

Constitutive Phosphorylation of STAT5A and STAT5B in Acute Myeloid Leukemia

Zhu Wang, Xiao Wei Yang, Martin Carroll, M.D.

Advisors: Xiao Wei Yang, Martin Carroll

Department of Hematology/Oncology, University of Pennsylvania School of Medicine

Acute Myeloid Leukemia (AML) is a malignant disease of the blood which is fatal in over half of adults diagnosed with the disease. It is hypothesized that dysregulated growth signaling may contribute to the pathogenesis of AML but the mechanism of growth dysregulation is poorly understood. Signal transducer and activator of transcription 5 (STAT5) is a transcription factor that regulates many aspects of cell growth, survival and differentiation. STAT5 protein has two different forms, STAT5A and STAT5B, which are encoded by two distinct, yet closely related genes. Constitutive activation of STAT5 has been identified in a number of hematopoietic malignancies including AML. However, it is unclear whether STAT5 activation in AML suggests the activation of STAT5A or STAT5B or both. In this study, activation status of both STAT5A and STAT5B proteins are examined respectively in AML cell lines and primary patient samples, by using co-immunoprecipitation with anti-STAT5A, STAT5B, and p-STAT5 (Tyr694/699) antibodies. Results show that all AML cells contain constitutively phosphorylated STAT5A except one line as well as constitutively phosphorylated STAT5B. To further determine the functional significance of the constitutive activation of STAT5A and STAT5B, knockdown strategy was employed to deplete STAT5A and STAT5B by using siRNA. Depleted expression of STAT5A/B in AML resulted in decreased survival and growth in liquid culture assays and colony forming cells (CFCs) assay. These results demonstrate that both STAT5A and STAT5B are required for AML cell growth and survival. Understanding the role of STAT5A/B should lead to novel therapies for AML and, likely, other malignancies that also contain constitutive activation of STAT5A/B.

Introduction

Leukemias are blood neoplasms that can be broadly classified into four main types based on their cell of origin and their natural progression (1). These malignancies involve the proliferation of the hematopoietic cells derived from either the lymphoid or myeloid lineage and can present in either an acute or a chronic phase (2-3). Thus, the four main types of leukemia are acute and chronic myelogenous leukemia (AML and CML), and acute and chronic lymphocytic leukemia (ALL and CLL). Acute leukemias are characterized by the accumulation of immature (or poorly differentiated) cells, known as blast cells, and tend to proliferate rapidly, are very aggressive and usually progress to a chemo refractory state (the recurrence of disease after 12 months from the last dose of chemotherapy) fairly quickly (4). Alternatively, chronic phase leukemias accumulate more mature cells of a

particular lineage, tend to have long latencies and have been described as slow growing, smoldering diseases. However, as chronic leukemias progress, they acquire additional genetic mutations that increase their rate of division and block differentiation, resulting in transition into an acute phase or blast crisis (5). AMLs are fast growing, highly aggressive, and refractory to chemotherapy hematological malignancies that accounts for approximately one third of all new leukemia cases. Presently, induction therapies are capable of achieving complete remission in 50-70% of patients (6). However, long-term survival rates remain low (<20%) due to a high frequency of relapse (6). Consequently, the development of more effective therapeutic strategies is essential.

STATs (signal transducers and activators of transcription) are members of the ubiquitously expressed family of transcription factors activated in response to growth factors and cytokines

(7-10). Activation of STATs requires phosphorylation of their tyrosine residues by either the receptors that often display an intrinsic tyrosine kinase activity or by receptor associated kinase. The activated STATs form dimers that translocate into the nucleus and initiate transcription of the growth factor/cytokine responsive genes. STATs play a critical role in promoting cell proliferation and survival, both normal and malignant. Several members of the STAT family have been identified and designated STAT1 to STAT6. Numerous biochemical and genetic studies have demonstrated that constitutive activation (activation with or without cytokine induction) of STATs, such as STAT53 and STAT5, is essential for cellular transformation and oncogenesis. Constitutive STAT activation might be due either to the constitutive activation of cytokine receptors activated by autocrine growth factors or due to mutations in specific upstream tyrosine kinases resulting in

constitutive activity of these kinases.

