

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Zbtb46 and Runx3 regulated blood cell differentiation from
pluripotent embryonic stem cells

by Pál Botó

Supervisor: István Szatmári PhD



UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR CELL AND IMMUNE BIOLOGY

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By Pál Botó, Molecular biology MSc

Supervisor: István Szatmári, PhD

Doctoral School of Molecular Cell and Immune Biology, University of Debrecen

Head of the Defense Committee:	Gábor Szabó, PhD, DSc
Reviewers:	Melinda Pírity, PhD Endre Kókai, PhD
Members of the Defense Committee:	Ágota Apáti, PhD György Vámosi, PhD

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1. Introduction

Immunotherapy represents a powerful weapon to treat solid tumors and hematopoietic malignancies. Moreover, this approach can be applied to mitigate autoimmune diseases and inflammatory disorders. For example, immune checkpoint inhibitor antibodies can be used to promote the T cell response by antagonizing co-inhibitory signals (e.g., PD1/PD-L1 or CTLA-4). Interestingly, durable immune responses were detected in numerous types of tumors upon the application of these checkpoint blocker antibodies. Moreover, adoptive cell therapies can be applied using tumor-infiltrating lymphocytes or peripheral T cells. These lymphocytes can be engineered to target cancer-specific antigens with T cell receptors or chimeric antigen receptors (CARs). It is worth mentioning that clinical trials with CD19-targeting CAR T cells have shown a 90% remission in patients with acute B lymphoblastic leukemia, indicating the great potential of this cell-based therapeutic approach. More related to this study, that *ex vivo*-produced dendritic cells (DCs) can be loaded with tumor antigens and injected back into cancer patients to stimulate the antitumor immune response. Some of these DC-based vaccines have shown promising clinical results.

DCs are usually generated by *in vitro* differentiation of peripheral blood monocytes for immunotherapy. However, there is a limitation in the number of the harvested monocytes, and the DC-differentiation capacity of these cells varies depending on the blood donor. In contrast to the monocytes, the pluripotent embryonic stem cells (ESCs) can provide an inexhaustible source for immune cell therapies because of their unlimited self-renewal activity and broad differentiation capacity. ESC-derived DCs (ES-DCs) can be made through *ex vivo* differentiation using well-defined protocols. However, it is still challenging to steer the differentiation of ESCs to functional cells because the end products often represent embryonic type or immature cells. In ESC-derived cells the embryonic developmental programs are readily activated, however, these gene regulatory networks usually do not guarantee the formation of fully mature cells. For proper maturation, further steps are needed which are missing from the existing *in vitro* differentiation protocols.

Genetically encoded tools like ectopically-expressed transcription factors allow cell engineers to enhance the maturation of the *ex vivo* generated immune cells. For example, the development of the pluripotent stem-cell-derived blood cells can be enhanced with ectopically expressed transcription factors. In my research project, I investigated the effects of two DC specific transcription factors (RUNX3 and ZBTB46) during the *ex vivo* differentiation of the pluripotent ESCs into DCs.

2. Aims of the study

To improve our understanding on ES-DC differentiation, we carefully examined the efficiency of DC generation from murine ESCs. It was found that ES-DCs showed a lower expression of numerous DC maturation specific markers after LPS treatment compared to bone marrow derived DCs (BM-DCs). Moreover, some DC specific transcription factors were barely detected in ES-DCs. Based on these data we aimed to examine the effect of two important regulators of the DC development, the RUNX3 and ZBTB46 transcription factors in mouse ESC derived DC progenitors.

We intended to assess the developmental and immunological properties of ES-DCs influenced by these factors in phenotypic level and also perform gene expression analysis to uncover the following:

- Characterize the RUNX3 driven ES-DC maturation process
- Reveal the modulatory role of RUNX3 in T cell activation and migratory potential of the ES-DCs
- Assess how ZBTB46 influences the early ESC-mesoderm transition and the myeloid cell development
- Determine how ZBTB46 influences the cell cycle and proliferation of ESCs
- Reveal how ZBTB46 alters the global gene expression pattern of ESC derived progenitors
- Prove the effect of ZBTB46 in the ESC derived erythroid development.

3. Materials and Methods

Embryonic stem cell culture

Mitomycin C (Merck) treated MEF cells as feeders were utilized for the maintenance of the ESCs in knockout DMEM (Thermo-Fisher Scientific) containing 1000 U/ml LIF (Merck), 15% FBS (Thermo-Fisher Scientific), 100 µg/ml streptomycin and 100 U/ml penicillin (Merck).

For expansion and reselection of transgenic cells before differentiation mouse ESCs were maintained without MEF feeder cells in knockout DMEM (Thermo Fisher Scientific) with 15% FBS (Thermo Fisher Scientific), 1000 U/ml LIF (Merck) in presence of 200-300 µg/ml G418 (Thermo Fisher Scientific).

