methyltransferases *(DNMT1, DNMT3A, DNMT3B)* were examined by RT-qPCR. Gene expressions in all three cell groups demonstrated a rather independent profile. It is very valuable to elucidate and understand the mechanism underlying the stem cells' ability to renew themselves and maintain this potent structure without differentiation. Various studies have been conducted to understand the fluctuations in the expression of genes such as *OCT4* and Nanog in MSCs, which act as core transcriptional factors for ESC maintenance, self-renewal and proliferation, and for the protection of pluripotent characteristics of cells. It has been demonstrated that they also play a regulatory role in multipotent adult cells (138, 139). In ESCs, expression of *OCT3/4* also regulates the expression of genes such as *SOX2* and *NANOG*, resulting in suppression of differentiation and persistence of pluripotency (86). Thus, suppression of *OCT4* expression pushes ESCs to abrupt differentiation and formation of trophoblast cells, while downregulation of *NANOG* expression differentiates them into cells of extra-embryonic lineage (140-142). Besides, *OCT4* and *NANOG* knockdown induced MSCs into the lineage-specific differentiation (143). Although the regulation of these genes is known to be essential for the maintenance of pluripotency and multipotency, the underlying mechanism requires further research.

As a result of our RT-qPCR analysis, we found a significant increase in ***OCT3/4*** gene expression of BM-MSCs at 10, 25 and 100 µm (1.5- , 2- and 2.5 fold respectively) methionine containing medium compared to the methionine-depleted medium. OCT3/4 expression was decreased in BM-MSCs for all conditions compared to control, and this difference was significant ($p<0.05$) for cells in the depleted medium. On the other hand, an increase was found in ***NANOG*** expression of BM-MSCs in 10, 25, 50 and 100 µM methionine supplemented medium condition. In consistent with our results, Matic et al. showed that expression of *OCT4* is extremely low in undifferentiated human BM-MSCs (144). In addition, Pierantozzi et al. could not detect *OCT3/4* and *SOX2* expression in BM-MSCs, and they suggested that *NANOG* expression does not directly regulate stem/progenitor

properties in cells, but adapts cells to the *in vitro* culture medium from the quiescence stage to the proliferative stage (145). Based on this study, 3-fold increase in *NANOG* expression of BM-MSCs in the medium containing methionine at all studied doses compared to methionine-depleted condition reveals the importance of methionine in terms of stemness and maintenance in these cells.

Similarly in UCB-MSCs, a significant 1.3-fold increase in OCT3/4 expression was observed in 10 µM condition compared to methionine-depleted condition. In addition, ***OCT3/4*** expression of UCB-MSCs was decreased at almost all doses compared to contol as in BM-MSCs. An increase of 1.5 and 1.8-fold ($p<0.05$) was found in the ***NANOG*** expression of UCB-MSCs under culture conditions containing 10 µm and 50 µm methionine compared to depleted condition, respectively. In BM-MSC ve UCB-MSCs, an increase in *OCT3/4* and *NANOG* gene expression levels was observed when 10 µM methionine is supplemented to culture medium. This data suggests that, low concentration of methionine application might keep MSCs in a more proliferative state.

It has been reported in 2021 that breast cancer is in the first three places in causing death in women among cancer cases in the United States (146). Secondary tumor development and malignancy after therapeutic targeting are explained by the CSC model, and therefore it seems reasonable to establish specific targeting to these cells by demonstrating their relationship with stem cell metabolism (147, 148). *OCT4,* as a pluripotency marker for CSCs, has revealed its necessity for proliferation, invasive properties and self-renewal of CSCs, both because of its ability to form complexes with other transcription factors and its involvement in various signaling pathways (149). According to our results, *OCT3/4* expressions were significantly increased by 50- and 160-fold ($p<0.05$) in CSC at 25 and 50 µM L-methionine supplemented culture medium, compared to the depleted medium. A similar tendency as 16-fold (p<0=5) increase in *OCT3/4* expression of CSCs was found in 50 µM L-methionine supplemented condition compared to control.

