Differential contributions of STAT5A and STAT5B to stress protection and tyrosine kinase inhibitor resistance of chronic myeloid leukemia stem/progenitor cells

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Abstract

STAT5 fulfills essential roles in hematopoietic stem cell (HSC) self-renewal and chronic myeloid leukemia (CML), a prototypical stem cell malignancy. However, the specific contributions of the two related genes STAT5A and STAT5B have not been determined. In this study, we used an RNAi-based strategy to establish participation of these genes to CML disease and persistence following targeted therapy. We showed that STAT5A/STAT5B double knockdown triggers CML cell apoptosis and suppresses both normal and CML HSC long-term clonogenic potential. STAT5A and STAT5B exhibited similar pro-survival activity, but STAT5A attenuation alone was ineffective at impairing growth of normal and CML CD34+ cells isolated at diagnosis. In contrast, STAT5A attenuation was sufficient to enhance basal oxidative stress and DNA damage of normal CD34+ and CML cells. Further, it weakened the ability to manage exogenous oxidative stress, increased p53 (TRP53)/CHK-2 (CHEK2) stress pathway activation and enhanced prolyl hydroxylase domain (PHD)-3 (EGLN3) mRNA expression. Only STAT5A and its transactivation domain-deficient mutant STAT5AD749 specifically rescued these activities. STAT5A attenuation was also active at inhibiting growth of CML CD34+ cells from patients with acquired resistance to imatinib. Our findings demonstrate that STAT5A has a selective role in contributing to stress resistance through unconventional mechanisms, offering new opportunities to eradicate the most primitive and TKI- resistant CML cells with an additional potential to eradicate persistent stem cell populations.
Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell malignancy characterized by the unique t(9:22) chromosome translocation. This generates the BCR-ABL oncogene, a constitutively activated tyrosine kinase which is the primary oncogenic event causing CML (1). This has made CML a model for targeted therapies by the use of first (Imatinib mesylate, IM) and second generation tyrosine kinase inhibitors (TKI). Currently, TKI administration leads rapidly to complete cytogenetic remission (CCyR) and major molecular response (MMR) in the majority of patients with CML. However, BCR-ABL mRNA remains detectable in the most immature stem/progenitor cells despite long-term TKI therapy, discontinuation of IM in patients with complete molecular remission results in molecular relapse in 60% patients, and clinical resistance to IM also occurs in approximately 15% patients (2, 3). These observations highlight the needs to approach mechanisms of CML persistence.

Activation of signal transduction and activator of transcription 5 (STAT5) is a signaling hallmark of CML, reported more than a decade ago (4). STAT5 plays a central role in the maintenance of murine and human HSC from healthy donors and patients with several types of acute leukemia (5). Inhibition with a dominant negative mutant of STAT5 and STAT5 knockdown impair survival of BCR-ABL expressing cell lines and short-term clonogenic potential of primary CML progenitors, respectively (6, 7). In vivo transplantation of BCR-ABL-expressing murine hematopoietic progenitor cells induces an acute so-called “CML-like” leukemia in recipient animals, which almost disappears in STAT5-deficient background. Moreover, STAT5 overexpression rendered v-ABL+ murine BM cells resistant to IM (8). These observations demonstrated that STAT5 is essential to the induction of CML-like leukemia by exogenous BCR-ABL and provides resistance to IM in murine models. Thus,
there is considerable interest in delineating STAT5 contribution to normal and CML HSC maintenance in human cells.

STAT5 consists of two separate proteins, STAT5A and STAT5B, which are encoded by two closely related genes. Most studies have made use of STAT5A and STAT5B double deficient mice to get insight into STAT5’s functions. However, single gene knockout mouse models have reported non-overlapping functions of the two STAT5s that have been linked to restricted expression of a single STAT5 in a few tissues, gene dosage effect or differential biochemical properties of the two factors (9, 10).

In the present study, in an attempt to approach mechanisms of CML persistence, we tracked human STAT5 activities by differentially knocking down the two STAT5 genes in normal and CML HSC/progenitor cells, using a RNA interference strategy.

