BRIEF COMMUNICATION

TCF4 promotes erythroid development

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Transcription factor 4 (TCF4) is implicated in lymphoid cell differentiation and its expression predicts outcome in acute myeloid leukemia. Here, we investigated the role of TCF4 in myelopoiesis. Overexpression of TCF4 (TCF4^{OE}) in umbilical cord blood (UCB) cells resulted in a twofold increase in erythroid colony forming units (CFU-Es), whereas knock-down (KD) of TCF4 (TCF4^{KD}) caused a dramatic decrease in the number of erythroid colonies. In megakaryocyte CFUs (CFU-MKs), both TCF4^{KD} and TCF4^{OE} inhibited MK colony formation. TCF4 did not have an impact on granulocyte, macrophage, or granulocyte−macrophage colonies or on the proportion of MK−erythrocyte progenitors (MEPs) in culture. Because TCF4 affects erythroid/MK development and these lineages are affected in myelodysplastic syndrome (MDS), we studied the impact of TCF4 expression in this disease. MDS patients with high (≥ median) TCF4 mRNA expression had higher hemoglobin (Hb) levels than MDS patients with low TCF4 expression (mean 9.0 vs. 8.55 g/dL, $p = 0.02$). Overall, TCF4 mRNA expression was lower in hematopoietic stem cells, common myeloid progenitors, and MEPs from MDS patients, but not in granulocyte−macrophage progenitors, compared with healthy controls. Therefore, in cell fractions with erythroid lineage potential, TCF4 is expressed less in MDS patients than in healthy controls. This correlates with the low overall Hb levels seen in MDS patients compared with healthy individuals and is consistent with the positive impact of TCF4 on erythroid development while not having impact on white colonies. These results indicate a role for TCF4 as a novel factor in erythroid−megakaryocytic differentiation. © 2018 Published by Elsevier Inc. on behalf of ISEH – Society for Hematology and Stem Cells.

Transcription factor 4 (TCF4, ITF2, E2-2) is a basic helix−loop−helix (bHLH) transcription factor that belongs to the family of E-box-binding proteins. These proteins recognize CANNTG (Ephrussi box) DNA sequences that are present in a variety of tissue-specific enhancers and promoters. E-proteins are widely expressed and can form both homodimers with other E-proteins or heterodimers with tissue-specific bHLH proteins. Dimerization of bHLH transcription factors results in the formation of a four-helix bundle, which allows the DNA-binding domains to associate with the E-box recognition site to regulate transcription [1]. TCF4 is important in normal development and Tcf4$^{−/−}$ mice die shortly after birth [1,2]. In hematopoiesis, TCF4 is involved in the development of B and T cells and is crucial for the development of plasmacytoid dendritic cells. Recently, TCF4 has been identified as

the master regulator of the oncogenic program in blastic plasmacytoid dendritic cell neoplasm [3]. Furthermore, *TCF4* is mutated (albeit at a low frequency) in acute myeloid leukemia (AML) [4] and myelodysplastic syndromes (MDS) [5,6]. Additionally, high mRNA expression levels of *TCF4* were found to be an independent adverse prognostic factor in AML [5]. The exact role of TCF4 in myelopoiesis, however, is largely unknown. Here, we investigated the role of TCF4 in myelopoiesis.

Methods

Primary cell isolation

Umbilical cord blood (UCB) was obtained after informed consent from healthy pregnant women in the obstetrics department of the Radboud University Medical Center (RadboudUMC) in Nijmegen, The Netherlands. Mononuclear cells were isolated using Ficoll (GE Healthcare) density isolation and CD34+ cells were enriched using magnetic bead isolation (STEMCELL Technologies).