Mesodermal and myeloid differentiation

For cell differentiation an OP9 cell based protocol was utilized. ESCs were cultivated on previously seeded feeder layer of OP9 stromal cells. An α -MEM (Thermo-Fisher Scientific) based 20% FBS (Thermo-Fisher Scientific), 100 U/ml penicillin and 100 µg/ml streptomycin (Merck) supplemented cell culture medium was used for the 5-day mesodermal differentiation. On 5 day differentiated cells were harvested using 0.25% trypsin-EDTA (Thermo-Fisher Scientific), then further cultivated for 3-6 days using fresh OP9 cell layer in α -MEM medium supplemented with 20% FBS, 50 ng/ml GM-CSF (PeproTech) and 50 µM 2-ME (β -mercaptoethanol; Merck).

Embryoid body differentiation (EBD) was assembled using IMDM (Thermo-Fisher Scientific) cell culture medium supplemented with 15% FBS, 200 ng/ml iron-saturated transferrin (Sigma), 4.5 mM monothioglycerol (Sigma Aldrich) and 50 ng/ml ascorbic acid (Sigma). ESCs were harvested and seeded in EBD medium using hanging drop formation and incubated in inverted bacterial culture dishes. At day 2 EBs were harvested and transferred into 6 cm culture dishes using a low-speed orbital shaker to avoid cell adherence to the culture dish. Cells were harvested either at day 4 or day 6. In some experiments the harvested cells were further cultured for 3 days on OP9 feeder layer in 6 well cell culture plates using α -MEM medium supplemented with 20% FBS, 50 ng/ml GM-CSF and 50 µM 2-ME.

Bone marrow cell isolation and differentiation

Mouse BM cells were isolated from 12-week-old male C57BL/6 or 129S1 animals. For BM-DC differentiation 500,000 BM cells were cultivated for 9 days in RPMI medium containing 10 % FBS (Life Technologies), GM-CSF (50 ng/ml) and 2-ME (50 µM) in 6-well tissue culture

plate. To promote DC activation, medium was replaced with fresh RPMI on day 8 and the cells were exposed with 100 ng/ml LPS.

Generation of chemically inducible ESC lines

To generate chemically inducible murine ES cell lines targeting vectors (p2lox) containing the coding sequences of the murine *Irf8*, *Zbtb46*, EGFP or bi-cistronic *Zbtb46*-T2A-EGFP were constructed. To produce chemically inducible cell lines a genetic engineered murine ESC line (ZX1) was utilized. In short, p2lox targeting vector was transfected into ZX1 (genetic background: 129/OlaHsd) ESCs by electroporation. After the electroporation step cells were seeded onto previously prepared geneticin/neomycin resistant MEF (EmbryoMax, Merck) layer, followed by the chemical selection of the resistant cells for 8 days in knockout DMEM containing 300 µg/ml G418.

Characterization of the transgenic ESC lines

To evaluate the inducibility of the generated cell clones mouse ESCs were cultured for 2 days in knockout DMEM (Thermo Fisher Scientific) with 15% FBS (Thermo Fisher Scientific), 1000 U/ml LIF (Merck), 100 U/ml penicillin and 100 µg/ml streptomycin (Merck) and treated with doxycycline 1 µg/ml for 72 hours. Inducibility of the transgene was quantified using quantitative real time PCR or cells were harvested with 0.25% trypsin-EDTA and analyzed with flow cytometry after intracellular ZBTB46 labeling.

mRNA sequencing

Illumina platform was utilized to perform the mRNA sequencing. Total RNA was extracted using TRI reagent. Library preparation was carried out using Ultra II RNA Sample Prep kit (New England BioLabs) using the manufacturer's recommendations. Sequencing runs were performed on Illumina NextSeq 500 using single-end 75 cycles sequencing. Medical Genomics and Bioinformatics Core Facility of the University of Debrecen performed the library preparation, sequencing and basic bioinformatics.

RNA isolation and quantitative reverse transcription polymerase gene reaction (RT-PCR)

For total RNA extractions TRI reagent was used. The reverse transcriptions were carried out with High-Capacity cDNA RT Kit (Thermo-Fisher Scientific). Quantitative real-time PCR reactions were performed using Roche LC480 or LC96 platform with the following conditions: 1 cycle (for denaturation) at 95°C for 60 sec; 40 cycles at 95°C for 10 sec and 60°C for 30 sec using Taqman (Thermo Fisher Scientific) hydrolysis probes. Comparative threshold cycle method was used to determine the relative gene expression levels normalized to ActB.

Western blot

Electrophoretic separation of the extracted proteins from 40,000 cells/sample were carried out using 10% polyacrylamide gel then samples were transferred to PVDF membrane (Pall Corporation). Anti-IRF8 polyclonal antibody (ab245607, 2000x dilution, Abcam) and anti-GAPDH monoclonal antibody (AM4300, 2000x dilution, Thermo-Fisher Scientific) were used to probe the membranes.

