Stem Cell Research 16 (2016) 677–681

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

Short report

GABP is necessary for stem/progenitor cell maintenance and myeloid differentiation in human hematopoiesis and chronic myeloid leukemia

Georgi Manukjan [a,*,1], Tim Ripperger [a], Letizia Venturini [b], Michael Stadler [b], Gudrun Göhring [a], Axel Schambach [c], Brigitte Schlegelberger [a], Doris Steinemann [a]

[a] Institute of Human Genetics, Hannover Medical School, Hannover, Germany
[b] Department of Hematology, Hemostasis, Oncology, and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany
[c] Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany

ARTICLE INFO

Article history:
Received 20 November 2015
Received in revised form 16 March 2016
Accepted 7 April 2016
Available online 12 April 2016

Keywords:
GABP
Transcription factor
Hematopoietic stem cell
Leukemic stem cell
CML/chronic myeloid leukemia

ABSTRACT

Maintenance of hematopoietic stem cells and their potential to give rise to progenitors of differentiated lymphoid and myeloid cells are accomplished by a network of regulatory processes. As a part of this network, the heteromeric transcription factor GA-binding protein (GABP) plays a crucial role in self-renewal of murine hematopoietic and leukemic stem cells. Here, we report the consequences of functional impairment of GABP in human hematopoietic and in leukemic stem/progenitor cells. Ectopic overexpression of a dominant-negative acting GABP mutant led to impaired myeloid differentiation of $CD34^+$ hematopoietic stem/progenitor cells obtained from healthy donors. Moreover, drastically reduced clonogenic capacity of leukemic stem/progenitor cells isolated from bone marrow aspirates of chronic myeloid leukemia (CML) patients underlines the importance of GABP on stem/progenitor cell maintenance and confirms the relevance of GABP for human myelopoiesis in healthy and diseased states.

1. Introduction

Transcriptional regulation is of particular relevance for self-renewal of hematopoietic stem cells and accurate hematopoietic differentiation. The potential to give rise to mature hematopoietic cells as well as to preserve stem cell properties is regulated by a set of transcription factors, of which only few are well-characterized (Orkin and Zon, 2008). The ETS transcription factor GA-binding protein (GABP) was recently shown to play a crucial role for myeloid differentiation in mice and humans (Yang et al., 2011; Yu et al., 2011; Ripperger et al., 2015). Moreover, GABP was reported to directly impact propagation of leukemic clones in mice transplanted with BCR-$ABL1^+$ murine leukemic stem cells, which resemble human chronic myeloid leukemia (CML) (Yu et al., 2012; Yang et al., 2013). Our recent work supported these observations by showing that GABP affects viability and imatinib sensitivity in human CML cell lines (Manukjan et al., 2015).

Here, we focus on the effects of GABP in human normal and leukemic hematopoietic stem/progenitor cells and demonstrate that GABP is required for proper myeloid differentiation in human primary cells. GABP functions as an obligate heterodimer, in which the alpha-subunit (GABPα) binds targeted DNA-motifs and the beta-subunit (GABPβ1) contributes to transcriptional regulation via its transactivation capacity (Rosmarin et al., 2004). Hence, ectopic overexpression of GABPβ1 lacking the transcriptional activation domain (TAD) results in a dominant-negative GABP, as we show here and have already demonstrated in the preliminary work (Manukjan et al., 2015). In this context, we further show that impaired GABP decreases leukemic stem/progenitor cell capacity of human $CD34^+$ cells derived from CML patients.