NANOG expression of CSCs was increased in 50 µm methionine supplemented medium compared to methionine-depleted and control conditions ($p<0.05$). According to *OCT3/*4 and *NANOG* expression profiles, CSCs express

stemness markers from 25 µM to 100 µM methionine supplementation, compared to lower doses. This data shows similarity with the postulation of Shiraki et al. as ESCs display their pluripotent characteristics in 25 µM methionine supplemented culture (14).

The genetic and epigenetic regulation mechanisms affecting the functions of cells and the DNA and histone modifications preserve the maintenance of the stem cells. DNA methyl transferases are divided into three: *DNMT1, DNMT3B* and *DNMT3B* in order to provide tissue-specific gene expression profile (150). Responsible for genome imprinting by maintaining the methylation pattern in hemi-methylated DNA during DNA replication, *DNMT1* is the major one among DNMTs (151, 152). *DNMT3A* and *DNMT3B* are known to provide *de novo* methylation and play an active role in the differentiation process by being highly expressed in ESCs (150). Not only does DNMT1 have a regulatory role on *NANOG* and *OCT3/4* expressions by CpG methylation on DNA, but also *OCT4* and *NANOG* directly bind to the *DNMT1* promoter region and upregulate *DNMT1* expression, allowing the regulation of differentiation-specific genes such as p16 and 21 during development (143). Consistent with this, the RT-qPCR results of *DNMT1* expressions, which we found as a result of our research, are in direct parallel with the increase in OCT3/4 and *NANOG* expressions in both BM-MSC and UCB-MSCs and show similar increases at the same doses. As shown in the literature, *DNMT1* and *DNMT3A* knockdown directed MSCs into spontaneous differentiation (153-155). So Ay et al. detected an increase in senescence-related p16 and p21 protein expressions and demethylation in the promoter regions of their genes in human UCB-MSCs as a result of inhibition of *DNMT3A* and *DNMT1*, but not *DNMT3B* (156). However, *DNMT3B* inhibition led cells to apoptosis directly. Based on this, we can interpret that in order to preserve their multipotent properties in the early passages, MSCs keep both the expression of DNMTs and the expression of stemness-related genes such as *OCT3/4* and *NANOG* high. Also, differentiation capacity, methylation profiles and metabolic markers of MSCs isolated from different tissues are naturally different (157). Since *DNMT3A* and *DNMT3B* are responsible for tissue-specific methylation and are highly expressed in the undifferentiated potent cells and ESCs, the approximately 5-fold ($p<0.05$, *DNMT3A*) and 4-fold (*DNMT3B*) increase in BM-

MSCs at 100 µM methionine containing medium compared to depleted condition, respectively, might indicate that this dose affected the methylation profile to keep BM-MSC in a more potent state (158-160).

Epigenetic mechanisms, particularly the necessity of *DNMT1* activity, for mammary gland development during adolescence and pregnancy have been demonstrated. In addition, *DNMT1*-mediated mammary gland development enriched breast tissue from basal and luminal breast stem cells (161). Aberrant hypermethylation of most CpG regions in tumor tissue, which are normally unmethylated in somatic cells, is also used as a biomarker for early detection of tumor cells. Therefore, there is also an increase in *DNMT3A* and *DNMT3B* expressions, which are responsible for these de novo methylation. Unfortunately, in cancer cells, most oncogene CpG sites are hypomethylated, while tumor suppressor genes are hypermethylated (162-165). In our findings, *DNMT1* expression, which was stated to be necessary for breast CSC maintenance, increased in parallel with pluripotency markers between doses similar as in MSCs (Figure 4.12.). As a result of the application of medium containing 25 and 50 µM methionine, higher expressions were observed in *OCT3/4, NANOG, DNMT1, DNMT3A* and *DNMT3B* expressions compared to other conditions.

Since MAT1A, which is one of the three different isotopes of MAT enzymes that perform methionine-SAM conversion, has liver-specific expression, we preferred to focus MAT2A and MAT2B expressions in this thesis (166). MAT2B, which has a higher affinity for methionine and ATP, synthesizes the MAT2A regulatory protein and regulates its activity (167, 168). It has been shown that MAT2A is cruicial for targeting CSCs to inhibits drug resistance and secondary tumorigenesis (56, 169). In the literature, both inhibition of MAT2A activity and methionine restriction, either separately or combined, demonstrated to decrease mammosphere formation , metastasis and tumor initiation potential of CSCs and induced apoptosis not only in breast tumors but also in stem cells of breast, pancreas and brain tumor types.