Flow cytometry and cell sorting

BD FACS Aria III (BD Biosciences) was used for flow cytometric analyses and cell sorting. The following anti-mouse antibodies were used for staining: CD45-FITC (30-F11), CD11b-BV711 (M1/70), Flk-1(CD309)-BV421 (Avas 12 α 1), MHC2-FITC (I-A/I-E; 2G9), CD80-APC (16-10A1), and CD86-APC (GL1) were purchased from BD Biosciences. F4/80–Alexa Fluor 488 (BM8) Ab was obtained from eBioscience (San Diego, CA). For sorting purposes 1 million cells were harvested and labeled with anti-mouse Flk1 antibody, then 50,000-100,000 Flk1+ cells were sorted. For intracellular ZBTB46 staining Transcription Factor Buffer Set (BD Biosciences) was used per manufacturer's recommendations, staining was carried out using anti-mouse ZBTB46-PE (U4-1374) antibody after surface labeling.

MACS cell separation

Flk1+ cells were purified using CD309 Microbead Kit (Miltenyi Biotec) according to the manufacturer's instructions.

Cell cycle and apoptosis assay

Apoptosis detection assay was carried out using FITC Annexin V Apoptosis Detection Kit with propidium iodide (Biolegend) per manufacturer's instructions. In short, harvested cells were resuspended in Annexin V binding buffer and incubated with Annexin V and propidium iodide, respectively. DNA staining with propidium iodide was utilized to assess the cell distribution in respective cell cycle compartments. Harvested samples were fixed using 70% ethanol then washed and treated with 50 μ l RNase (100 μ g/ml) and 200 μ l propidium iodide (20 μ g/ml) solutions. The samples were analyzed on a BD FACS Aria III instrument.

Hematopoietic colony forming assay

To assess the colony forming potential of the ES derived cells Colony Forming Unit (CFU) assay was utilized using Methocult GF M3434 semisolid medium (Stemcell Technologies). Colonies were identified and counted after 8 days of cultivation with EVOS XL Cell Imaging System (Thermo Fisher Scientific). The following type of blood cell colonies were detected in

the ESC derived progenitors: erythroid, GM (granulocyte and macrophage) and GEMM (granulocyte, erythrocyte, macrophage and megakaryocyte) colonies.

Statistical analysis

Student's t-test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) was used to assess and calculate statistical significance.

DC migration assay

Transwell migration assays were utilized for the assessment of the CCL19 (C-C motif chemokine ligand 19) and CCL21 (C-C motif chemokine ligand 21) chemokine induced DC migration. 24 well plates with 5 μm pore size (Corning-Sigma) were used.

T cell proliferation analysis

T cells were isolated from spleens obtained from 12-week-old male BALB/c mice by the Miltenyi Pan T cell isolation kit II (Miltenyi Biotec). For allogeneic MLR (mixed leukocyte reaction) 10^3 or 10^4 ES-DCs (genetic background: 129/OlaHsd) as stimulator were co-cultured with 10^5 T cells using 96-well round-bottomed culture plates for 5 days. BrdU (5-bromo-2'-deoxyuridine) was added upon the last 12 hours of the culture. The BrdU incorporation was assessed with a Cell proliferation Assay kit (Merck) according to the manufacturer's recommendations.

4. Results

Enhanced DC maturation by RUNX3

To improve our understanding on ES-DC development, my colleagues examined the efficiency of DC generation from mouse ESCs and analyzed the phenotype of the obtained antigen presenting cells. Interestingly, two DC maturation markers (MHCII and CD80) were poorly expressed upon LPS administration in ES-DCs (less than 25% MHCII/CD80+ cells were detected). In contrast, more than 50% percent of MHCII/CD80+ cells were obtained from BM-DCs. In addition, they observed that the LPS-activated ES-DCs showed a heterogeneous CD86 expression, in contrast, most of the LPS-treated BM-DCs were CD86+. Of note, both the CD80 and CD86 are DC specific maturation markers which can provide costimulatory signals necessary for T cell activation and survival. These findings revealed that ES-DCs represent a distinct myeloid cell type with a limited maturation capacity. To further characterize these immune cells the transcript levels of numerous transcription factors in ES-DCs were directly compared to BM-DCs. Analysis of Erika Takács revealed that three genes (*Spi-B*, *Irf4* and *Runx3*) exhibited a significantly lower expression in ES-DCs versus BM-DCs. In line with the transcript profile, detectable level of RUNX3 protein was measured from BM-DCs, in contrast, this transcription factor was barely detected from ES-DCs. This altered gene expression prompted us to test the effects of the RUNX3 transcription factor in ES-DCs and their progenitors using chemically inducible transgenic ESCs.