2. Material & methods

Mobilized $CD34^+$ cells were purified from peripheral blood leukapheresis material from three individuals in hematological remission after treatment for acute myeloid leukemia (AML). Bone marrow $CD34^+$ cells were obtained from healthy donors. Bone marrow $CD34^+$ cells from five CML patients (three females; two males; age range at diagnosis: 44–87 years) in chronic phase were obtained at the time of diagnosis. Patients did not receive tyrosine kinase inhibitor therapy prior to sampling. Diagnosis of CML was confirmed by standard cytogenetic and molecular genetic analyses to detect translocation t(9;22)(q34;q11) and detection and quantification of p210 BCR-ABL1 fusion transcripts as described previously (Emig et al., 1999; Hughes et al., 2006). The investigation was approved by the Hannover Medical

* Corresponding author at: Institute of Human Genetics, Hannover Medical School, Carl-Neuberg-Str. 1, 30625, Hannover, Germany.
E-mail addresses: manukjan.georgi@mh-hannover.de, manukjan_g@ukw.de (G. Manukjan).
[1] Present address: Department of Experimental Biomedicine, Experimental Hemostaseology, University Hospital of Würzburg, Josef-Schneider-Str. 2, 97080, Würzburg, Germany.

http://dx.doi.org/10.1016/j.scr.2016.04.007

School Ethics Committee (No. 2899) and written consent was obtained from each patient in accordance with the Declaration of Helsinki. In all cases, $CD34^+$ cells were purified by magnetic separation according to the manufacturer's recommendations (Miltenyi Biotec, Bergisch Gladbach, Germany).

Cultivation, transduction and chemical treatment (DMSO and imatinib) of K-562 cells (ACC-10, DSMZ, Braunschweig, Germany) were performed as described earlier (Manukjan et al., 2015).

The pRSF91.IRES.dTomato.pre* vector was used to ectopically overexpress *GABPB1*.ΔTAD, following published protocols (Ripperger et al., 2015). $CD34^+$ cells were transduced with VSV-G pseudotyped retroviral particles using a RetroNectin protocol (Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) and a multiplicity of infection (MOI) of 50. Quantitative RT-PCR was performed using the QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Hilden, Germany) under standard conditions. Relative expression was calculated by the $\Delta\Delta C_t$ method in correlation to *SDHA*. Primer sequences are as follows: *GABPB1* (forward: 5′-GGT CAA GAT GAT GAA GTT CG-3′; reverse: 5′-CTG GCA TCT CTG CTC ACA C-3′); *SDHA* (forward: 5′-GCC ATC CAC TAC ATG ACG-3′; reverse: 5′-TCC ATA TAA GGT GTG CAA TAG C-3′).

Following fluorescence activated cell sorting (FACS) for dTomato-reporter expression, HSPCs were maintained for ten days in liquid culture using StemSpan medium (StemCell Technologies, Grenoble, France) supplemented with 1% penicillin/streptomycin, 1% L-glutamine (Biochrom AG, Berlin, Germany) and the following recombinant human cytokines: 100 ng/ml M-CSF, 50 ng/ml FLT3-L, 20 ng/ml SCF, and 20 ng/ml IL-6 (all PeproTech, Hamburg, Germany). For flow cytometry, the following antibodies were used: anti-human CD11b APC (ICRF44) (eBioscience, Frankfurt am Main, Germany); anti-human CD14 APC (555399) (BD Pharmingen, Heidelberg, Germany). Colony formation assays were performed two days after transduction with 5000 FACS-purified reporter-positive cells per 35 mm^2 culture dish using MethoCult™ H4230 methylcellulose (StemCell Technologies) supplemented with 1% penicillin/streptomycin (Biochrom AG), as well as a recombinant human cytokine cocktail containing 100 ng/ml SCF, 20 ng/ml G-CSF, 20 ng/ml GM-CSF, 20 ng/ml IL-3, and 20 ng/ml IL-6 (PeproTech). Granulocyte/macrophage colony-forming units (CFU-GM) were determined after 14 days of cultivation under standard conditions.

