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TET2 is upregulated during erythroid differentiation of CD34⁺ cells from healthy donors and myelodysplastic syndrome patients

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Abstract

Background: Tet methylcytosine dioxygenase 2 (TET2) is frequently mutated and/or downregulated in myeloid neoplasm, including myelodysplastic syndromes. Despite the extensive studies, the specific contribution of TET2 in disease phenotype of myeloid neoplasms is not fully elucidated. Recent findings have grown attention on the role of TET2 in normal and malignant erythropoiesis.

Methods: In the present study, we investigated *TET2* mRNA levels by quantitative PCR during erythropoietin-induced erythroid differentiation CD34⁺ cells from healthy donor and myelodysplastic syndrome patients. Statistical analyses were performed using the ANOVA and Bonferroni post hoc test and a *p*-value <0.05 was considered statically significant.

Results: *TET2* expression is upregulated during erythroid differentiation of CD34⁺ cells from healthy donor and myelodysplastic syndrome patients.

Conclusions: Our findings corroborate that TET2 is involved in the erythrocyte differentiation.

Keywords: TET2, Tet methylcytosine dioxygenase 2, Myelodysplastic syndromes, Erythropoiesis, Erythroid differentiation

Background

Myelodysplastic syndromes (MDS) are clonal hematopoietic neoplasms characterized by bone marrow dysplasia and peripheral blood cytopenias [1]. In MDS, ineffective erythropoiesis leads to anemia, which has a prognostic impact (e.g. Revised International Prognostic Scoring System [2]). Tet methylcytosine dioxygenase 2 (TET2; also know as Ten-Eleven-Translocation 2) is a methyl cytosine dioxygenase frequently mutated and/or downregulated in myeloid neoplasm, including MDS [3–5]. Experiments in loss-of-function of TET2 using human hematopoietic stem cells and murine models indicate that this protein contributes to myeloid transformation [6, 7]. Despite extensive studies, the specific contribution of TET2 in disease phenotype of myeloid neoplasms is not fully elucidated. Recent findings have

brought attention to the role of TET2 in normal and malignant erythropoiesis [8, 9].

To provide additional evidence of TET2 enrolment in erythropoiesis, we investigate *TET2* mRNA levels in CD34⁺ cells from healthy donor and MDS patients during erythropoietin-induced erythroid differentiation.

Methods

Primary samples

Bone marrow samples were collected from seven MDS patients (refractory cytopenia with multilineage dysplasia *n* = 6 and refractory anemia with ringed sideroblasts *n* = 1, according to the World Health Organization (WHO) 2008 classification [10]). Bone marrow (*n* = 3) or buffy coat preparation from peripheral blood samples (*n* = 6) were obtained from nine healthy donors. The study was approved by the Ethics Committee of the University of Campinas and informed written consent was obtained from healthy donors and MDS patients. Patients' characteristics are described in Table 1.

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Table 1 MDS patients' characteristics

Factors	Number
Gender, n (%)	
Male	6
Female	1
Age y, median (range)	67 (36–75)
Bone marrow blasts (%), median (range)	2.5 (0–4.5)
WHO 2008	
RCMD/RARS	6/1
Karyotype, n	
Normal	7
Peripheral blood counts, median (range)	
Hemoglobin, g/dL	12.8 (7.0–14.2)
Neutrophils, $\times 10^9/L$	1.1 (0.2–1.7)
Platelet count, $\times 10^9/L$	112 (39–259)

Abbreviations: MDS, myelodysplastic syndromes; WHO, World Health Organization; RCMD, refractory cytopenia with multilineage dysplasia; RARS, refractory anemia with ringed sideroblasts

Erythroid cell differentiation

CD34⁺ cells were separated using MIDI-MACS immunoaffinity columns (Miltenyi Biotec, Auburn, CA, USA) and submitted to erythroid differentiation, as previously described [11, 12]. In brief, CD34⁺ cells were plated in methylcellulose medium containing 3 U/mL erythropoietin (EPO), 50 ng/mL stem cell factor, and 30 ng/mL interleukin 3, and cultured for 6 days (cell expansion period). The resulting cells (BFU-E and CFU-E derived colony cells and proerythroblasts) were then transferred to alpha MEM (Gibco BR, Carisbad, CA, USA) containing 30% fetal bovine serum (Sigma, St. Louis, MO, USA), 10^{-5} M 2-mercaptoethanol (Sigma), 2 U/mL EPO, 300 mg/mL holotransferrin (Sigma), and 1% bovine serum albumin (Calbiochem, Darmstadt, Germany) for an additional 6 days (cell differentiation period). Cells were submitted to RNA extraction followed by quantitative PCR (qPCR) on days 6, 8 and 12 or to immunophenotyping on days 6 and 12. The experimental design is illustrated in Fig. 1.