In this study first, we tested the effect of the transgenic *Runx3* on the later stage of the *ex vivo* DC differentiation. This transcription factor was overexpressed between day 5 and day 19 and cells were also treated with LPS at day 18 to generate mature DCs and to boost their activation. To characterize the maturation capacity of our ESC derived terminally differentiated DCs, we assessed their MHCII and CD80 expression pattern at day 19. In line with our expectations an elevated MHCII/CD80 double positive subpopulation was observed as response to the LPS treatment. Although these cells still showed lower MHCII/CD80 expression compared to the BM-DCs, our findings indicate the enhanced maturation potential of ESC derived DCs, *ex vivo* by RUNX3.

Improved migratory capacity and T cell activation potential of ES-DCs by RUNX3

Next, we tested if RUNX3 can modulate the functional attributes of our ESC derived DCs. We analyzed the CCL19/CCL21 based migratory potential in our cells utilizing Transwell migration assay. Our data showed the migratory potential of the RUNX3 driven ES-DCs was adequate, however our ES-DCs without RUNX3 showed an impaired capacity even in company

with CCL19 and CCL21. As a benchmark we also evaluated the migration capacity of BM derived DCs. Strikingly these cells showed higher level migratory potential even without the addition of the cytokines mentioned above. Our results demonstrated the elevated migration potential of our RUNX3 driven DCs, however the level of this cell function was still below the benchmark level set by BM-DCs.

Finally, we monitored the T cell activation potential of the *Runx3* transgenic ES-DCs by MLR reactions. In these experiments the ES-DCs were co-cultured with T cells possessed different genetic background (allogeneic stimulation). In line with the improved maturation potential, we detected an enhanced T cell proliferation rate as response to *Runx3* induction in ES-DCs. In conclusion, our results suggest that RUNX3 enhances the migration potential and T cell activation capability of the ESC derived antigen presenting cells. Our analysis of the phenotype supported by the evaluation of the DC function showed that *Runx3* overexpression enhances the immunogenic properties of our ESC derived DCs. Our observation that compared to BM-DCs only partial reconstitution of DC differentiation could be achieved prompted us to examine the function of other transcription factors.

ZBTB46 dependent impaired myeloid cell differentiation

Our colleagues have compared the mRNA expression level of 17 different DC specific transcription factors in ES derived *ex vivo* generated DCs. Some of these factors showed highly elevated mRNA expression, other transcripts were however poorly represented. Similar to RUNX3, the cDC marker ZBTB46 was barely expressed in ES-DCs and their progenitors. Therefore, in this study we also aimed to reveal the role of this transcription factor in the ES-DC differentiation using a similar gain of function approach as we applied for the RUNX3 study. Chemically inducible *Zbtb46* expressing ESCs were constructed in a genetically engineered system and several stable *Zbtb46* expressing clones were established. Two ESC clones (C2 and C4) were selected, further expanded and characterized. Our experimental data shows the mRNA expression level of *Zbtb46* highly elevated after 3 days of doxycycline treatment and ZBTB46 was also successfully detected in the protein level in 95% of the induced cells. These together proved the ZBTB46 transcription factor can be uniformly induced in our ESC based transgenic system.

The validation process of the newly generated clones was followed by the assessment of this factor in *ex vivo* ESC differentiation. To obtain ES-DC progenitors we utilized an OP9 cell-based culture method. Overexpression of the transgene started at day 5 and the progenitor cells were harvested in 3 different timepoints: at days 8, day 11 or day 14. Interestingly the

CD45⁺/CD11b⁺ fraction was greatly diminished by ZBTB46 in 8 day differentiated state of our ES derived cells. It is worth mentioning that CD45 is a receptor-linked protein tyrosine phosphatase that is expressed on all leucocytes including myeloid blood cells. In contrast, CD11b is a myeloid cell specific marker. This result suggests the strong repressive effect of ZBTB46 on myeloid commitment. The 11 day differentiated progenitors also showed a lowered myeloid potential upon ZBTB46 however this effect was slightly moderate. The impaired myeloid cell formation was also observed at day 14.

We also tested the effect of ZBTB46 in progenitors derived from EBs. These three-dimensional cell aggregates were grown for 6 days and the disaggregated EB derived progenitors were further cultured with OP9 cells for 3 days. Similar to the monolayer differentiation, the EB derived cells poorly converted to CD11b⁺/CD45⁺ myeloid blood cells in the presence of ZBTB46. These results further support the general myelosuppressive role of ZBTB46 in our murine ES derived transgenic system.