**Material and Methods (543)**

**Cell lines and primary samples**

Umbilical cord blood (CB) or adult peripheral blood (PB) samples from healthy donors and CML patients were collected after informed consent, in accordance with the declaration of Helsinki. Patient samples were collected at time of initial diagnosis or after >18 months of IM (Novartis) treatment. Mononuclear cells were isolated by density-gradient separation on Ficoll-Paque Plus (GE Healthcare). CD34^+ cells were isolated using a positive magnetic bead selection procedure (Miltenyi Biotech) and were maintained in IMDM medium supplemented with 15% BIT (Stem cell Technologies), stem cell factor (100 ng/mL, Amgen;), Flt3 ligand (100 ng/mL), interleukin-3 (50 ng/mL), and thrombopoietin mimetic peptide (10 nM; Sigma-Aldrich) (4F-medium). For long-term culture assays, CD34^+ cells were first transduced in 4F-medium and transferred 24 hours later on MS5 murine stromal cells in LTC-IC conditioned medium before FACS-sorting GFP^+ cells at day 4. GFP^+ cells were maintained on MS5 cells...
for one or five weeks before cell plating for colony assays using standard procedures. IM (1.5 μM) was added to the medium of IM-resistant cells. The resulting colony forming cells (CFC) were scored after 14 days of incubation. CML cell lines (MEG01, LAMA84, and K562) were purchased from cell depository banks. IM-resistant LAMA84 or MEG01 cells were generated using escalating concentrations of IM (from 0.05 to 1 μM) over four months. Resistant cells were maintained in IM (1μM)-supplemented medium.

**Lentiviral constructs, transductions**

The shC, shS5A, shS5B and shS5A/B sequences (see Supplementary Information) were inserted downstream of the H1 promoter into pTRIPΔU3-EF1α which encodes green fluorescent protein (GFP) or human CD4 as reporters. Murine STAT5A or STAT5B cDNA were introduced into pTRIPΔU3-EF1α ahead of IRES<sub> EMCV</sub>-GFP sequence. VSV-G-pseudotyped lentiviral particles were generated as described (11). 85-98% and 70-90% transduction with CML cell lines and CD34<sup>+</sup> cells, respectively, were obtained as measured by GFP expression. Cells were FACS-sorted when required. All subsequent assays were performed at day 7 post transduction. For rescue assays, cells were transduced with shRNA/CD4 encoding vectors and maintained in culture for 4 days before adding the cDNA/GFP rescue vectors. Assays were performed at day 3-5 post 2<sup>nd</sup> transduction.

**Cytofluorometric analysis**

Cell viability and cell cycle were assessed after staining with AnnexinV/7AAD, or propidium iodide (PI). Cell oxidative stress was assessed by incubating cells for 15 min at room temperature in the dark in the presence of dihydroethidium (DHE, 5 μM, Life Technologies) and, where indicated, cyclosporine A (20 μM, Novartis). Cells were pre-incubated with H<sub>2</sub>O<sub>2</sub> for 6hrs or VAS2870 (Sigma) for 1 hr. Flow cytometry was conducted on FACS-Canto II or
Accuri (Becton Dickinson) using anti-CD34-PC5, anti-CD4-PC7, AnnexinV-PE, 7-AAD, Propidium iodide reagents, all from BD Biosciences.

**Western blot analysis**

Cell extracts were analyzed by standard procedures using commercially available primary and peroxidase-labeled secondary antibodies (see Supplementary Information). Protein signals were detected by chemiluminescence (GE Healthcare) and quantified using a Fuji-LAS4000 image analyzer.

**Immunofluorescence**

Immunofluorescence assays were performed by standard procedures using anti-γH2Ax (Cell Signaling Technology) or anti-RAD51 (Pharmingen) antibody and Alexa 546-conjugated anti-rabbit Ig.

**Q-PCR**

Total RNA was extracted using RNeasy mini kit (Qiagen); Quantitative PCR was performed using SYBR green kit on a Lightcycler (Roche). Data were normalized to HPRT mRNA expression.