According to our data, cells in methionine-depleted medium have approximately 10-fold ($p<0.05$) higher expression of MAT2A compared to CSCs in

the control condition. The condition detected in CSC was also observed in UCB-MSC cells as a 3-fold ($p<0.05$) increase in MAT2A expression in the methionine-depleted condition compared to cells in the medium containing all other doses. The fact that high SAM levels and MAT2A activities were still observed in the methionine-depleted condition could be interpreted as the cycle may have also followed a route to provide Met by Hcy remethylation via MTHFR activity of folate cycle. It has been reported that after methionine restriction in mammary tumour and HepG2 cell cultures increased MAT2A induction and increases its mRNA and protein levels (56, 170). Furthermore, after siRNA-mediated MAT2A silencing, mammosphere formation of CSCs was decreased and cells go to apoptosis (56).

Contrary to MAT2A expression profile in CSC and UCB-MSCs in depleted medium culture, BM-MSCs in the same condition did not show any similar result. On the other hand, the increase in pluripotency markers, DNMT and MAT in BM-MSCs at 100 μM methionine condition was strikingly not observed in other cell groups. The reverse pattern of expression in these cells compared to other groups suggests that BM-MSCs were less sensitive to low methionine concentrations in their culture responded at higher dose (100 μM) in 5h. MAT2B expressions exhibited a similar profile as MAT2A expressions in BM-MSC and UCB-MSCs, depending on methionine dose application. On the other hand, this profile was not observed in CSCs and MAT2B expressions were lower than MAT2A.

Protein isolation was made from UCB-MSC, BM-MSC and CSCs, which were exposed to 0, 10, 25, 50 and 100 μM methionine treatment for 5h and SAM & SAH concentrations were determined by ELISA measurement. At this point, the lowest SAM concentration was detected in BM-MSC cells in the control group (3.7±3.4), while SAM formation was determined to be maximum in culture conditions containing 0 μm (18.8±5.2) and 100 μm (18.8±2.5) methionine (Table 4.4). The increase of SAM levels in BM-MSCs cultured in 100 μM methionine-containing medium were also detected to have high MAT2A expression. This result indicates methionine cycle upregulation at 100 μM methionine supplementation in BM-MSCs.

The increase in H3K4me3 expression levels in BM-MSCs cultured with 0, 50 and 100 μM methionine doses was found as similar as reported by Shiraki et al (14). This result is correlated with the ELISA results that we obtained for SAM/SAH. Since intracellular SAM is the methyl group provider for DNMT and histone methyltransferases, an increase in SAM levels increased H3K4me3 levels along with H3K27me3 levels. The SAM/SAH ratio is also known as the 'methylation index' of the cell, and a decrease in this ratio leads undermethylation of DNA since DNMTs has higher binding affinity for SAH than SAM (8). Measurement of SAM and SAH in UCB-MSC does not offer any informative results. Unlike ESCs, the SAM/SAH ratio of cells at 25 μM concentration in CSCs was found to be lower than at other concentrations (14). Similar to BM-MSCs in 100 μM methionine-containing medium, CSCs in 25 μM methionine condition lowered its H3K4me3 levels in accordance with SAM levels. However, this effect was improved by 50 and 100 μM methionine supplementation to CSC culture medium. Together, this data support that intracellular SAM levels are the regulator of H3K4me3 levels CSCs and BM-MSCs as ESCs. The increase in H3K4me3 and H3K27me3 levels in cells treated with 50 and 100 μM methionine in CSC parallels the increase in SAM. We also determined the effect of SAM, which Shiraki suggested, on histone methylation by measuring protein levels (14).

