A New Transcription Factor in Hematopoiesis

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Abstract
Background: Hematopoiesis, is all the cells within the blood system being formed in a specific hierarchy from a small community of hematopoietic stem cells. The aim of this study was to identify the Tbx3 protein levels at stages of physiological development of blood cells in hematopoiesis.

Methods: The isolation of hematopoietic stem cell (HSC) was performed from the umbilical cord blood and cultured for differentiation to myeloid series for 14 days. The expression levels of CD34, CD13, CD45, ALDH (aldehyde dehydrogenase) in hematopoietic progenitor cells isolated from cord blood and myeloid series were checked. The levels of Tbx3 in myeloid series and HSC analyzed with flow cytometry. For statistical analysis student’s t test was used and p values ≤ 0.05 were considered statistically significant.

Results: CD34, CD45 and ALDH levels were high in hematopoietic progenitor cells. After the differentiation CD13 levels increased, whereas CD34, CD45, ALDH levels were decreased. Tbx3 protein was shown in the hematopoietic stem cells obtained from umbilical cord blood and myeloid series cells. There was no significant difference between Tbx3 protein levels of the umbilical cord blood and myeloid series cells.

Conclusion: The presence of Tbx3 transcription factor in hematopoiesis has been shown for the first time with this study.

Key words: transcription factor, Tbx3, cord blood, hematopoiesis, hematopoietic stem cell

DOI: 10.7176/JHMN/75-05

1.INTRODUCTION
Hematopoiesis means that all the cells in the blood system being formed in a specific hierarchy from a small community of hematopoietic stem cells (HSC). In this process, the HSC community primarily forms progenitor cells, later these cells continue to differentiate and specialise to form completely differentiated mature blood cells. Shortly, hematopoiesis appears when the events of cell division and differentiation occur sequentially. Cell division and differentiation takes place with symmetric and asymmetric division. In symmetric cell division, the cells are morphologically and genetically identical cells but in asymmetric division, there are asymmetry in surface molecules, cell organelles, transcription factors, cofactors and genetic material and these are completely different cells [1]. HSC undergoes asymmetric division to renew itself and keep the stem cell pool stable and also it carries out symmetric division to differentiate to form mature cells. Niche determines whether the cell will be divided, differentiated end kept silent. HSC niche’s signalisation network, cytokines and it’s receptors, adhesion molecules, matrix proteins and extrinsic signals work with one another within the intrinsic mechanism to organize the silence of HSC, it’s self renovation, proliferation and differentiation [2, 3]. HSCs
generating healthy blood cells are subject to the physiological control mechanisms working properly. One of these control mechanisms is the "transcriptional control" which enables suitable genes to be identified and regulated at the right time, with an adequate amount and in a sufficient time. The transcription factors, which are the main elements in this control mechanism are proteins that increase or repress gene expression when attaching to a specific DNA index. The activity of the transcription factors involved in stem cell division and differentiation decreases as the degree of differentiation in the cell increases and disappears completely in a mature cell. Therefore, transcription factors involved in embryological development are suppressed in healthy cells of adult individuals. The disappearance or damage of physiological control in the process of hematopoiesis results in the uncontrolled proliferation of cells and specialised cells return to their unspecialised form which then causes cancer to be formed. In previous studies, the peripheral blood and bone marrow samples of patients with different types of haematological cancer were shown to have high levels of Tbx3 named transcription factor. The Tbx3 protein has been found to be increased in not only haematological cancer, but many cancer types [4–9]. Tbx3 has important tasks in the process of organogenesis during the embryonic period. While the TBX3 genes homozygote mutations ended with death for mice, genes homozygote mutations on humans is undefined. Heterozygote mutations in humans can cause breast hypoplasia, lack of apocrine, damage on tooth, hair and genitalia and UMS (Ulnar Mammary Syndrome) that is characterised with damage to front extremities [10,11].