Myeloid lineage suppression by ZBTB46 in Flk1⁺ mesodermal cells

Next, we assessed the early mesodermal development of our *Zbtb46* expressing, transgenic cell lines between day 3-5 of the differentiation. Our results showed introduction of *Zbtb46* resulted in an impaired Flk1⁺ cell formation at day 5 demonstrating the negative impact of this transcription factor on not only the myelopoiesis but also the ESC-mesoderm transition. This observation opened the question if the limited capacity to give rise myeloid cells is a direct effect of the impaired mesodermal cell development. Cells in pre-mesodermal state can possibly exist among our 5 day differentiated cells which cannot transition into Flk1⁺ myeloid precursors. To test these possibilities 5 day differentiated Flk1⁺ cells were purified using MACS cell separation and differentiated for 3 days feederless with the overexpression of our transgenic *Zbtb46*. Strikingly, similar to the cells without magnetic separation the ratio of the CD45 expressing cells were greatly reduced in presence of ZBTB46 in our magnetically separated Flk1⁺ myeloid precursors. It was also concluded the ZBTB46 driven suppression can also be observed in feederless cell cultures, without the use of OP9 cells.

ZBTB46 induced the cell cycle changes in ESCs

Along with the previously uncovered attributes of the ZBTB46 driven ES cell differentiation an altered cell number was also observed as a result of this transcription factor. Lower number of cells were assessed from our differentiated cells between day 0-5 or at day 8 and day 11. This phenomenon was even observed in ESC cultures upon introduction of *Zbtb46*.

These results indicated that ZBTB46 impacts the cell viability or the proliferation capacity of our undifferentiated cells or ESC derived progenitors. To test these possibilities, we determined the rate of cell death in our transgene driven system and also assessed the cell cycle profile to uncover any possible changes indicated by ZBTB46. Strikingly, we observed lower propidium iodide and annexin V positivity in ZBTB46 instructed cells compared to the control (without *Zbtb46* induction), non-differentiated ESCs indicating neither viability nor the formation of apoptotic cells can be accounted for the decreased cell number. Moreover, an altered cell cycle profile was observed upon introduction of *Zbtb46*, the ratio of G0/G1 cells was lowered however the fraction of cells in G2/M phase was elevated in ZBTB46 driven ESCs. In the light of our result, we concluded this delay in the cell cycle process can be accounted for the negative impact on cell proliferation.

Gene expression pattern altered by ZBTB46

In order to reveal the ZBTB46 driven gene expression changes we optimized our protocol to achieve homogenous cell populations. Five day differentiated and MACS purified Flk1+ cells were cultured feederless in presence or absence of doxycycline. We harvested the cells at 24 hours to observe the early effect of ZBTB46 and sampling was also taken place after 72 hours. Genome wide gene expression analysis was performed using RNA sequencing to test 3 biological replicates per experimental condition (total of 15 samples).

First, global gene expression analysis was performed using principal component analysis which showed the 5 day differentiated Flk1+ cells has a unique gene expression pattern compared to the 6 or 8 day differentiated cells. This might be an indication of the moderate effect to the global expression signature by doxycycline (*Zbtb46* induction). In addition, ANOVA test was used on the preselected genes to determine the differential gene expression within the tested 5 experimental conditions. Our analysis revealed 3030 changing genes of the 15371 expressed genes showing a dynamic profile at this differentiation stage. We observed a specific set of genes with increased expression at day 8, however interestingly most of these high expressors were downregulated by ZBTB46. This result was in line with the negative phenotypic effect of the transgene after 3 days of doxycycline administration.

We further analyzed the transcripts repressed by ZBTB46 using a new approach including only the transgenic effect of ZBTB46 in our ESC derived 6 and 8 day differentiated cells. Our analysis showed decreased expression of 726 transcripts in 6 or 8 day differentiated cells instructed by ZBTB46 and more interestingly as our cluster analysis pointed out several of these were involved in both day 6 and day 8. To determine the gene ontology (GO) groups among

these genes, gene set enrichment analysis was performed. Notably, GO categories associated with immune cell were highly represented over other classes. For example, the category “immune system process” include 148 transcripts involving *Itgal* (*DC11a*), *Itgam* (*CD11b*) and *Irf8*. Strikingly all these genes were repressed even more prominently at day 8 suggesting several genes associated with immune and myeloid cell development are negatively regulated in those cells instructed by ZBTB46. Validation of the impaired expression of *Irf8* at day 8 was carried out using quantitative PCR and Western blot with independent sets of samples. In line with our flow cytometric assessments, ZBTB46 greatly repressed the majority of genes involved in the myelopoiesis and our genome-wide transcript analysis served as evidence to the suppressive role of ZBTB46 in the myeloid cell development.

Following these, we assessed those transcript sets with elevated expression level upon ZBTB46 with the help of the same method we used to determine the downregulated genes. We uncovered 361 genes which were positively regulated by ZBTB46, among these entities, our RT-PCR analyses confirmed two genes (*Cyp26b1* and *Ramp3*) which were upregulated in the ZBTB46 induced cells.