3. Results & discussion

To study the impact of GABP during myeloid differentiation and self-renewal of primary human hematopoietic stem/progenitor cells (HSPCs), GABP function was impaired by ectopic overexpression of a GABPβ1 subunit lacking the TAD (*GABPB1*.ΔTAD, shown schematically in Fig. 1A) (Ripperger et al., 2015). *GABPB1*.ΔTAD ectopic overexpression was performed since knockdown efficiencies of shRNAs targeting GABP subunits were not sufficient in HSPCs (data not shown). Initially, effects of *GABPB1*.ΔTAD ectopic overexpression were studied in the $BCR\text{-}ABL1^+$ K-562 cell line in combination with imatinib treatment to confirm the dominant-negative action of the mutant protein (Fig. 1B). A similar proliferation behavior in terms of elevated sensitivity to imatinib upon *GABPB1*.ΔTAD ectopic overexpression was observed as compared to our previous investigations applying shRNA-mediated knockdown of *GABPA* in K-562 cells (Manukjan et al., 2015). Hence, the approach is feasible to be used in primary HSPCs particularly as overexpression efficiency could be proven by qPCR (Fig. 1C).

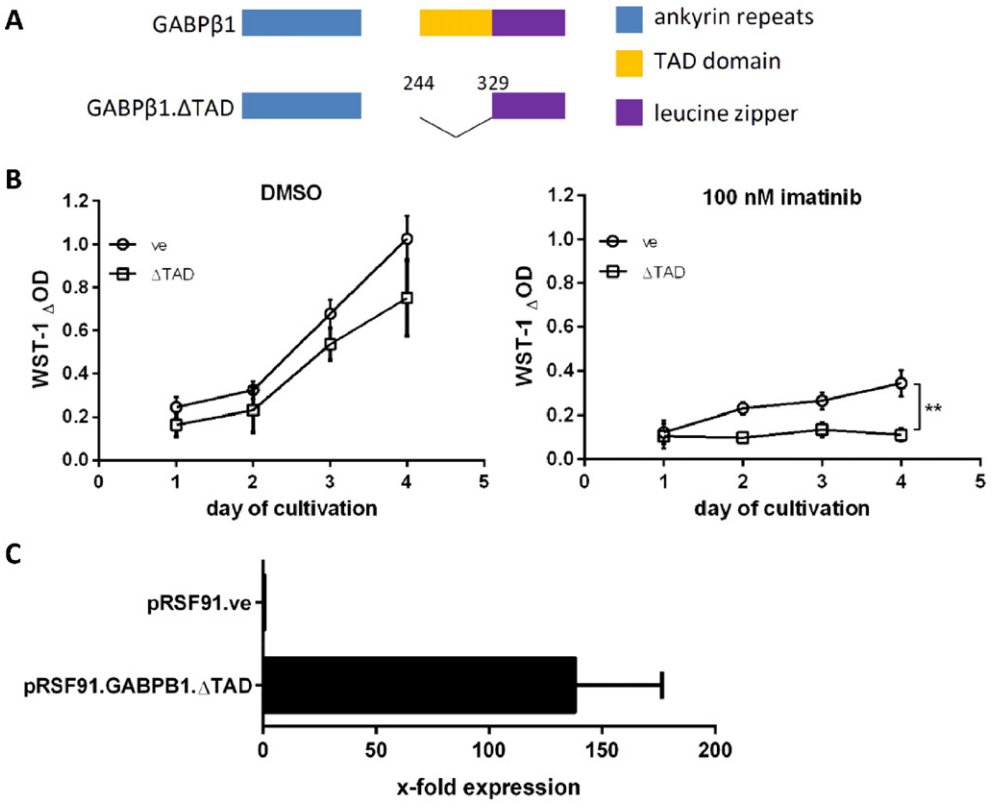

Fig. 1. *GABPB1*.ΔTAD acts as dominant-negative mutant. (A) Schematic representation of GABPβ1 subunit domains. The transactivation domain (TAD) of *GABPB1* subunit, transcript variant beta-2 (NM_016654.4) was deleted by restriction digestion to create the *GABPB1*.ΔTAD construct (bottom) used for ectopic overexpression. Numbers indicate amino acid residues. (B) WST-1 proliferation assay on K-562 cells. Cells were FACS-purified for dTomato expression upon transduction with the ectopic overexpression vector (pRSF91.IRES.dTomato.pre*) either as empty vector control (ve) or containing a *GABPB1*.