Cancer stem cells are pluripotent stem cells that have the ability to renew themselves and differentiate into different cells at the same time. Cancer stem cells and physiological stem cells are similar in many respects, in particular their transcription factors. These findings show that if Tbx3 is found in cancer cells, it should also be present in physiological stem cells, and as the stem cell differentiates, its amount should decrease. The aim of this study was to determinate the presence of Tbx3 transcription factor in the differentiation process of HSC up to the common myloid progenitor and also to present the Tbx3 protein at which stages it stars and ends.

2. MATERIALS AND METHODS
This study conducted with twenty healthy pregnant women who applied to Akdeniz University, Medical Faculty, Department of Gynaecological Diseases. In accordance with the Helsinki Declaration Rules, this study was submitted to the Clinical Research Ethics Committee of Akdeniz University, Medical Faculty and required permissions were obtained (Decision no: 20.09.2017/554). Individuals who accepted to participate in the study were read the “informed consent form” and their signatures were taken to show their acceptance of participation in the study. 20 bags of 30-60 ml cord blood from medical waste cord tissue was taken to transfer GMP (good manufacturing practice) laboratory, after a total of 20 healthy vaginal/cesarean deliveries. The mononuclear cells (MNCs) were isolated and then frozen in a 5 ml cryotup with a graded freezer (1°C/min). Thawing for cord blood tube is kept in a 37°C water bath for 2 minutes. The cell suspension was centrifuged at 300xg for 10 minutes. Cell pellet was slowly resuspended. For MNC isolation 3 ml of histopaque 1077 was slowly added to the cell pellet then centrifuged at 1100 rpm for 30 minutes. According to the density gradient the mononuclear cells were separated. For hematopoietic progenitor cell isolation target-specific 400 μl mAb coated (CD117) A-pluriBeads® used. Differentiation medium (MethoCult™ H4034 Optimum) and 1000 CD34 + cells were added to per well of a 6 well-plate and incubated for 14 days in a 37 °C, 5% CO2 incubator. Differentiation medium was changed every 3 days.

2.1. Determination of Tbx3 protein level
Isolated cells were identified by CD45 gate and intracellularly labeled with Tbx3 (Anti-TBX3 + DyLight 488 goat anti-mouse) antibody (Table 1). The mean fluorescence concentration (MFI) was assessed in the entire population.

| Tube 1 | Tube 2 | Isotypic control |
|--------|--------|-----------------|
| CD34   | CD13   | IgG1-DyLight488 |
| CD45   | CD45   | IgG1-PE-Cy7     |
| Anti-Tbx3 + DyLight 488 goat anti-mouse antibody | Anti-Tbx3 + DyLight 488 goat anti- mouse antibody | IgG1-APC |
Flow cytometric analyses were performed using the BD Accuri C6 instrument. The Alde Red ALDH detection kit used for characterization of HSC and myeloid series by flow cytometry. ALDH is an enzyme that causes the oxidation of aldehyde to carboxylic acid molecules. This enzyme product retinoic acid and it plays role in maintaining the stem cell feature by changing gene expression [12].

2.2. Statistical analyzes
Data are expressed as mean ± standard Deviation. Student’s t test was used. P ≤ 0.05 were considered statistically significant.

3. RESULTS
The expression levels of CD34, CD13, CD45 and Tbx3 hematopoietic progenitor cells isolated from cord blood checked. The viability of hematopoietic progenitor cells that were isolated from cord blood were analysed. Viability analysis were performed by flow cytometry with 7AAD dye. 7AAD positive cells were evaluated as dead and negative cells as alive (Figure 1a,1b).

![Flow Cytometry Image](image1.png)

**Figure 1.** Flow cytometric viability of the cells
a) dot b) histogram image viability ≥ 98% SSC: side scatter

In order to determine the Tbx3 protein expression in the initial stage of the cells, firstly it was determined immune phenotypically these cells were primarily hematopoietic progenitor cells. The CD34 marker was found to be positive in the hematopoietic progenitor cells (Figure 1b). Tbx3 protein expression MFI values were determined in CD34 + cells (Figure 7 and 8).