Cloning, lentiviral virus production, and infection

A TCF4 lentiviral overexpression construct was made by cloning TCF4 (DNASU Plasmid Repository, Arizona State University) into pRRL-PGK-IRES-GFP. TCF4 lentiviral KD constructs were made by cloning a TCF4-short hairpin (sh) into a pLKO1 backbone (TCF4^KD1 TTGCCACATTGCTT-CATTAAA;TCF4^KD2 TCTTCGGAGGACAAGAAATTA; nontargeting [NT] CAACAAGATGAAGAGCACCAA). Lentiviral particles were produced by transfection of 293FT cells using calcium phosphate precipitates. Medium was refreshed 16 hours later and the lentiviral supernatants were collected 48 hours after that. KD virus was aliquoted and frozen immediately. To concentrate the overexpression virus, polyethylene-glycol and natrium chloride were added to a final concentration of 5% and 0.15 mmol/L, respectively. Twenty-four to 48 hours later, the overexpression lentiviral particles were spun down at maximal speed for 30 minutes to be used directly or to be stored at −80°C until further use. UCB CD34+ cells were cultured for 48−72 hours in StemSpan ACF medium (STEMCELL Technologies) with recombinant human (rh) stem cell factor (100 ng/μL, Immunotools), rh thrombopoietin (50 ng/μL, Immunotools), rh interleukin-3 (rhIL-3, 10 ng/μL, Immunotools), rh-IL-6 (10 ng/μL, Immunotools), and penicillin−streptomycin (Life Technologies) and subsequently transduced for 72 hours.

Flow cytometry

Immunostaining was performed with phycoerythrin (PE)-conjugated antihuman CD34 (Beckman Coulter) for colony assays. For progenitor staining, Brilliant Violet 421-conjugated anti-CD34 (BioLegend), PE-cy7-conjugated anti-CD38 (Beckman Coulter), allophycocyanin (APC)-conjugated anti-CD45RA (Beckman Coulter), and PE-conjugated anti-CD123 (BD Biosciences) were used. Live cells were gated based on forward and sideward scatter. Cells were analyzed and sorted using a FACSAria flow cytometer (BD Biosciences).

In vitro functional assays

A total of 1000−1500 green fluorescent protein (GFP)/CD34+ double-positive cells were sorted into methylcellulose (Miltenyi Biotec) containing cytokines for colony-forming unit (CFU)-granulocyte, erythrocyte, monocyte/macrophage, megakaryocyte (GEMM) assays or CFU-granulocyte/monocyte/macrophage (GM) assays (STEMCELL Technologies). To the CFU-GM assay, 0, 0.1, 0.5, 1, or 3 units of erythropoietin (EPO)-beta (Neorecormon Roche) was added. For CFU-megakaryocyte (MK) assays, 10,000−15,000 GFP and CD34 positive cells were sorted into Megacult medium (STEMCELL Technologies). After 10−14 days, colonies were counted.

### MDS patients and quantitative reverse transcription polymerase chain reaction

For quantitative reverse transcription polymerase chain reaction (qRT-PCR), RNA of MDS patient bone marrow (BM) samples treated according to the HOVON89 study (EudraCT: 2008-002195-10) were isolated from mononuclear cells after Ficoll (GE Healthcare) density isolation. cDNA was synthesized using reverse transcriptase (Invitrogen). *TCF4* qRT-PCR was performed (forward primer TCCAGGTTTGC-CATCTTCAGT and reverse primer GCCTGGCGAGTCCC-TATTG) using an Applied Biosystems 7500 real-time PCR machine with the 9600 emulation. Measurements were performed in triplicate and Ct values were normalized for input using the GAPDH (Applied Biosystems) housekeeping gene. Ninety-four patients with available hemoglobin (Hb), red blood cell transplantation status, and *TCF4* expression were selected. Hb levels were stratified according to the World Health Organization (WHO) anemia classification [7] as follows: normal Hb is ≥12.0/13.0 g/dL (female/male), mild anemia is 11.0−12.0/13.0 g/dL (female/male), moderate anemia is <11.0 g/dL, and severe anemia is <8.0 g/dL.

Statistics

A Mann−Whitney test was used to assess differences in Hb levels in the different TCF4 expression groups and the difference in TCF4 expression in the MDS samples. Colony data were analyzed using a paired t test.