ΔTAD (ΔTAD) cDNA cassette and subsequently treated with 100 nM imatinib or 0.1% DMSO as solvent control. The WST-1 assay was performed every 24 h. The background-corrected optical density ($_\Delta$OD) is plotted over time (mean ± s.d., $n = 3$; unpaired t-test; $^{**}P < 0.01$). (C) Quantitative PCR results of three independent experiments performed two days after transduction of HSPCs comparing *GABPB1*.ΔTAD with the empty vector control (pRSF91.ve) (mean + s.d.). Primers allow amplification of both, endogenous wild-type *GABPB1* and ectopically expressed *GABPB1*.ΔTAD.

Subsequently, liquid culture assays on mobilized peripheral blood (mPB) CD34$^+$ cells were performed after transduction with the empty vector control or *GABPB1*.ΔTAD. CD34$^+$ cells were achieved by leukapheresis of AML patients at remission. Upon transduction with *GABPB1*.ΔTAD, CD11b and CD14 expressions were significantly decreased in mPB CD34$^+$ HSPCs after ten days in liquid culture containing a myeloid cytokine cocktail (Fig. 2A). CD11b and CD14 are defined cell-surface markers of the mature myelo-monocytic compartment. The significant reduction of CD11b$^+$ and CD14$^+$ cells indicates that functional GABP is necessary to perform proper myelopoiesis in human CD34$^+$ HSPCs. In this context, GABP was recently shown to directly regulate expression of *ITGAM* (integrin alpha M), which codes for CD11b (Ripperger et al., 2015). Remarkably, after 14 days in liquid culture containing a myeloid cytokine cocktail, fewer total cell numbers were detected in the *GABPB1*.ΔTAD group (Fig. 2B). This confirms the lowered myelopoietic potential of mPB CD34$^+$ HSPCs with functionally impaired GABP. In addition, we performed colony formation assays focusing on granulocyte/macrophage (GM)