Once again at the initial stage, CD13 expression levels which are myeloid serial markers were also evaluated. CD13 expression levels increase during the differentiation process from hematopoietic progenitor to myeloid series [13]. In order to demonstrate this, CD13 expression levels were determined in the initial and subsequent differentiation stages in our study. Very low CD13 positivity was detected at baseline (Figure 2e).

After the determination of the expression levels of CD34, CD13, CD45 and Tbx3, hematopoietic progenitor cells were cultured in myeloid series differentiation medium for 14 days. The expression levels of CD34, CD13, CD45 and Tbx3 were evaluated after the culturing process (Figure 3a-3e). Differentiation from hematopoietic progenitors to myeloid series was demonstrated by decrease in CD34 expression that was parallel to rise CD13 expression. ALDH enzyme levels were found to be high in HSCs and progenitor cells with high self-renewal properties; it has been detected that as the level of self-renewal decreases with differentiation so does ALDH enzyme levels. The transformation in this expression pattern proves that differentiation into myeloid series, in vitro, has been achieved successfully. In addition, colony formation was morphologically detected (Figure 4).
Figure 2. Hematopoietic progenitor CD34, CD45, CD13 and Tbx3 levels
a) FSC/SSC dot plot distribution of isolated cells. b) Histogram image to identify CD34 expression of cells in R2 gate, 99% ≥ CD34+ c) Histogram image to identify CD45 expression of cells in R2 gate, 97% ≥ CD45+ d) Histogram image showing the levels of Tbx3 expression of CD34+ cells (MFI values are given graphically in the text.) e) Histogram image showing CD13 expression levels of CD34+ cells.
Figure 3. CFU-GM CD34, CD45, CD13 and Tbx3 levels
a) FSC/SSC dot plot distribution image of isolated cells. b) Histogram image to identify CD34 expression of cells in R2 gate, 10.1% CD34+ c) Histogram image to identify CD45 expression of cells in R2 gate, 80% ≥ CD45+ d) Histogram image showing Tbx3 expression levels of CD34+ cells (MFI values are given graphically in the text.) e) Histogram image showing CD13 expression levels of cells in R2 gate.
**Figure 4.** Hematopoietic progenitor cells and CFU-GM  
a) macroscopic view b) 4x microscopic c) 10x microscopic d) 40x microscopic.

**Figure 5.** ALDH expression levels  
a) right after isolation b) after culturing in differentiation medium for 14 days.
Figure 6. Tbx3 expression levels
Green line: Initial moment, Red line: Tbx3 expression level at the end of 14th day

Figure 7. Mean fluorescent intensity
Blue line: Initial moment  Red line: Tbx3 protein expression after 14 days (MFI) (p = 0.088)
4. DISCUSSION

Control of the cell cycle transcription factors is vital for the division and proliferation of the HSCs. There are numerous studies that have demonstrated the functions and behaviours of HSCs are regulated by transcription factors. 50 different transcription factors have identified on a key role in hematopoiesis [14]. Shivdashi defined Scl as a key regulatory transcription factor of hematopoiesis [15]. GATA1, GATA2, Runx1, Gfi, c-Myc are just a few of them [16, 23]. The presence of a new transcription factor in hematopoiesis has been shown with this study. Tbx3 protein was shown in the two stages of hematopoiesis that umbilical cord blood derived hematopoietic stem cells and myeloid series cells. Transcription factors are structures with at least two functional sites, a specific DNA-binding site to which they bind to DNA, and the protein site responsible for activation or suppression [24]. Transcriptional regulation is achieved by nucleated cells under physiological conditions being warned by external stimulus and therefore transcription factors coherently working together. In the HSC, the relationship between microenvironment and the transcription factor, especially the regulation of genes responsible for cell cycle on the transcriptional level, carries vital importance. Errors occurring in one or more steps of these regulatory mechanisms lead to the formation of hematological malignancies. Jamieson and Krause et al. defined Tbx3 in hematological malignencies [25, 26]. Tbx3 protein is also involved in hematologic malignancies but its role in hematopoiesis has not been physiologically defined yet. Chapman et al. pointed out that the Tbx3 protein takes on important roles in the development of the nervous system, skeleton, eye, heart, kidney, lung, pancreas and breast tissue in the organogenesis stage of embryonic development [27]. In addition to Jamieson and Krause and Chapman, our study also shows the Tbx3 protein in hematopoiesis and thus we have established a step that can play a role in hematopoiesis.