Results and discussion

To determine the role of TCF4 in myelopoiesis, we overexpressed TCF4 (TCF4^OE) and downregulated TCF4 (TCF4^KD) in UCB-derived CD34+ cells (Supplementary Figure E1A, online only, available at www.exphem.org). TCF4^OE or TCF4^KD had no impact on granulocyte, macrophage, or granulocyte−macrophage (white) colony formation compared with the control (empty vector [EV] or NT sh) (Figure 1A). Interestingly, TCF4^OE enhanced the formation of erythroid colonies by twofold, whereas TCF4^KD inhibited the formation of these colonies by more than fivefold (Figure 1B, mean EV/NT set on 1; TCF4^OE mean 2.05, $p = 0.055$; TCF4^KD1 mean 0.15, $p = 0.033$; TCF4^KD2 mean 0.20, $p = 0.036$) without affecting their size (data not shown). To determine whether TCF4^OE might reduce EPO dependence, we performed CFU-GM
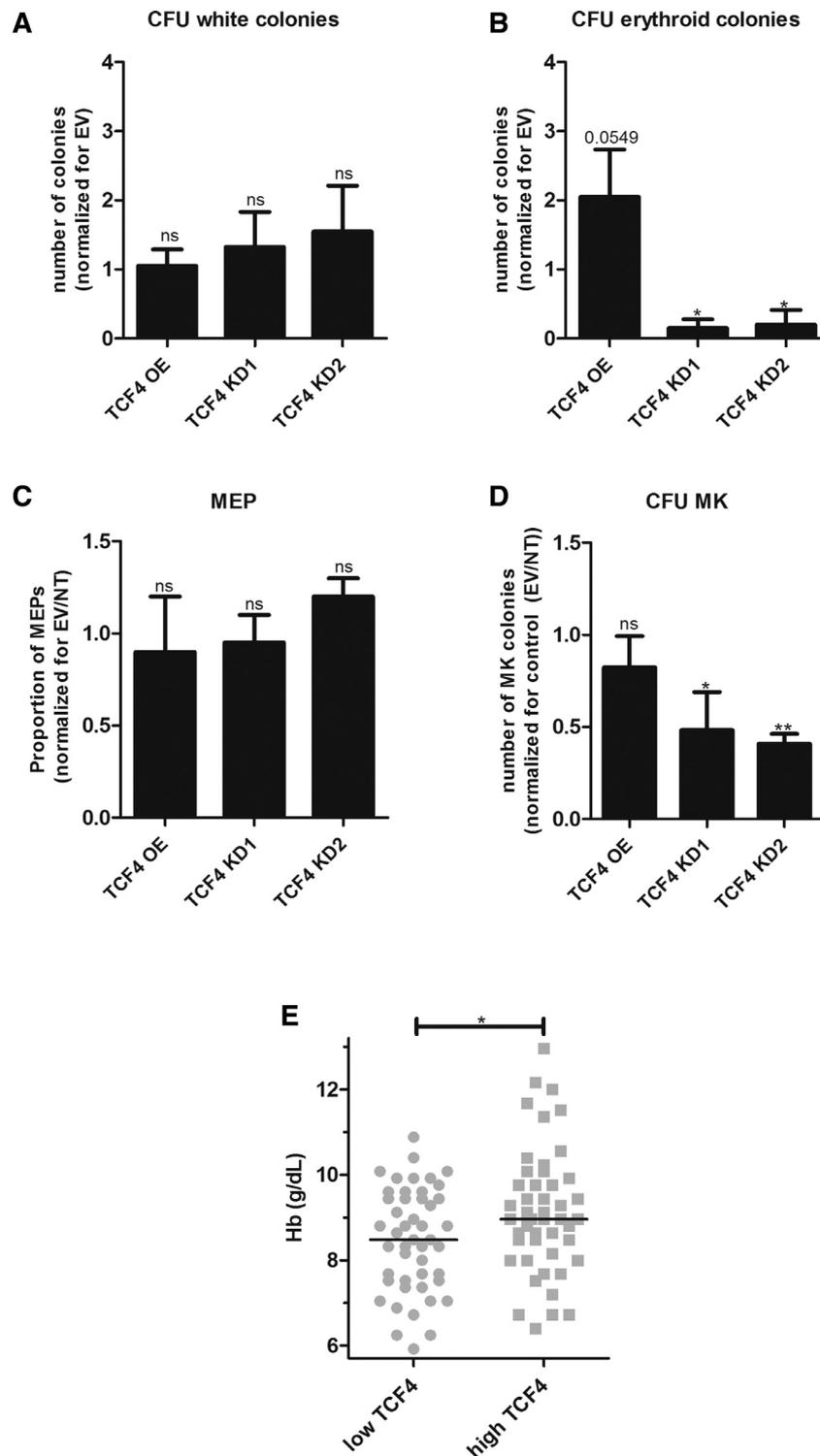
Figure 1. Impact of TCF4^{OE} and TCF4^{KD} on colony formation. The numbers of colonies were normalized to the EV. **(A)** White colonies of CFU-GEMM counted blindly 10−14 days after 1000−1500 GFP$^+$ and CD34$^+$ UCB cells were seeded in methylcellulose ($n = 4$). Paired t test compared the conditions with their matching control (EV or NT sh). **(B)** Erythroid colonies of CFU-GEMM counted blindly 10−14 days after 1000−1500 GFP$^+$ and CD34$^+$ UCB cells were seeded in methylcellulose ($n = 4$). Paired t test compared the conditions with their matching control (EV or NT). **(C)** Proportion of MEPs after transduction of UCB CD34$^+$ cells after 7 days of culture ($n = 3$). **(D)** MK colonies of CFU-MK counted blindly 10−12 days after 10,000−15,000 GFP$^+$ and CD34$^+$ UCB cells were seeded in Megacult medium ($n = 6$). **(E)** Hb levels (g/dL) in MDS patients with low *TCF4* expression ($n = 47$) or high TCF4 expression ($n = 47$). *$p = 0.01−0.05$, **$p = 0.001−0.01$. ns = not significant