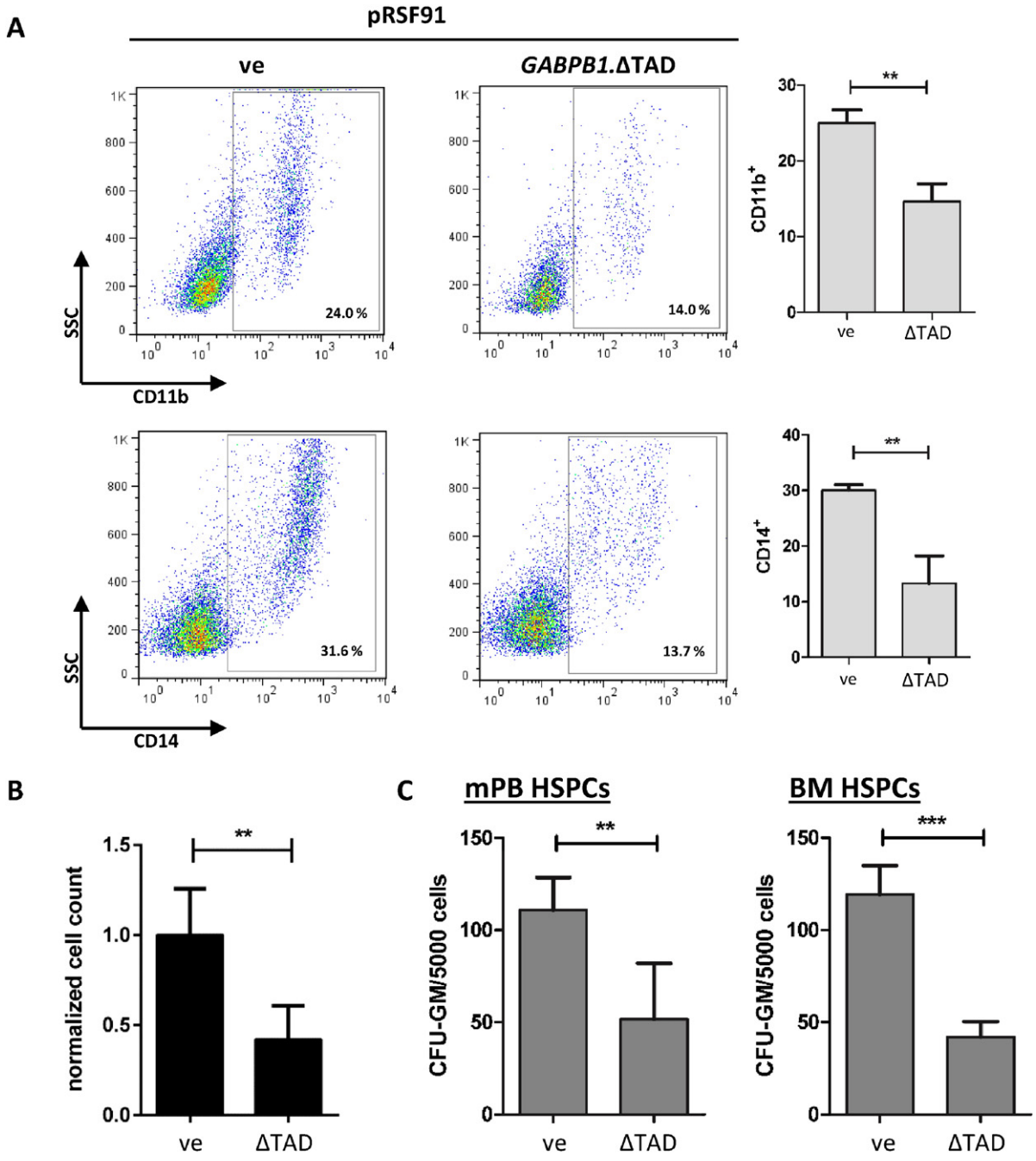

Fig. 2. Ectopic overexpression of *GABPB1*.ΔTAD in human CD34$^+$ HSPCs reduces myeloid differentiation potential. (A) Liquid culture of mobilized peripheral blood (mPB) CD34$^+$ cells for ten days after transduction with the ectopic overexpression vector (pRSF91.IRES.dTomato.pre*) either as empty vector control (ve) or containing a *GABPB1*.ΔTAD cDNA cassette. Left: Exemplary CD11b and CD14 flow cytometric analyses plotted against the side scatter (SSC). Right: Bar graphs summarizing three independent experiments each of one different individual for quantification of CD11b and CD14 expression, respectively (mean + s.d.; unpaired t-test; $^{**}P < 0.01$). (B) Normalized total cell count after 14 days in liquid culture comparing cells bearing the empty vector (ve) or the *GABPB1*.ΔTAD (ΔTAD) cDNA cassette. Cells were counted using a Neubauer counting chamber. Bar graphs are representing six independent experiments normalized to the empty vector control (mean + s.d.; unpaired t-test; $^{**}P < 0.01$). (C) Colony formation assays performed on mPB and bone marrow (BM) CD34$^+$ cells after transduction with the empty vector (ve) or the *GABPB1*.ΔTAD (ΔTAD) cDNA cassette. Three independent experiments using cells of three different donors were performed in triplicates and summarized as bar graphs (mean + s.d.; unpaired t-test; $^{**}P < 0.01$; $^{***}P < 0.001$).