The mechanism of the formation of diseases that have a direct or indirect relationship with this system cannot be fully understood without to explain this physiological process. Tatyana et al. preferred bone marrow derived HSC, we have been attempted to be clarified the role of Tbx3 in the different stages of hematopoiesis by using cord blood-derived hematopoietic cells. Unlike Tatyana et al. one of the reasons why we prefer cord blood is to exclude the effect of bone microenvironment while other reasons are difficulties in obtaining bone marrow from healthy individuals and ethical concerns [28]. It is known that CD13 expression in the hematopoietic progenitor cell is very slightly expressed and with the myeloid series differentiation this expression increases considerably [13]. Similarly, in our differentiation experiment, it was determined that CD34 expression decreased while CD13 expression increased. The differentiation process of in vitro conditions were supported by immunophenotypical...
methods. The morphological image of the colony forming units in the culture medium and the results of immunophenotyping showed that differentiation from hematopoietic progenitor cell to myeloid series occurred (Figure 4). This result within the scope of this study, provided the opportunity for the expression pattern of Tbx3 protein during the myeloid series differentiation to be evaluated in two different cases, the initial and differentiation stages. As is known, ALDH is a parameter expressed by stem cells and the expression level decreases rapidly during the maturation phase and finally disappears [29]. In our study, it was similarly found that before the differentiation of hematopoietic progenitor cells occurred. ALDH were initially overexpressed, after 14 days of culture, myeloid series differentiation occurred and the expressions were decreased rapidly (Figure 5).

The expression level of Tbx3 protein was determined by flow cytometry at least two different stages of hematopoiesis. Our results should be a new method in this field. Numerical evaluation of CFU-GM colonies using methylcellulose containing semi-solid media is frequently used, particularly in toxicity studies [30]. Efficient isolation and immunophenotyping of differentiated cells of the CFU-GM in semi-solid medium was firstly introduced in this study in order to evaluate the Tbx3 protein. The choice of semi-solid medium in the preparation stage allowed morphological identification of cells. Transcriptional regulation and protein expression may also vary depending on the time and activation state under environmental stimuli. The most appropriate system for this situation is the hematopoietic system and the blood tissue cells that mature by differentiating from this series in the next stage [31]. Changes in structure and function can be observed in certain time periods with internal and external stimuli, both in immune response and in physiopathological processes such as trauma [32]. In our study, Tbx3 expression did not show a statistically significant difference in terms of expression pattern at the beginning and at the end of the 14th day. It is known that Tbx3 protein expression is increased by stimulating different cell lines with Phorbol 12-myristate 13-acetate (PMA) as in vitro [33]. However, in our study, the change in Tbx3 expression due to any stimulus could not be evaluated. The existence of “cancer stem cells” among cancer causing mechanisms has been known for many years [34, 35]. Cancer stem cells are pluripotent stem cells that have the unlimited ability to renew itself and differentiate into different cells. Cancer stem cells and physiological stem cells are similar in many aspects. Since both types of stem cells are cells that are capable of division and differentiation, the signalling pathways and gene products which induce cell division used by these cells show similarities. Therefore, by detecting the Tbx3 expression during differentiation of hematopoietic stem cells, we have taken a step forward in explaining the development of leukemia/lymphoma. However, in order to determine the formation of blastic form in hematologic malignancies and the contribution of Tbx3 expression level in this formation, it is necessary to evaluate Tbx3 level in cells at rest and after maturation.

5. CONCLUSION
In this study, the expression level of Tbx3 protein was determined by flow cytometry at least two different stages of hematopoiesis. We demonstrated the presence of Tbx3 transcription factor for the first time in hematopoietic stem cells obtained from cord blood and myeloid series cells.

Acknowledgement
This work was supported by The Scientific Research Projects Coordination Unit of Akdeniz University (Project Number: TTU-2018-3034).

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