Increased erythroid development by ZBTB46 in ESC originated cells

In this study our transcript analysis revealed the inhibitory role of ZBTB46 to the myelopoiesis in ESC based system. ESC derived progenitors can give rise to other blood cell lineages, for examples, these cells have a known potential to erythroid cell formation. Interestingly, our genome-wide analysis highlighted the increased expression of embryonic and adult hemoglobin genes in 6 and 8 day differentiated cells in comparison with the 5-day differentiated Flk1+ purified cells. In addition to this in 8 day differentiated cell we also identified an elevated transcript levels of *Hbb1* and *Hbb2* upon priming with ZBTB46 indicating the effect of this transcription factor to the development of red blood cells. Our extended RT-PCR based analysis uncovered the upregulated state of the adult beta globin gene (*Hbb-b1*) in 8 and 11-day differentiated, ZBTB46 instructed cells as well as EBs and their progenitor cells upon overexpression of *Zbtb46*. Altogether these results indicate the increased expression of the beta globin gene in ZBTB46 instructed ESC derived cells.

Following these assessments, we examined the hematopoietic colony forming potential of these cells generated *ex vivo*. The 8 day differentiated cells were harvested then seeded into Methocult M3434 semisolid medium and further cultured for an additional 8 days. At the end the various blood colonies were identified and counted. Strikingly, regardless whether the presence or absence of our transgene, low number of myeloid (GM) and mixed (GEMM) colonies were

observed. Contrary to this finding an enhanced number of erythroid colonies were detected upon *Zbtb46* induction. Similarly elevated erythroid cell formation was observed in 11-day differentiated cells. Development of mixed colonies (GEMM) was also enhanced after *Zbtb46* induction in case of the 11-day differentiated cells. We also observed an impaired number of myeloid colonies (GM) at day 11 and 14 upon introduction of *Zbtb46*. Altogether these results suggest that overexpression of *Zbtb46* drives the blood cell development from myeloid to erythroid lineage commitment in our ESC derived progenitor cells.

Interestingly, the major regulators of the erythroid differentiation (KLF1, LMO2, GATA1 and LDB1) however were either repressed or remained unaltered in the presence of ZBTB46 suggesting the enhanced erythroid development cannot be interpreted solely as result of the general upregulation of the specific regulatory genes and their products. Instead, we observed enhanced CD105 (Endoglin) expression in both the 8-day differentiated cells and EB derived cells upon priming with ZBTB46. This Endoglin positive population was well separated from the CD45+ cells and these two markers were regulated oppositely by ZBTB46. Being a known marker of erythroid progenitors the enhanced formation of the CD105+ subset can promote the elevated erythroid potential.

5. Discussion

In this study we assessed the developmental potential of two DC specific transcription factors, ZBTB46 and RUNX3 during blood cell differentiation of ESCs. Transcription factor assisted cell engineering is frequently applied to modify the cellular identity. This approach can be used to develop special cells for regenerative medicine or immunotherapies. With transcription factors somatic cell can be dedifferentiated into pluripotent cells (iPSC reprogramming) or transdifferentiated into other cell types. In addition, directed differentiation of PSCs can also be facilitated with transcription factors. In case of directed differentiation one can try to recapitulate the embryonic development *ex vivo* towards the desired *in vivo* functional cell types. Why do we want to enhance the ES-DC differentiation with transcription factors? There are several protocols for DC differentiation from ESCs or iPSCs. These methods employ various cytokines to direct the immune cell formation, especially, GM-CSF are applied to facilitate the myeloid and DC differentiation. Although, it was documented in numerous studies that ES- or iPS-DCs were able to stimulate antitumor immune responses. However, it was also observed that these ESC or iPSC derived DCs possessed a lower T-cell activation potential than the adult progenitor derived DCs. Consistent with these findings our colleagues also found that ES-DCs showed a lower expression of several maturation specific cell surface markers (MHCII, CD80 and CD86) after LPS exposure compared to BM-DCs. These results suggest that some factors are missing from the *ex vivo* generated ESC derived immune cells. Therefore, our colleagues systematically compared the gene expression patterns of numerous DC affiliated transcription factors in ES- and BM-DCs. With RT-PCR analyses Erika Takacs identified three genes (*Spi-B*, *Irf4* and *Runx3*) which were underexpressed in ES-DCs versus BM-DCs. These results inspired us to use chemically inducible ESC lines to test the effect of these three transcription factors in ES-DCs and their progenitors. To achieve this goal our colleagues engineered Runx3, Irf4 and Spi-B inducible ESC clones using a Cre-mediated site-specific recombination. I analyzed the effects of the transgenic *Spi-B* and *Runx3* on ES-DC activation. Interestingly, the mature ES-DC phenotype remained unaltered in the presence of Irf4 suggesting that this factor has a minimal impact on DC activation. On the other hand, an elevated ratio of MHCII/CD80 double positive cells was found in the RUNX3 instructed ES-DCs in response to LPS priming. Moreover, a unique MHCII expressing cell population was also detected even without LPS activation in the RUNX3 programmed cells. Of note, it was published that the MHCII expression is directly regulated by RUNX3. Our results are consistent with this observation because elevated MHCII positivity was detected in the RUNX3 primed

in ES-DCs. However, in our cells the RUNX3-induced phenotypic changes are not confined to the overexpression of MHCII because augmented expression of CD86 was also detected indicating that RUNX3 generally promotes the maturation capacity of ES-DC.