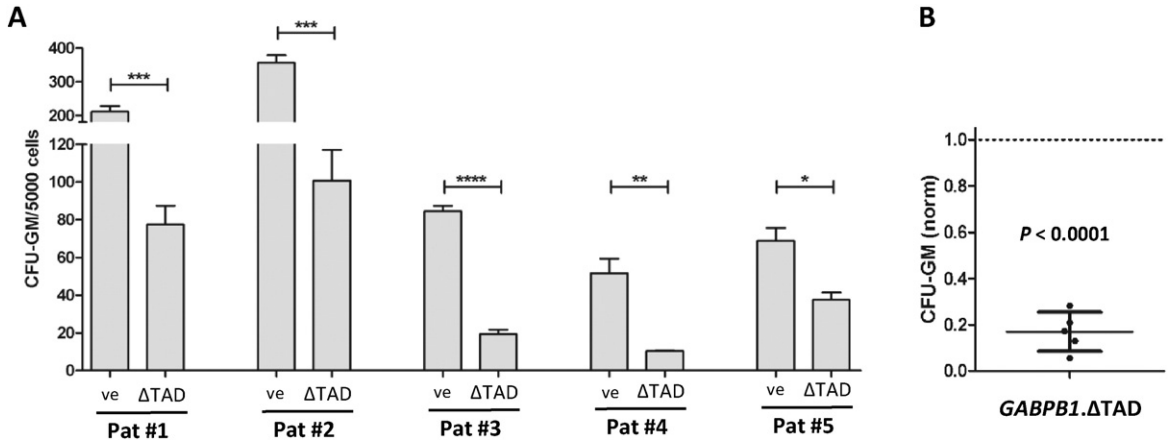

Fig. 3. Clonogenic capacity is decreased in human CML stem/progenitor cells upon GABP impairment. (A) Colony formation assays were performed on BM-derived CD34$^+$ cells from five CML patients (Pat #1–5) after transduction with the empty vector (ve) or the *GABPB1*.ΔTAD (ΔTAD) cDNA cassette. For each patient, cells were plated in triplicates and granulocytic/macrophage colony-forming units (CFU-GM) were determined after 14 days of cultivation (mean + s.d.; unpaired t-test; $^*P < 0.1$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$). (B) Dot plot of the *GABPB1*.ΔTAD overexpression group relative to the mean of the empty vector control group summarizing all five experiments (mean ± s.d.; paired t-test).

colonies. Upon ectopic overexpression of *GABPB1*.ΔTAD, colony forming capacity of CD34$^+$ HSPCs derived from mPB and healthy bone marrow (BM) was significantly decreased (Fig. 2C). Apart from GABP's effect on myeloid differentiation, these results indicate its crucial role for human hematopoietic self-renewal potential.

To address the question whether GABP also contributes to the propagation of human leukemic stem/progenitor cells, we tested CD34$^+$ cells obtained from five CML patients at diagnosis. Cytogenetic and molecular genetic analyses showed the presence of BCR-ABL1 fusion and indicated high tumor cell burden. Colony formation assays were performed with CD34$^+$ cells from each individual and showed remarkably decreased colony forming capacity (>80%) upon overexpression of *GABPB1*.ΔTAD (Fig. 3). Noteworthy, colony forming capacities were heterogeneous among individual patient samples. This observation did not correlate with the *BCR-ABL1/ABL1* ratio, cytogenetic aberrations, sex, age at diagnosis or any other known traits. It remains speculative whether this is due to pre-programmed cell-extrinsic effects or if undetected genomic alterations may be responsible. Moreover, by applying qPCR on colony forming cells maintained in methylcellulose for 14 days, initially present overexpression of *GABPB1*.ΔTAD was lacking (data not shown). In line with this, expression of the dTomato reporter was decreased in *GABPB1*.ΔTAD overexpressing cells to nearly undetectable levels indicating transgene silencing in those cells (Zhang et al., 2007). This hints towards a negative selective pressure on cells overexpressing *GABPB1*.ΔTAD confirming the necessity of functional GABP during leukemic stem cell maintenance.