**Statistical analysis**

Data were expressed as mean ± SEM. Student’s t-test was used for statistical analysis.
Results and discussion

**STAT5 is required for long-term maintenance of normal and CML HSC**

We identified three small hairpin RNAs (shRNAs) that differentially knockdown human STAT5A and STAT5B factors, thereafter named shS5A, shS5B and shS5A/B. These were stably introduced into three CML cell lines (K562, LAMA84, and MEG01) using lentiviral vectors that co-encode the GFP dye, and immunoblot analyses of the transduced cells were performed (Fig. 1A and supplementary Fig. S1). ShS5A decreased STAT5A gene expression only, with no impact on STAT5B, as compared to untransduced (uT) or control shRNA (shC) expressing cells. ShS5B inhibited STAT5B and partially STAT5A expression, whereas shS5A/B inhibited both STAT5s. Our attempts to select for other STAT5A- or STAT5B-restricted shRNAs have been unsuccessful. The shRNAs did not change STAT1/3/6 expression or STAT1/3 tyrosine phosphorylation (Fig.1A, Supplementary Fig. S1 and data not shown), as opposed to the compensatory STAT1/3 activation observed in some murine STAT5A/B double deficient cells (9). Such compensations may need complete STAT5A/B knockout to occur. Growth properties of the transduced cells were evaluated by analyzing GFP+cells along time of culture (Fig. 1B and supplementary Fig. S2). ShS5A and shC had no impact on CML cell growth, regardless of the virus to cell ratio used. In contrast, shS5B+ and shS5A/B+ CML cells progressively disappeared. Expression of sh5B and shS5A/B had no impact on cell cycle phase transitions (data not shown); however, it induced cell apoptosis, as assessed by AnnexinV staining and poly(ADP-ribose) polymerase-1 cleavage (Fig. 1C and supplementary Fig. S1). ShS5B activity was confirmed by performing rescue experiments. Murine STAT5B which is insensitive to shS5B reversed STAT5B expression to endogenous levels in shS5B−cells (Fig. 1D, inset); it simultaneously rescued shS5B−cell survival, as assessed by the maintenance of mSTAT5−shS5B− doubly-transduced cells in culture (Fig. 1D). These observations assessed that shS5B activities were not “off-target” effects. Murine
STAT5A was then introduced in place of STAT5B and similarly rescued shS5B+ cell growth (Fig. 1D). These results indicated that the two STAT5s commonly sustain CML cell survival. Because shS5A—which inhibited STAT5A only—was inactive at altering CML cell growth, as opposed to shS5B which inhibited both STAT5B and STAT5A, the data further suggested that CML cell growth is sensitive to STAT5 gene dosage.

We next examined STAT5 activities in the context of primary CML stem/progenitor cells. ShRNAs were introduced in CML CD34+ cells that were then assayed for their clonogenic potential. ShS5B strongly impaired the short-term clonogenic properties of CML CD34+ cells (P<0.01), whereas shS5A had no or only weakly positive effects (Fig. 2A). Also, shS5B+ but not shS5A+ CD34+ CML cells showed strongly reduced long-term colony initiating cell (LTC-IC) potential, the most stringent in vitro assays of human HSC (Fig. 2B). We extended these experiments to normal (wt) CD34+ cells collected from umbilical cord (CB) and adult peripheral (PB) blood samples. ShS5B—but not shS5A—similarly suppressed LTC-IC potential of wtCD34+ cells and further inhibited CD34+ cell growth upon in vitro culture in a minimal four-cytokine serum-free growth medium (Fig. 2C-D). Altogether these data indicated that STAT5 activities sustain long-term maintenance of both healthy and CML HSC. These findings clearly extend previous reports and demonstrate for the first time in the context of CML stem cells, the crucial role of human STAT5 in HSC maintenance. BCR-ABL activity was shown to provide expansion of CML progenitors/precursors in a potentially STAT5-dependent manner (6). However, recent reports have demonstrated that primitive CML HSCs exhibit a BCR-ABL-independent phenotype that mimics normal microenvironment-dependent HSC (12); these CML HSCs are refractory to TKI treatment and sustain residual disease. Our findings now indicate that STAT5 may mediate such crucial stroma-derived signals. STAT5 thus could be a novel attractive therapeutic target to eliminate the most immature BCR-ABL-independent CML HSCs.
STAT5A protects cells from stress