To assess whether RUNX3 can contribute to the functional attributes of our ES-DCs we tested the migratory capacity of the LPS activated ES-DCs using Transwell migration assays. Without *Runx3* induction ES-DCs showed an impaired transmigration even in the presence of CCL19 and CCL21. In contrast, the RUNX3 primed cells exhibited a CCL19 and CCL21 dependent enhanced transmigration. We also examined the T cell activation potential of our RUNX3 instructed ES-DCs using MRL reactions. The allogenic stimulation (our antigen presenting cells were co-cultured with T cells which carry a different genetic background) showed an elevated T cell proliferation rate as a response to RUNX3 induced ES-DCs. These results showed that forced induction of *Runx3* enhanced the T cell activation potential of the ES-DCs. In conclusion, our *ex vivo* analyses revealed that ES-DCs had an inferior migratory and maturation capacity. However, enforced expression of the *Runx3* transgene was sufficient to impart ES-DCs with superior maturation, chemotactic and T cell activation potential. These results suggest that a single transcription factor is enough to improve the ES-DCs maturation and immunogenicity. It is important to mention that adult progenitor derived DCs (BM-DCs) still contained higher percent of MHCII/CD80 and CD86 positive cells and BM-DCs showed a superior migratory capacity than the RUNX3-primed ES-DCs. These results indicated that RUNX3-induced ES-DCs remained less immunogenic compared to the adult DCs. These results prompt us to test additional transcriptional factors and cytokines along with the RUNX3, to further enhance the immunogenicity of the RUNX3-primed pluripotent stem cell-derived DCs.

In the second part of this study, we investigated the effects of the ZBTB46 protein on ESC differentiation. Similar to RUNX3, the classical DC marker *Zbtb46* was barely expressed in ES-DCs and their progenitors this result inspired us to investigate the effect of this transgene. To uncover the role of this factor in ES-DC development we used the same gain of function approach as we utilized for the RUNX3 analysis. We found that ectopic expression of *Zbtb46* invoked a strong repressive effect during the early hematopoietic development. The ESC derived ZBTB46-primed cells inefficiently formed CD45⁺/CD11b⁺ myeloid progenitors suggesting that the myeloid blood cell development was repressed by this transcription factor. It is worth mentioning that some inhibitory functions of the ZBTB46 have already been observed. For example, enforced expression of *Zbtb46* in BM derived hematopoietic progenitors negatively regulated the myeloid granulocyte development and skewed the cell

differentiation towards cDC like cells. Moreover, huge number of monocyte development related genes were upregulated in *Zbtb46* null DCs suggesting that this transcription factor negatively modulates the myeloid/monocytic gene regulatory networks. We also found a reduced myeloid blood cell formation in ESC derived progenitors, however, we did not observe any DC like markers after the overexpression of *Zbtb46*. These results suggest that ZBTB46 can provoke a general inhibitory effect on the myeloid blood cell development but the direction of the alternative cell development is context dependent. We proposed that multiple genes and pathways can be repressed by ZBTB46 during the ESC differentiation. Therefore, we examined the genomic impact of this gene regulatory protein with mRNA sequencing. In agreement with the repressive function, we found that more than 700 genes were negatively regulated upon the forced expression of this transcription factor in the 6- or 8-day differentiated cells. Importantly among these transcripts, several myeloid specific mRNAs were detected, including *Irf8*, *CD14*, and *Itgam* (*CD11b*). It is possible that some of these myeloid specific genes are directly repressed by ZBTB46. Of note, it was already published that numerous myeloid/monocyte specific gene regulatory regions can be occupied with ZBTB46.

Despite of this strong repressive effects, our genome-wide transcriptional analysis highlighted the elevated expression of embryonic and adult hemoglobin genes in 6 and 8 day differentiated cells compared to the *Flk1+* cells differentiated for 5 days. Moreover, elevated level of *Hbb1* and *Hbb2* was observed upon priming with ZBTB46. Consistent with this finding, elevated number of erythroid colonies was detected when we tested ZBTB46 primed 8 or 11 day differentiated cells on a colony forming assay. Together these results indicate that overexpression of *Zbtb46* enhances the erythroid blood cell lineage formation in our ESC based system. Strikingly, the key erythroid regulators (*KLF1*, *LMO2*, *GATA1* and *LDB1*) were either unaltered or repressed by ZBTB46. We concluded that the enhanced erythroid development cannot be explained as a result of the upregulation of the erythropoiesis specific transcription factors by ZBTB46. However, we observed an elevated *CD105* (Endoglin) expression during the early stage of differentiation in the presence of ZBTB46. The *CD105+* cell population was well separated from the *CD45+* population and these two markers are oppositely regulated by ZBTB46. It was described that erythroid progenitors express *CD105* and it was also found that the ESC-derived erythroid development is positively modulated by the forced expression of *CD105*. Therefore, the ZBTB46 dependent expanded *CD105+* cell population might be connected with the enhanced erythropoiesis. Obviously, further studies will be necessary to characterize the ZBTB46 mediated erythroid development in ESC derived differentiated cells.