The present results demonstrate that GABP is important for myeloid differentiation confirming the recently reported observations by us and others in hematopoietic cell lines and murine models, respectively (Yang et al., 2011; Yu et al., 2011; Ripperger et al., 2015). Here, for the first time, human primary hematopoietic cells were used to address GABP's crucial impact on myeloid differentiation and maintenance of hematopoietic stem/progenitor cells. A putative leukemogenic role of GABP was already postulated based upon a murine model, in which GABP was indicated to contribute to the establishment of leukemic stem/progenitor cell clones after transplantation of BCR-ABL1$^+$ leukemic stem cells (Yu et al., 2012; Yang et al., 2013). In line with this, we demonstrated that a properly functioning heteromeric GABP complex is also necessary for human CML HSPCs to give rise to myeloid colonies. Colonies of either healthy or leukemic stem cell origin are usually formed during multiple cell division cycles prior to maturation into respective hematopoietic lineages (Marley and Gordon, 2005). Hence, the colony formation assay used here is a helpful indicator for self-renewal potential and the myeloid differentiation competence of HSPCs.

GABP dysfunction resulted in diminished clonogenic capacity of healthy and CML CD34$^+$ HSPCs. This could not be overcome by the intrinsic leukemogenic potency of BCR-ABL1$^+$ cells, thus confirming the importance of GABP as a regulator of crucial effectors in human CML. Several signaling pathways are defined to co-operate with the BCR-ABL1 fusion protein to establish a transforming and anti-apoptotic phenotype (Steelman et al., 2004). However, whether GABP acts downstream of BCR-ABL1 and/or influences BCR-ABL1 co-operating signaling pathways still needs to be addressed. In our former study, we showed expression correlation of protein kinase D2 (*PRKD2*) to GABP expression levels in the K-562 cell line and CML patients at diagnosis (Manukjan et al., 2015). This was in line with mouse studies showing GABP's impact on PRKD2 in the context of murine CML-like disease (Yang et al., 2013). However, by applying qPCR on cells two days after transduction as well as after 14 days in methylcellulose, no expression alteration could be detected after overexpression of *GABPB1*.ΔTAD (data not shown). Possibly, PRKD2-regulation by GABP plays rather a role in the native situation or during progression to blast crisis. However, possible effects on *PRKD2* expression in primary human cells should be investigated in more detail to address the question of a druggable target in CML, since PRKD2 is susceptible to kinase inhibitors (Yang et al., 2013).

In summary, these results indicate GABP to be an indispensable ETS transcription factor during normal hematopoiesis and leukemogenesis. Further mechanistic investigations are needed to address the time and context dependent molecular function of GABP in myeloid differentiation. Even though, we observed lowered cell counts after GABP functional impairment, it remains speculative to what extent the loss of cells is acquired by differentiation block, cell cycle arrest or apoptosis. Since myeloid cytokine cocktails were used to force cells into myelopoiesis, the present study could not answer whether the functional impairment of GABP could cause compensatory expansion of other hematopoietic lineages. Nonetheless, this study is supportive to former data and has the potential to initiate further analyses on GABP in the context of human hematopoietic stem cell maintenance, differentiation and CML.

Acknowledgment

The authors thank Dr. Michael Morgan for his support in editing the manuscript. Moreover, many thanks to the Hannover Medical School's cell sorting core facility for FACS purification of transduced cells and the Cellular Therapy Center (CTC) for providing leukapheresis material. GM and TR were supported by a scholarship from Hannover Biomedical Research School (HBRS), PhD Program Molecular Medicine, and DS, GM, GG and AS by the German Research Foundation (DFG; Cluster of Excellence REBIRTH).

References

Emig, M., Saussele, S., Wittor, H., Weisser, A., Reiter, A., Willer, A., Berger, U., Hehlmann, R., Cross, N.C., Hochhaus, A., 1999. Accurate and rapid analysis of residual disease in patients with CML using specific fluorescent hybridization probes for real time quantitative RT-PCR. Leukemia 13, 1825–1832.