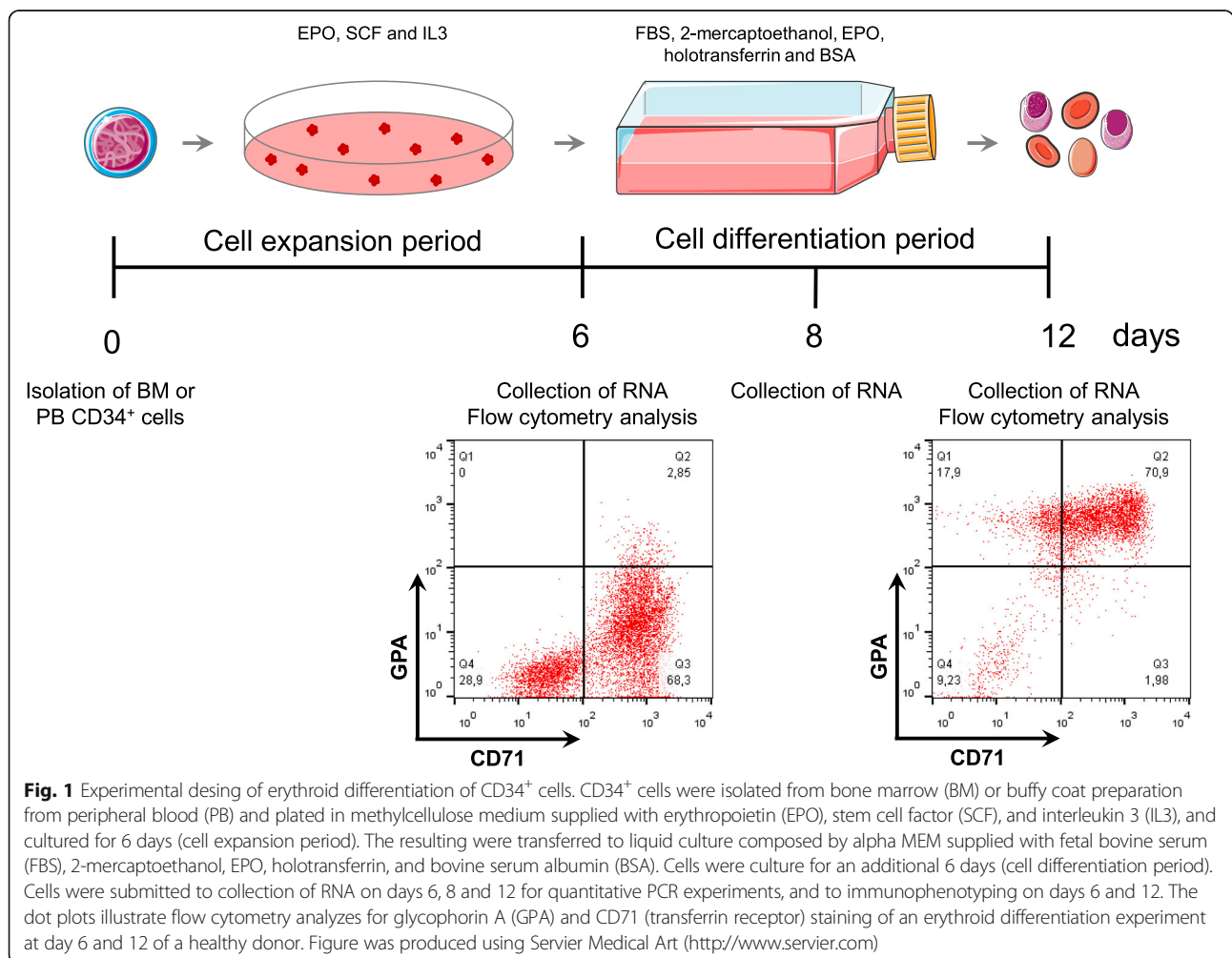


Fig. 1 Experimental desing of erythroid differentiation of CD34⁺ cells. CD34⁺ cells were isolated from bone marrow (BM) or buffy coat preparation from peripheral blood (PB) and plated in methylcellulose medium supplied with erythropoietin (EPO), stem cell factor (SCF), and interleukin 3 (IL3), and cultured for 6 days (cell expansion period). The resulting were transferred to liquid culture composed by alpha MEM supplied with fetal bovine serum (FBS), 2-mercaptoethanol, EPO, holotransferrin, and bovine serum albumin (BSA). Cells were culture for an additional 6 days (cell differentiation period). Cells were submitted to collection of RNA on days 6, 8 and 12 for quantitative PCR experiments, and to immunophenotyping on days 6 and 12. The dot plots illustrate flow cytometry analyzes for glycophorin A (GPA) and CD71 (transferrin receptor) staining of an erythroid differentiation experiment at day 6 and 12 of a healthy donor. Figure was produced using Servier Medical Art (<http://www.servier.com>)

Table 2 Primer sequences and concentrations

Gene	Sequence	Concentration
<i>TET2</i>	FW: 5'-ACGCAAGCCAGGCTAAACA-3' RV: 5'-GCTGGGACTGCTGCATGA-3'	300 nM
<i>HPRT1</i>	FW: 5'-GAACGTCTTGCTCGAGATGTGA-3' RV: 5'-TCCAGCAGGTCAGCAAAGAAT-3'	150 nM

Quantitative PCR