As a summary, our analysis illustrate that a single transcription factor (ZBTB46) can overwrite the blood cell developmental program. The overexpression of this transcription factor in mouse ESC-derived cells was sufficient to inhibit the myeloid gene regulatory networks and activate the erythroid pathway. The robust impact of ZBTB46 in ESC derived progenitors suggests that it would be interesting to test other members of the ZBTB proteins alone or in combination to facilitate directed differentiation of ESCs in the future. Moreover, our genome-wide gene expression profiling provides a catalogue for exploration of the ZBTB46 regulatory network in ESC derived progenitors.

6. Summary

The *ex vivo* generated monocyte-derived DCs have been often applied for antitumor immunotherapies. Some of these DC-based vaccines have shown promising clinical results. However, there is a limitation in the number of the harvested monocytes, and the DC-differentiation capacity of these immune cells varies. In contrast to monocytes, the pluripotent ESCs could represent an unlimited source for immunotherapies because of their immortality and broad differentiation capacity. However, it is very challenging to drive the ESC differentiation into DCs because the end products are often immature cells with limited immunogenicity. For ES-DC maturation, further steps are needed which are missing from the existing ESC differentiation protocols. We hypothesized that ectopically-expressed DC specific transcription factors can enhance the maturation of these *ex vivo* differentiated immune cells. In our study we examined the effect of the RUNX3 and the ZBTB46 transcription factors during the mouse ES-DC differentiation and concluded the following results:

1. Mouse ESC derived DCs has a limited maturation capacity, but it can be enhanced by the forced expression of *Runx3*. We obtained more MHCII/CD80 double positive cells as well as more CD86+ cells as response to LPS treatment of the RUNX3-primed cells. In contrast, we found that overexpression of *Spi-B* did not alter the ES-DC maturation.
2. In contrast to the enhanced expression of DC maturation related cell surface markers, RUNX3 cannot influence the LPS induced cytokine production of ES-DCs. However, our results showed an elevated migratory potential of the ES-DCs upon introduction of *Runx3*, but this was still below the benchmark level set by BM-DCs. Importantly, we also found that RUNX3 enhanced the T cell activation capacity of our ES-DCs.
3. We also tested the effect of ZBTB46 during the ES-DC differentiation and failed to generate any fully differentiated DCs in the presence of this transcription factor. Instead of we observed that the enforced expression of *Zbtb46* was associated with a profound inhibition of the myeloid blood cell formation during the ESC differentiation.
4. The mesodermal development was also negatively regulated by ZBTB46, moreover, we revealed that ZBTB46 also negatively influence the cell proliferation probably due to the altered cell cycle profile.
5. Consistent with the suppressed myeloid cell differentiation, our global transcript analysis revealed that several myeloid cell specific genes (for example, *Irf8*, *Itgal* and *Itgam*) exhibited an impaired expression in the ZBTB46-programmed cells. In addition, we identified

numerous mRNA transcripts (for example *Cyp26b1* and *Ramp3*) which were upregulated in the ZBTB46-primed differentiated cells.

6. Finally, we observed that overexpression of *Zbtb46* steers the blood cell development from myeloid to the erythroid lineage commitment in ESC derived progenitors. Moreover, we also found that the adult version of the beta globin gene showed an elevated expression in the ZBTB46-instructed ESC-derived progenitors.

In summary, ESC derived DCs had a compromised maturation ability and immunogenicity. However, forced expression of *Runx3* acts as an instructive tool for generation of mature ES-DCs with enhanced immunogenicity. Our findings also illustrate that a single transcription factor (ZBTB46) can overwrite the blood cell development program in ESC derived progenitor: ZBTB46 is sufficient to suppress the myeloid gene regulatory networks and activate the erythroid development.

7. Publication list



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Registry number: DEENK/120/2022.PL
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Candidate: Pál Botó

Doctoral School: Doctoral School of Molecular Cellular and Immune Biology

List of publications related to the dissertation

1. Botó, P., Gerzsenyi, T. B., Lengyel, A., Szúnyog, B., Szatmári, I.: Zbtb46-dependent altered developmental program in embryonic stem cell-derived blood cell progenitors.
Stem Cells. 39 (10), 1322-1334, 2021.
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* These authors contributed equally this work.
IF: 4.539

List of other publications

3. Douida, A., Batista, F., Botó, P., Regdon, Z., Robaszkiewicz, A., Tar, K.: Cells Lacking PA200 Adapt to Mitochondrial Dysfunction by Enhancing Glycolysis via Distinct Opa1 Processing.
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