Hughes, T., Deininger, M., Hochhaus, A., Branford, S., Radich, J., Kaeda, J., Baccarani, M., Cortes, J., Cross, N.C., Druker, B.J., Gabert, J., Grimwade, D., Hehlmann, R., Kamel-Reid, S., Lipton, J.H., Longtine, J., Martinelli, G., Saglio, G., Soverini, S., Stock, W., Goldman, J.M., 2006. Monitoring CML patients responding to treatment with tyrosine kinase inhibitors: review and recommendations for harmonizing current methodology for detecting BCR-ABL transcripts and kinase domain mutations and for expressing results. Blood 108, 28–37.

Manukjan, G., Ripperger, T., Santer, L., von Neuhoff, N., Ganser, A., Schambach, A., Schlegelberger, B., Steinemann, D., 2015. Expression of the ETS transcription factor GABPalpha is positively correlated to the BCR-ABL1/ABL1 ratio in CML patients and affects imatinib sensitivity in vitro. Exp. Hematol. 43, 880–890.

Marley, S.B., Gordon, M.Y., 2005. Chronic myeloid leukaemia: stem cell derived but progenitor cell driven. Clin. Sci. (Lond.) 109, 13–25.

Orkin, S.H., Zon, L.I., 2008. Hematopoiesis: an evolving paradigm for stem cell biology. Cell 132, 631–644.

Ripperger, T., Manukjan, G., Meyer, J., Wolter, S., Schambach, A., Bohne, J., Modlich, U., Li, Z., Skawran, B., Schlegelberger, B., Steinemann, D., 2015. The heteromeric transcription factor GABP activates the ITGAM/CD11b promoter and induces myeloid differentiation. Biochim. Biophys. Acta 1849, 1145–1154.

Rosmarin, A.G., Resendes, K.K., Yang, Z., McMillan, J.N., Fleming, S.L., 2004. GA-binding protein transcription factor: a review of GABP as an integrator of intracellular signaling and protein–protein interactions. Blood Cells Mol. Dis. 32, 143–154.

Steelman, L.S., Pohnert, S.C., Shelton, J.G., Franklin, R.A., Bertrand, F.E., McCubrey, J.A., 2004. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia 18, 189–218.

Yang, Z.F., Drumea, K., Cormier, J., Wang, J., Zhu, X., Rosmarin, A.G., 2011. GABP transcription factor is required for myeloid differentiation, in part, through its control of Gfi-1 expression. Blood 118, 2243–2253.

Yang, Z.F., Zhang, H., Ma, L., Peng, C., Chen, Y., Wang, J., Green, M.R., Li, S., Rosmarin, A.G., 2013. GABP transcription factor is required for development of chronic myelogenous leukemia via its control of PRKD2. Proc. Natl. Acad. Sci. U. S. A. 110, 2312–2317.

Yu, S., Cui, K., Jothi, R., Zhao, D.M., Jing, X., Zhao, K., Xue, H.H., 2011. GABP controls a critical transcription regulatory module that is essential for maintenance and differentiation of hematopoietic stem/progenitor cells. Blood 117, 2166–2178.

Yu, S., Jing, X., Colgan, J.D., Zhao, D.M., Xue, H.H., 2012. Targeting tetramer-forming GABPbeta isoforms impairs self-renewal of hematopoietic and leukemic stem cells. Cell Stem Cell 11, 207–219.

Zhang, F., Thornhill, S.I., Howe, S.J., Ulaganathan, M., Schambach, A., Sinclair, J., Kinnon, C., Gaspar, H.B., Antoniou, M., Thrasher, A.L., 2007. Lentiviral vectors containing an enhancer-less ubiquitously acting chromatin opening element (UCOE) provide highly reproducible and stable transgene expression in hematopoietic cells. Blood 110, 1448–1457.