colony assays with increasing concentrations of EPO. Without EPO, both the EV-transduced and TCF4^{OE} cells were unable to form erythroid colonies, indicating that TCF4^{OE} did not induce EPO independence. However, in low and higher concentrations of EPO, TCF4^{OE} also enhanced the number of erythroid colonies (data not shown). To further understand whether the increased number of red colonies could be a result of an enhanced expansion of the MK−erythrocyte progenitor (MEP) compartment, we performed immunophenotypical profiling after 1 week of cell culture. Our analyses showed no difference between the control and TCF4^{OE} or TCF4^{KD} in the proportion of cells within the MEP compartment (CD34$^+$CD38$^+$CD123$^-$CD45RA$^-$) compared with the common myeloid progenitor (CMP) compartment (CD34$^+$CD38$^+$CD123$^+$CD45RA$^-$) and the GM progenitor (GMP) compartment (CD34$^+$CD38$^+$CD123$^+$CD45RA$^+$) after 7 days of liquid culture (Figure 1C, Supplementary Figure E2, online only, available at www.exphem.org). Because MEPs have the potential to differentiate into both erythroid and megakaryocytic lineages, we subsequently investigated MK development by performing a CFU-MK assay. Both TCF4^{OE} and TCF4^{KD} in CD34$^+$ UCB hampered MK colony formation compared with their control (Figure 1D, NT set on 1/NT set on 1, mean TCF4^{OE} 0.82, $p = 0.051$; mean TCF4^{KD1} 0.48, $p = 0.049$; mean TCF4^{KD2} 0.41, $p = 0.003$). This suggests that TCF4 does not drive differentiation toward the MEP compartment, but rather enhances differentiation of MEPs toward erythroid differentiation. Apparently, the dosage of TCF4 for MK development has to be regulated very accurately because TCF4^{OE} and TCF4^{KD} both had a negative effect on MK differentiation. These results indicate a role for TCF4 as a novel factor in erythroid−MK differentiation.