Recent studies on stem cells have demonstrated the importance of epigenetic control for self-newal and differentiation. For instance, H3K27me3 and H3K4me3 expressions are carried together in the promoter regions of development-related genes by bivalent histone modification in ESCs (171, 172). It can be said that this is necessary for the priming of the cells and to allow for lineage-specific abrupt changes. H3K4me3 generally shows the activity state in the gene regions where it is located and is commonly found in the *OCT4, SOX2* and *NANOG* promoter regions of ESCs (173). In our results, the protein level of H3K27me3 in UCB-MSCs was increased as 4-fold ($p<0.05$) in depleted medium compared to control condition. On the other hand, H3K4me3 protein level was increased in 25 μM methionine condition among other doses. At the same time, the H3K27me3 protein level was increased in UCB-MSCs in depleted and control medium among other conditions.

6. CONCLUSION and FUTURE PERSPECTIVE

This study is a pioneering study showing the changing stemness characteristics depending on methionine in medium of stem cell groups reflecting different developmental stages. Within the scope of this thesis, we aimed to investigate the effect of adding/removing methionine in culture medium. As a conclusion, we obtained the following results:

- It was determined that stem cell groups at different stages did not change their viability depending on time with different doses of methionine.

- Five hour methionine administration, in which metabolic and epigenetic differences can be observed, was chosen for further studies for all cell groups.

- S phase transition is observed in different stem cell groups with different methionine dose applications. However, in general the cells were arrested in the G0/G1 phase for all culture conditions and all cell groups.

- It was observed that BM-MSCs increased all gene expressions, SAM and SAH levels in culture medium containing 100 µM methionine.

- In UCB-MSCs, *OCT3/4, NANOG* and *DNMT1* gene expressions of cells were increased in contrast to decreased MAT2A and MAT2B expression in culture medium containing 10 µM methionine.

- *OCT3/4, NANOG, DNMT1* and *MAT2B* gene expressions of CSCs were increased by 25-100 µM methionine-supplemented condition, and a similar trend was observed in H3K4me3 protein expression as a result of 50 and 100 µM methionine supplementation.

- Intracellular SAM levels were increased approximately 5-fold in BM-MSCs in methionine-supplemented culture condition compared to the control group. In addition, SAM levels were highest after 0 and 100 µM Met administration. On the other hand, after 25 µM met supplementation, CSCs were reduced SAM levels by half compared to other groups.

- This study demonstrates that methionine plays a critical role in metabolism and epigenetic regulation in different stem cell groups. Thus, methionine provides the maintenance of stem cells by regulating gene and protein expression in the cell, especially pluripotency-related genes.

As a future perspective, elucidating the effect of prolonged SAM deprivation by inhibiting MAT2A and MAT2B enzyme activities, which are involved in the methionine cycle, by using antagonist molecules and siRNA-mediated gene silencing methods, will provide more detailed information on the effect of metabolism in these cells. In addition, when the selected dose and time intervals are considered separately for each cell group, it will be possible to detail the effect of 1-C metabolism in these cells. Furthermore, in terms of determining the dose that will keep MSCs in a more potent stage or healthy prolonged culture conditions, comparative analysis by determining the differentiation tendency of MSCs towards adipogenic, osteogenic, chondrogenic lineages and analyzing CpG methylation at promoter regions of specific markers related to pluripotency by bisulfite sequencing method might be open to further investigation. By bisulfite sequencing, the gene and promoter regions of the cells involved in stemness/metabolism should be investigated. In addition, as a result of metabolomic analysis, intercellular differences can be revealed more comprehensively.