Total RNA was extracted from cells using the illustra RNAspin Mini Kit (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) according manufacture's instruction. The reverse transcription reaction was performed using RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany). *TET2* mRNA level was detected by Maxima Sybr green qPCR master mix (MBI Fermentas) in the ABI 7500 Sequence Detection System (Thermo Fisher Scientific, Fairlawn, NJ, USA) using specific primers. *HPRT1* (*hypoxanthine phosphoribosyltransferase 1*) was used as the reference gene. Primers sequences and concentration are described in Table 2. The relative quantification value was calculated using the eq. $2^{-\Delta\Delta CT}$ [13]. Undifferentiated cells after culture expansion period (day 6) were used as calibrator sample for each experiment. A negative 'No Template Control' was included for each primer pair. The dissociation protocol was performed at the end of each run to check for non-specific amplifications. Three replicas were run on the same plate for each sample.

Statistical analysis

Statistical analyses were performed using the ANOVA and Bonferroni post hoc test and GraphPad Prism 5 software (GraphPad Software, Inc., San. Diego, CA, USA). A p -value <0.05 was considered statistically significant.

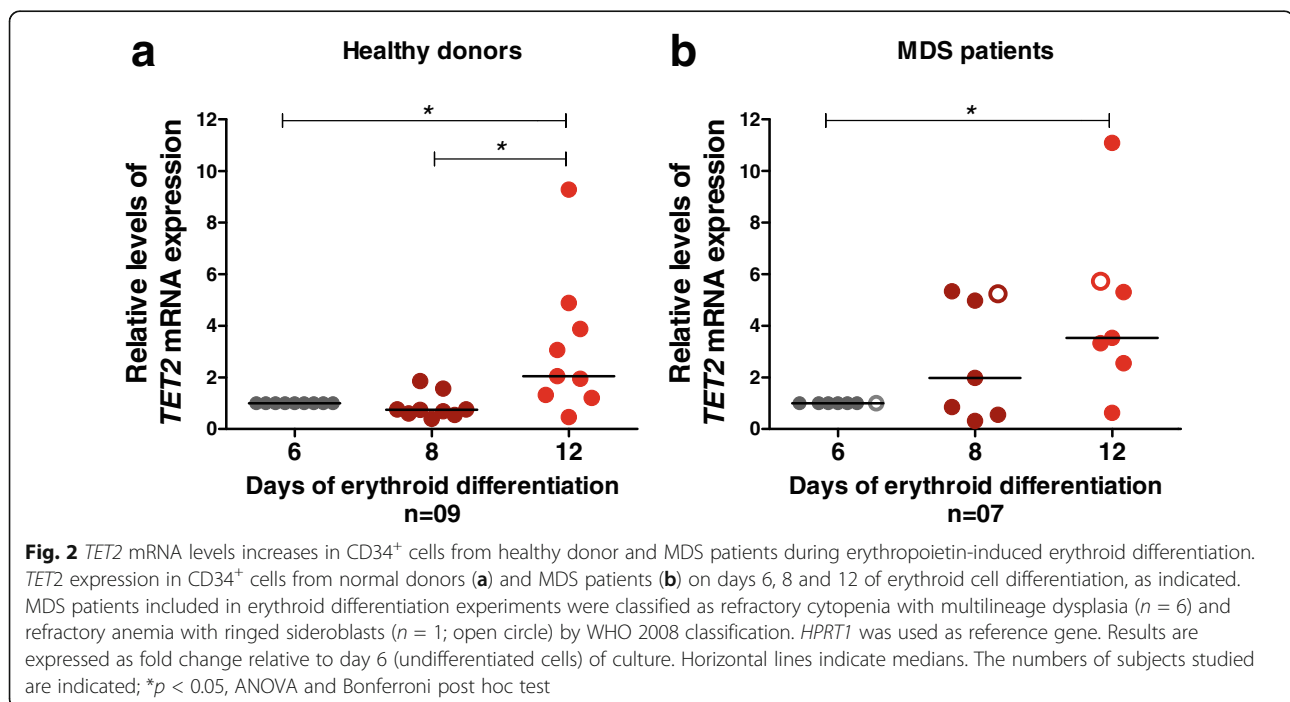
Results***TET2* mRNA levels increases during erythroid differentiation**