To investigate whether the role of TCF4 in erythropoiesis might have clinical value, we measured the TCF4 expression by qRT-PCR in BM samples of 95 MDS patients treated in the HOVON89 protocol. Interestingly, the absolute Hb value was higher in patients with high *TCF4* expression (above median) compared with patients with low *TCF4* expression (Figure 1E, mean 9.0 vs. 8.55 g/dL, $p = 0.02$, respectively). Moreover, of the six patients in this cohort with normal Hb or only a mild anemia, all had a high *TCF4* expression (Supplementary Table E1, online only, available at www.exphem.org). Furthermore, after dividing Hb levels based on WHO guidelines into no, mild, moderate, and severe anemia [7], we found that patients with low *TCF4* expression were twice as likely to have severe anemia compared with patients with higher *TCF4* expression (Supplementary Table E1, online only, available at www.exphem.org, 33% vs. 17% $p = 0.04$, respectively). We did not see a correlation between *TCF4* expression and platelet counts nor absolute neutrophil count (data not shown). To further explore which role TCF4 plays in myelopoiesis in MDS patients, we sorted hematopoietic stem cells (HSCs, CD34$^+$CD38$^-$), CMPs, MEPs, and GMPs from nine MDS patients and seven healthy controls and measured the *TCF4* mRNA expression levels. *TCF4* expression was lower in HSCs, CMPs, and MEPs in MDS patients compared with controls, but not in GMPs (Figure 2, HSC median 0.7 vs. 3.1, $p = 0.091$; CMP median 0.2 vs. 1.2, $p = 0.012$; MEP median 0.2 vs. 3.0, $p = 0.005$; GMP median 0.20 vs. 0.22, $p = 0.607$, respectively). This indicates that, in cell fractions with erythroid lineage potential, *TCF4* is expressed less in MDS patients than in healthy controls. This correlates with the low overall Hb levels seen in MDS patients compared with healthy individuals and is consistent with the positive impact of TCF4 on erythroid colony development.
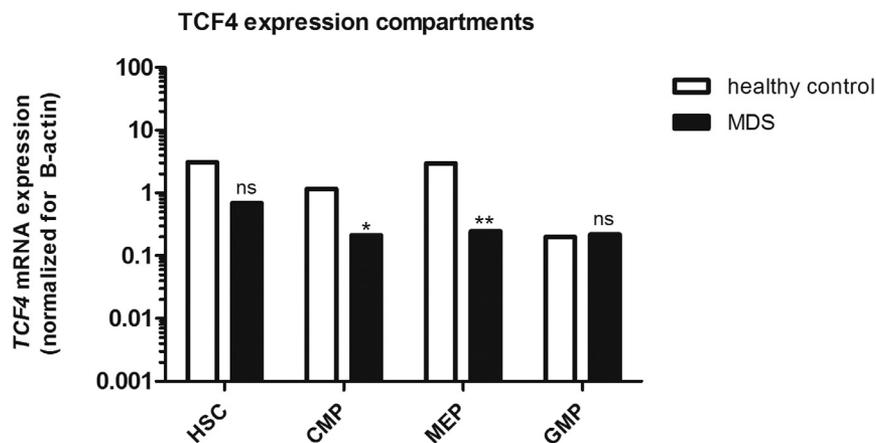
Figure 2. *TCF4* mRNA expression in HSC (CD34$^+$CD38$^-$), CMP (CD34$^+$CD38$^+$CD123$^+$CD45RA$^-$), MEP (CD34$^+$CD38$^+$CD123$^-$CD45RA$^-$), and GMP (CD34$^+$CD38$^+$CD123$^+$CD45RA$^+$) compartments of MDS patients ($n = 9$) compared with healthy controls ($n = 7$). ns = not significant. *$p = 0.01−0.05$, **$p = 0.001−0.01$.

Although TCF4 has not been previously implicated in the regulation of erythropoiesis, other bHLH proteins have [8]. For example TAL1 (SCL), a hematopoiesis-specific bHLH protein, has been shown to play a fundamental role in the regulation of erythroid and MK-specific gene expression programs [8−10]. TAL1 participates in the erythropoiesis-stimulating multiprotein complex with GATA1, LMO2, and LDB1. This complex binds DNA elements containing an E-box and a GATA-specific site separated from each other by nine to 10 base pairs [9,11]. In this multiprotein complex, TCF3 is commonly identified as the DNA-binding E-protein [8,9,12], but TCF4 has also been reported as one of the proteins in this complex [13,14]. Furthermore, direct binding of TAL1 and TCF4 has been reported [15] and enforced expression of TAL1 alone also enhances erythroid differentiation [16]. Possibly, the enhancing effect of enforced TCF4 expression on erythroid colony formation could be explained by the interaction of TAL1 and TCF4. Alternatively, TCF4 could enhance erythroid differentiation through one of its downstream targets such as BCL11a. BCL11a is a major determinant in fetal-to-adult Hb switching [17−19] and has been identified as a direct downstream target of TCF4 [3,20]. Further, $Bcl11a^{cko/cko}$ mice have a mild anemic phenotype [21]. Enforced expression of TCF4 in UCB CD34$^+$ cells could lead to more effective erythropoiesis due to upregulation of BCL11a. Interestingly, BCL11a has also been suggested as a downstream target of TAL1 [22], which makes cooperation between both suggested mechanisms possible. These data suggest that TCF4 plays a role in erythroid −MK differentiation.