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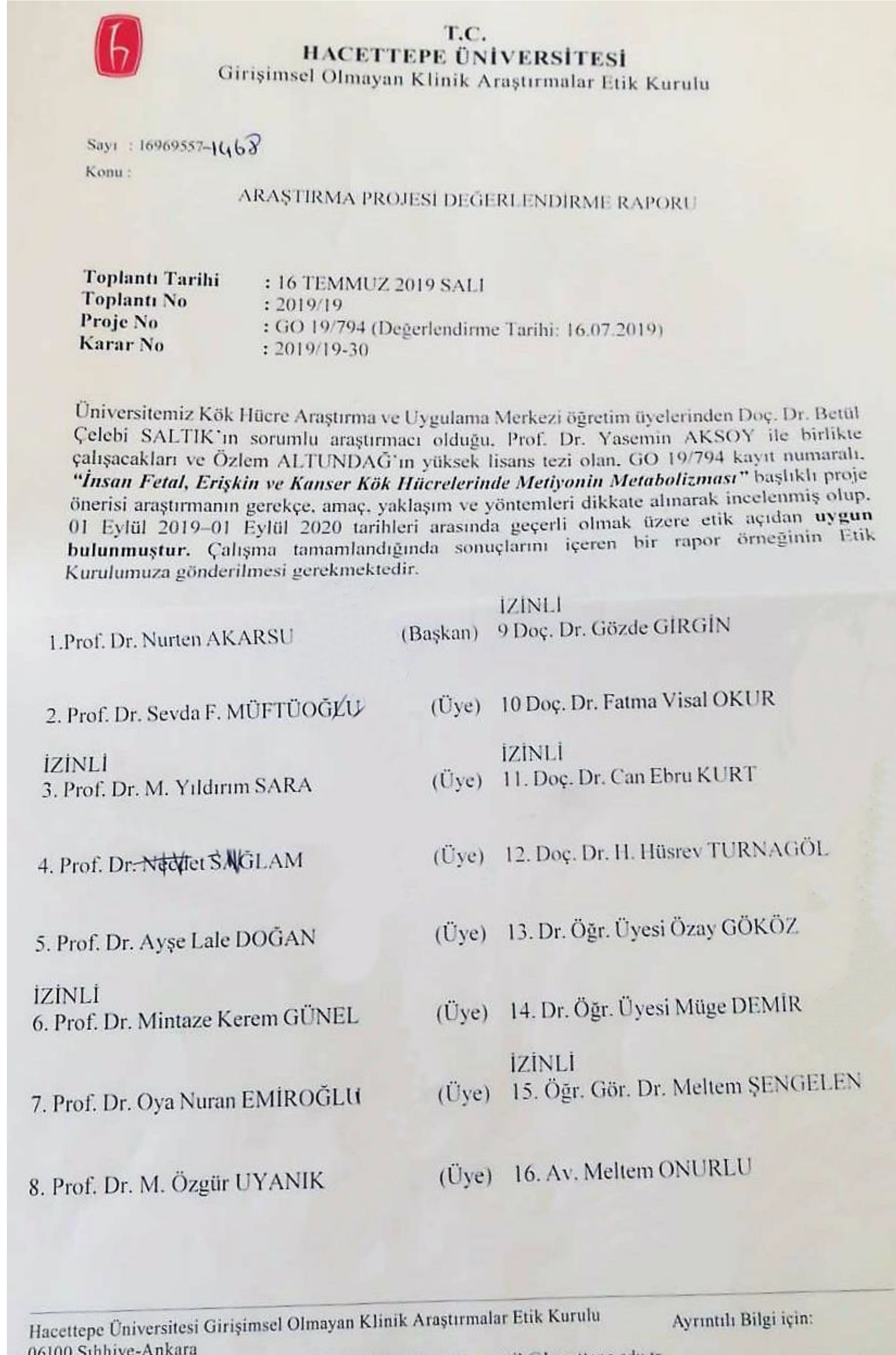
8. APPENDIX

APPENDIX 1. Ethical Approval



T.C.
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ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Toplantı Tarihi : 16 TEMMUZ 2019 SALI
Toplantı No : 2019/19
Proje No : GO 19/794 (Değerlendirme Tarihi: 16.07.2019)
Karar No : 2019/19-30

Üniversitemiz Kök Hücre Araştırma ve Uygulama Merkezi öğretim üyelerinden Doç. Dr. Betül Çelebi SALTIK'ın sorumlu araştırmacı olduğu, Prof. Dr. Yasemin AKSOY ile birlikte çalışacakları ve Özlem ALTUNDAĞ'ın yüksek lisans tezi olan, GO 19/794 kayıt numaralı, ***"İnsan Fetal, Erişkin ve Kanser Kök Hücrelerinde Metiyonin Metabolizması"*** başlıklı proje önerisi araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş olup, 01 Eylül 2019–01 Eylül 2020 tarihleri arasında geçerli olmak üzere etik açıdan **uygun bulunmuştur.** Çalışma tamamlandığında sonuçlarını içeren bir rapor örneğinin Etik *Kurulumuza gönderilmesi gerekmektedir.*