Because shS5A did not affect CML cell apoptosis under standard conditions, we used this tool to address additional STAT5 roles. shRNA\(^+\)-CML cell lines and -wtCD34\(^+\)cells were subjected to oxidative stress by short hydrogen peroxide (H\(_2\)O\(_2\)) treatment and intracellular reactive oxygen species (ROS) were evaluated by dihydroxyethidium (DHE) staining. shS5A\(^+\)-CML and -wtCD34\(^+\)cells exhibited enhanced ROS levels in the presence -and also in absence- of H\(_2\)O\(_2\), as compared to controls. Co-incubating DHE with cyclosporine A, a drug efflux inhibitor, improved ROS detection. Basal production of ROS increased along time post shS5A transduction, which was partially suppressed by the NADPH oxidase (Nox)-restricted inhibitor VAS2870, in absence of mitochondria dysfunctions (Fig. 3A and supplementary Fig. S3A-B). ROS trigger genomic stress with basal p53-mediated DNA damage response (DDR), histone H2AX phosphorylation on Ser\(_{139}\), and the assembly of γH2AX and RAD51 positive DNA repair foci. ShS5A\(^+\)-CML cell lines and -wtCD34\(^+\)cells showed an increased proportion of cells with γH2AX\(^+\) and RAD51\(^+\) repair foci, as compared to shC\(^+\) cells, without RAD51 expression change (Fig. 3B and supplementary Fig. S3C). As another source of stress, wtCD34\(^+\) cells were allowed to proliferate at very high concentration for a few days, without medium change. Cell stress response was analyzed by probing activation of the well-known ATM-ATR/CHK/p53 stress signaling pathway. Over-concentrated shS5A\(^+\) cells showed a strong increase in p53 expression, which was present under its active [pSer\(_{15}\)]-p53 form; cells also exhibited enhanced [pThr\(_{68}\)]-CHK2 level (Fig. 3C). Activation of this p53/CHK2 stress response pathway was not detectable by this approach in normally growing shS5A\(^+\) cells. Overall, these data indicated that shS5A enhances cell oxidative/replicative/genomic stress, as compared to controls, and Nox activity contributes to this action. Nox plays critical roles in the production of ROS in leukemic cell lines and normal CD34\(^+\)cells; ROS activate STAT5.
that associates with the central Nox regulator Rac1 (13, 14). STAT5A may thus participate to a negative regulatory loop that limits cellular ROS. Among ROS cellular sensors is the oxygen-dependent prolyl hydroxylase domain (PHD) family. All three shS5A+ CML cell lines showed enhanced PHD3 expression as compared to controls. This activity was reversed upon shS5A-resistant murine STAT5A and transcription-deficient mutant STAT5AΔ749, but not STAT5B, expression; it was not duplicated by shS5B that weakly inhibits STAT5A (Fig. 3D). Collectively, our results indicated that STAT5A exhibits the restricted property to limit CML and normal stem/progenitor cell stress, independently of its canonical transcriptional activity. In line with these data, STAT5 down-regulates ROS production in pre-B leukemic cell lines, in absence of detectable STAT5 tyrosine phosphorylation; oncogenic activation of STAT5 shows preferential cytoplasmic localization and function in myeloid leukemias including CML cells (15, 16). Moreover, SRC/ABL kinases differentially affect nuclear translocation of STAT5A and STAT5B; accumulation of ROS correlates with reduced STAT5A -but not STAT5B- activity in aged macrophages; STAT5A shows differential tetramerization potential and selective post-translational modifications (9, 17, 18).