Acknowledgments

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Supplementary data
Figure E1 and E2; Table E1

A.
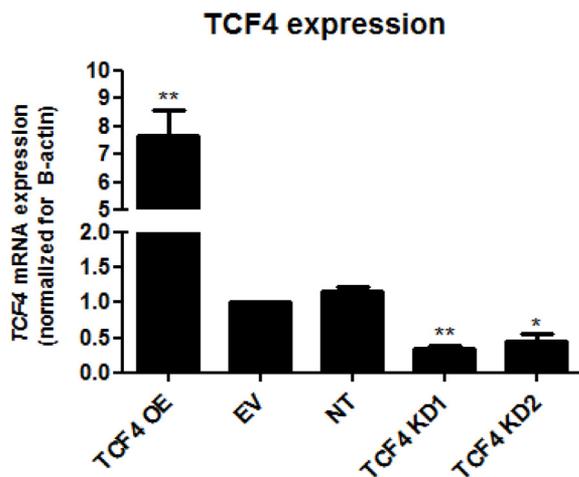
TCF4 expression

Figure E1. TCF4 mRNA expression of harvested CFU-GEMM colonies (n=2)

B.
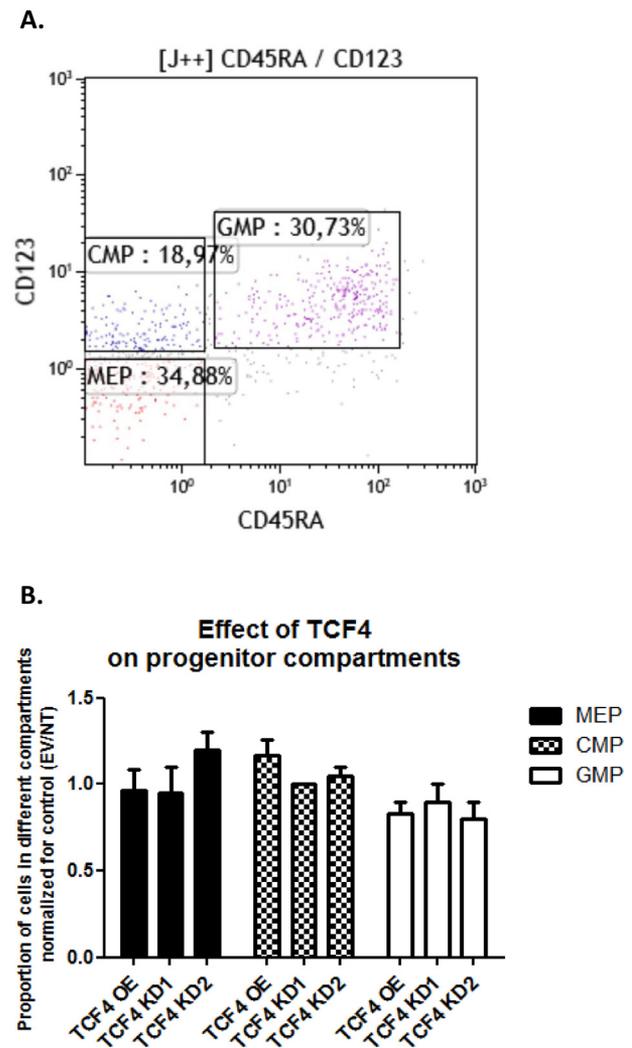
Figure E2. A. Example of flow cytometry dotplot of CD45RA and CD123, gated on GFP$^+$ and CD34$^+$/CD38$^+$ cells. **B.** Proportion of CD34+/CD38+ cells in different compartments normalized for control (EV for TCF4^{OE} and NT for TCF4^{KD}) (n=3 for TCF4^{OE}; n=2 for TCF4^{KD})

Table E1. Percentage of patients with normal, mild, moderate and severe anemia in low and intermediate-I MDS patients with low and high *TCF4* expression levels.

	Normal Hb	Mild anemia	Moderate anemia	Severe anemia	p-value
low *TCF4*	0 (0%)	0 (0%)	32 (67%)	16 (33%)	0.035
high *TCF4*	2 (4%)	4 (9%)	32 (70%)	8 (17%)	