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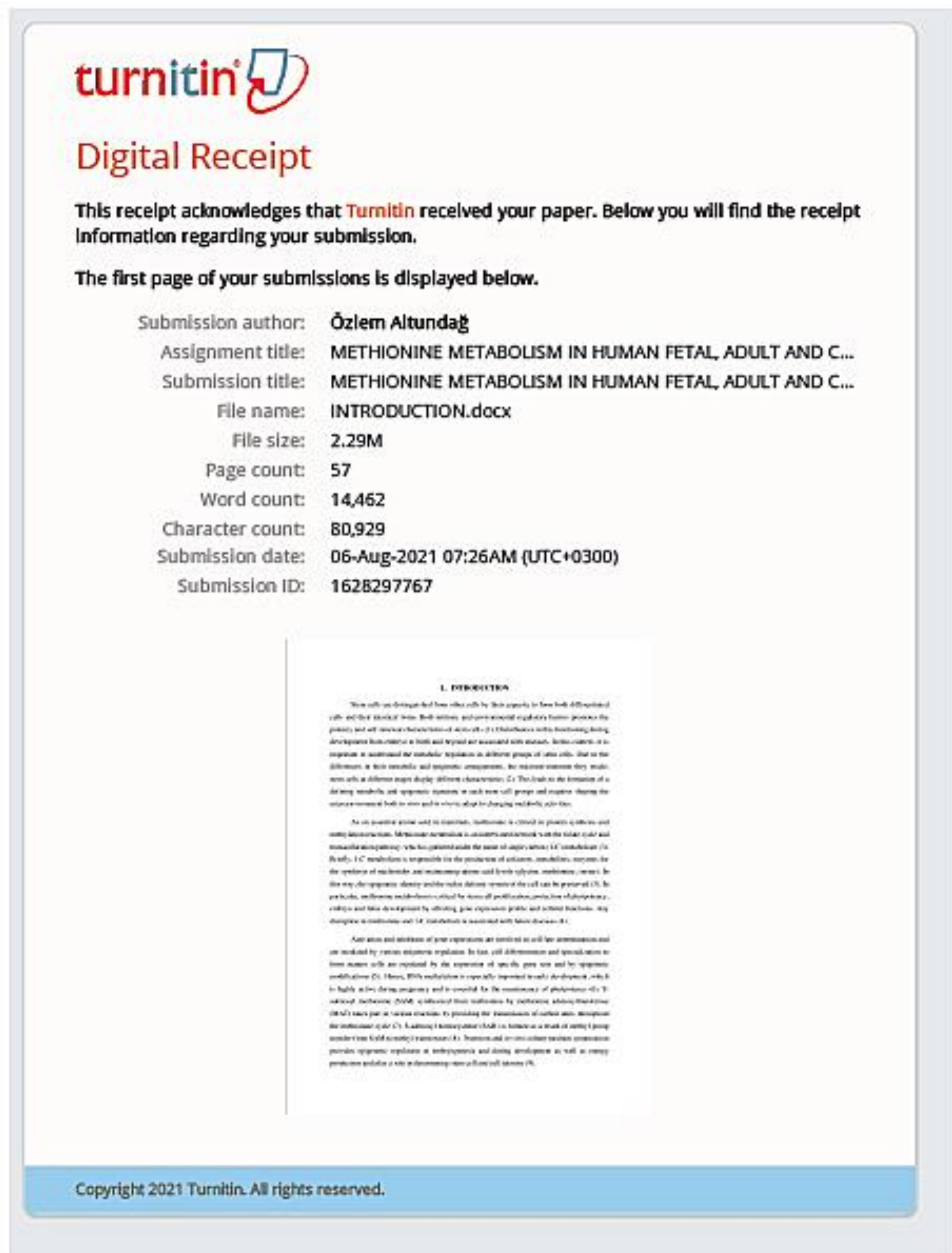

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APPENDIX 3. Originality Report

METHIONINE METABOLISM IN HUMAN FETAL, ADULT AND CANCER STEM CELLS Özlem Altundağ Yüksek Lisans Tezi

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