**TKI resistant CML cells show enhanced STAT5 dependence.**

Leukemic cell growth relies on proper oxidative stress control. STAT5A to STAT5B ratios were enhanced in all CML cell lines and patient cells as compared to normal counterparts (Fig.4A). STAT5A anti-stress activity may further help CML cells face exogenous insults. ShC+ and shS5A+ CML cells were treated with increasing concentrations of IM for two days. As compared to untreated cells, shS5A reduced cell viability of IM-treated cells (Fig. 4B). Stable IM-resistant cells were further generated from two CML (LAMA84 and MEG01) cell lines by increasing IM concentration in culture medium over four months. The resistant cells did not exhibit over-expressed or overactive BCR-ABL, increased multidrug resistance pump
activity, and over-expressed or over-active STAT5 (Supplementary Fig. S4). However, shS5A greatly affected growth of IM-resistant but not parental CML cells (Fig. 4C). Secondary IM-resistant patients were further selected that i) show no BCR-ABL over-expression or over-activity (no BCR-ABL mutations); ii) exhibit IM-resistant CD34+ cell clonogenic properties. These IM-resistant CD34+ cells did not express enhanced STAT5A/STAT5B mRNA levels and STA5A/STAT5B protein ratios (Fig. 4A and Supplementary Fig. S5). They, however, showed decreased clonogenic potential upon STAT5A knockdown (Fig. 4D), as opposed to CML CD34+ cells at diagnosis (Fig. 2A).

Altogether, our data indicated that IM-resistant cells have acquired enhanced STAT5-dependence, as compared to their sensitive counterparts, by being sensitive to STAT5A single knockdown. STAT5A has been identified as one of the four predictors of secondary IM resistance in CML (19). IM-resistant CML cells exhibit increased susceptibility to oxidant treatment due to mitochondrial dysfunction and high endogenous ROS levels (20). Our findings now indicate that human STAT5A could be one of these novel attractive therapeutic targets to hit ROS oversensitive CML cells and counteract development of long-term resistance to targeted therapy.

Collectively, our findings reveal STAT5s as doubly active pro-survival and stress protective factors, in primary normal and CML hematopoietic stem/progenitor cells; they further show that IM-resistance is associated with enhanced STAT5-dependence of patient stem/progenitor cells. These data highlight the central role of human STAT5s as pleiotropic oncogenic mediators of potential clinical importance in the treatment of CML and its recurrence. Our results could guide new strategies, based on STAT5 inhibition, for targeting drug resistant CML stem cell population. These novel STAT5 activities may also favor the maintenance, development and possible drug-resistance of other known STAT5-dependent
myeloproliferative syndromes, leukemia, as well as solid tumors (e.g. hepatocarcinoma and prostate cancer).

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References


**FIGURE LEGENDS**
Figure 1. STAT5 knockdown suppresses CML cell growth. (A) Immunoblot analysis of the indicated proteins in CML (K562) cells at day 7 post shRNA/GFP lentiviral transduction. ShC stands for control shRNA directed against luciferase; uT stands for untransduced cells. (B) FACS analysis of GFP expression in CML (K562, LAMA84, MEG01) cells as a function of time post shRNA/GFP transduction (day 0). One representative experiment is shown (n=4). (C) FACS analysis of Annexin V labeling in the indicated CML cells at day 7 post shRNA/GFP transduction. The percentage of Annexin V+ cells is indicated (mean ± standard error of the mean [SEM], n = 4). (D) FACS analysis of GFP and CD4 expression in CML (K562) cells upon double (shRNA/CD4 and cDNA/GFP) transduction. ctl stands for empty/GFP lentiviral vector; numbers indicate the multiplicity of infection used for ctl/cDNA vectors. Cells were analyzed at day 5 post 2nd transduction. Two independent experiments (Exp) are shown (n = 4); inset: immunoblot analysis of STAT5A (S5A), STAT5B (S5B) and β-tubulin (β-tub) expression in the FACS-analyzed doubly transduced CML (K562) cells. β-tubulin served as a loading control.