In order to provide additional evidence of *TET2* participation during normal and myelodysplastic human erythropoiesis, we investigated *TET2* expression during erythropoietin-induced erythroid differentiation in $CD34^+$ cells from healthy donor and MDS patients. As previously described, the number of cells derived from BFU-E and CFU-E, as well as the pattern of erythroid differentiation did not differ among $CD34^+$ cells from healthy donors and MDS patients [11, 12].

In the present study, qPCR experiments evidenced that *TET2* transcripts were significantly increased on day 12 of erythroid differentiation in normal $CD34^+$ cells (median: 2.05-fold (minimum: 0.47 – maximum: 9.28), $p < 0.05$; Fig. 2a) and in MDS $CD34^+$ cells (median: 3.59-fold (minimum: 0.63 – maximum: 11.09) $p < 0.05$; Fig. 2b).

Discussion

Herein, we demonstrated that *TET2* expression increases during erythroid differentiation of primary hematopoietic progenitors from healthy donors and MDS patients.



Pronier et al. [8] demonstrated that the TET2 silencing increased granulomonocytic differentiation in detriment of erythroid differentiation in normal CD34⁺ cells. Yan et al. [9], similarly demonstrated that TET2 inhibition led to MDS-like dyserythropoiesis. Of note, TET2 deletion leads to erythroid dysplasia and anemia in zebrafish model [14], suggesting a conserved function for TET2 in erythrocytes production.

Recently, Guo and colleagues [15] reported that *TET2* mRNA levels was increased in mature erythroid cells from murine fetal liver, and in MEL and K562 cell lines upon chemically-induced erythroid differentiation. Using functional assays, the authors also established that TET2 plays a cytoprotective function in iron homeostasis against oxidative stress during erythropoiesis. In contrast, Inokura and colleagues [16], using cell sorting of murine bone marrow erythroid populations, observed that *Tet2* mRNA levels is reduced at latter erythroid differentiation stages. However, the researchers observed that *Tet2* knockdown mice present mild normocytic anemia, and downregulation/methylation of heme biosynthesis and iron metabolism-related genes [16], corroborating the hypothesis that TET2 plays a key role during normal erythropoiesis.

Conclusions

Taken together, our findings corroborate the data that *TET2* is involved in erythrocyte differentiation and further mechanistic studies are necessary to elucidate the importance of TET2 for dyserythropoiesis in MDS.

Abbreviations

BFU-E: Erythroid burst-forming units; CFU-E: Colony forming unit-erythrocyte; HPRT1: Hypoxanthine phosphoribosyltransferase 1; MDS: Myelodysplastic syndromes; q-PCR: Quantitative polymerase chain reaction; TET2: Tet methylcytosine dioxygenase 2; WHO: World Health Organization

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Availability of data and materials

Please contact author for data requests.

Authors' contributions

RS-R and JAM-N performed the experiments, statistical analyses, manuscript preparation, completion and final approval. ASSD performed the experiments of erythroid differentiation, manuscript editing and final approval. FFC and STOS participated in revised the diagnoses, patient follow up, manuscript editing and final approval. FT participated in the overall design of the study and experiments, statistical analyses, patient follow up, manuscript preparation, editing, completion and final approval. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the ethics committee of the University of Campinas in accordance with the Helsinki Declaration. Written informed

consent was obtained from all healthy donors and MDS patients who participated in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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