Figure 2. STAT5 is essential for long-term HSC maintenance. (A) Colony forming cell (CFC) assays were performed on 7 newly diagnosed CML CD34+ blood samples upon shRNA/GFP transduction and GFP sorting. Results are expressed as the number of clones per 10^3 initial GFP+CD34+ cells (**, p < 0.01). (B) LTC-IC assays of shRNA/GFP+ CD34+ CML cells; Total colony forming cells (CFC) from LTC-IC were scored; CD34+ CML cells were isolated from blood samples at diagnosis (n = 4). (C) Long-term colony initiating cell (LTC-IC) assays of shRNA/GFP+ peripheral blood CD34+ cells. Total colony forming cells (CFC) from LTC-IC were scored (*, P<0.05). (D) FACS analysis of GFP expression in cord blood CD34+ cells as a function of time post shRNA/GFP transduction (mean ± SEM, n=3). Wt stands for cells collected from healthy donors.
Figure 3. STAT5 protects normal and CML cells from stress. (A) FACS analysis of DHE labeling in the indicated shRNA/GFP+ cells following H$_2$O$_2$ (6hr) or VAS2870 (1hr) pre-treatment. DHE was co-incubated with cyclosporine A (CsA, 20 µM) where indicated (mean ± SEM, n = 4). (B) Immunofluorescence microscopy of γH2Ax+ and RAD51+ foci in the indicated shRNA/GFP+ sorted cells. The percentage of positive cells (>3 foci per cell) is presented (mean± SEM, n = 5); left: one representative experiment in transduced GFP+CD34+ cells. (C) Immunoblot of the indicated proteins in over-proliferating shRNA/GFP+ cells at day 7 post CB CD34+ cell transduction. β-tubulin served as a loading control. (D) QRT-PCR analysis of PHD3 transcripts in the indicated shRNA+ CML cell lines at day 7 post shRNA transduction. Results are normalized to HPRT mRNA and expressed relative to PHD3 levels in shC+ cells (left) or shS5A+/empty (ctl) vector+ doubly transduced cells (right) (n = 3, mean ± SEM).

Figure 4. IM-resistant CML cells show enhanced STAT5 dependence. (A) Immunoblot analysis of the indicated proteins in normal and CML cells at day 7 post shRNA/GFP transduction. Samples were from three CB CD34+ cell lots, two CML CD34+ cells from secondary IM-resistant patients with no BCRABL over-activity, one CML CD34+ cells at diagnosis and the three CML (K562, LAMA84 and MEG01) cell lines. Long electrophoretic runs and anti-total STAT5 antibody were used. STAT5A and STAT5B signals were quantified for each sample, expressed as STAT5A/STAT5B ratios and grouped as indicated. (B) FACS analysis of propidium iodide labeling in shRNA/GFP+ CML (LAMA84) cells after a 2 day-treatment with imatinib mesylate (IM) at the indicated concentration. (C) Number of parental (LAMA84S: MEG01S) and IM-resistant CML (LAMA84 R, MEG01R) cells during a
5-day culture, between day 7 and day 12 post shRNA transduction. The proliferative index was calculated (mean ± SEM, n = 3). (D) Colony assay of shRNA/GFP⁺ secondary IM-resistant leukemic CD34⁺ cells post shRNA transduction and GFP sorting (mean ± SEM, n = 3). Results are expressed as the number of CFC per 2x10³ initial GFP⁺CD34⁺ cells (*p <0.05; ** p < 0.01, *** p<0.01).
Casetti et al, Figure 2

**CML-CD34**

(A) Colony number

(B) CFC from LTC-IC

(C) wt CD34

(D) wt CD34

4 6 10 14 18 days of culture
Casetti et al, Figure 3

**A**

- **-CsA**
  - shS5A-K562
  - shS5A- wt CD34^+
  - shC-K562
  - shC- wt CD34^+

- **+CsA**
  - DHE^+ GFP^+ cells (%) vs. 
  - H2O2 (µM)
  - VAS (µM)

**B**

- K562
- wt CD34^+
- γH2A_x RAD51
- γH2A_x

Positive cells (%) vs.
- shC
- shS5A
- shC
- shS5A

**C**

- wt CD34^+
- shC
- shS5A

- STAT5A
- p53
- P-Ser^15 p53
- P-Thr^68 CHK2
- β-tubulin

**D**

- K562 LAMA84 MEG01
- K562

- mRNA rel. expression vs.
- shC
- shS5A
- shS5A+5A
- shS5A+5B
- shS5A+5A+5B

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Casetti et al, Figure 4

A

B

C

D

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