STAT5 comprises two closely related but distinct STATs designated STAT5A and STAT5B. Despite the extensive similarity (94% identical at the protein level) between STAT5 proteins (11), gene knock-out mouse models clearly demonstrated that STAT5A and STAT5B possess some distinct, non-overlapping functional properties (11,12). Although the constitutive activation of STAT5 has been reported in AML, the exact role of STAT5A versus STAT5B in malignant cell transformation is at present much less understood (13).

The lack of clear distinction of the role of STAT5A versus STAT5B in the cell biology and pathology partly stems from the broad usage for the activation status analysis of a phospho-specific antibody that reacts with both STAT5A and STAT5B. Here, we report that both STAT5A and STAT5B become constitutively phosphorylated in AML. Furthermore, the activation of STAT5A and STAT5B is required for AML cell growth and survival. Understanding the role of STAT5A/B should lead to novel therapies for AML and, likely, other malignancies that also contain constitutive activation of STAT5A/B.

Materials and Method

Cell line culture and primary cell isolation

Human AML and CML cells were grown in RPMI-1640 medium (Bio-Whittaker; Walkersville, MD, USA) with 10% fetal bovine serum (Cellgro, Mediatech). Stock cultures were maintained at 37°C in a humidified 5% CO₂ incubator. Primary leukemia samples were obtained from the Stem Cell and Leukemia Core Facility at the University of Pennsylvania. Samples were obtained from patients presenting with acute leukemia seen at the Hospital of the University of Pennsylvania after informed consent in accordance with institutional guidelines. Bone marrow or peripheral blood samples were collected, and samples were prepared by Ficoll gradient centrifugation. Mono-

nuclear cells were frozen as viable cells in fetal calf serum and 10% dimethyl sulfoxide (DMSO) and stored in liquid nitrogen.

Immunoprecipitation and Western blot

Cells were lysed for 30 min in ice-cold lysis buffer (10x lysis buffer, Cell Signaling), then centrifuged at 15,000 rpm and the supernatant were pre-cleared overnight at 4°C with protein A-agarose (Sigma). 2 µg of STAT5A or STAT5B antibody (Santa Cruz Biotechnology) were added into 500 µg of protein and incubated for 90 min on ice. Then the protein A-agarose was added to precipitate the immune complexes for 2 hr or overnight at 4°C. The immunoprecipitates were washed, boiled, suspended in reducing SDS loading buffer, separated on a 10% polyacrylamide SDS gel, and transferred electrophoretically to hybridization transfer membranes. The membranes were blocked with 5% milk in TBST buffer (10 mM Tris HCl, pH 7.4/75 mM NaCl/1 mM EDTA/0.1% Tween 20) for at least 1 hr at room temperature or overnight in a cold room, incubated with the relevant primary antibody, washed, incubated with peroxidase-conjugated secondary antibody, and washed again. Blots were developed using the ECL chemiluminescence reagents (Amersham).

Short interfering RNA (siRNA)

STAT5 siRNA was designed based upon 50% GC contents and asymmetry of siRNA sequence. STAT5 siRNA has the sequence: sense strand 5'-GCAAGUGGUC-CCUGAGUUUdTdT-3', antisense strand 5'-AAACUCAGGGACCAC-UUGCdTdT-3'. Scramble siRNA has the sequence: sense strand 5'-AUGGA-CAACUUUCAGACCCdTdT-3', antisense strand 5'-GGGUCUGAAAGUU-GUCCAU-3'. Fluorescence-labeled siRNA has the same sequence as siRNA, tagged with Cy3 at 5' end.

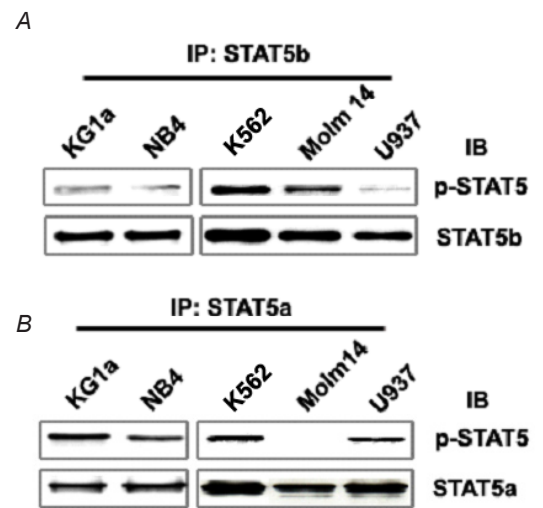


Figure 1. Constitutive phosphorylation of STAT5A and STAT5B in AML cell lines. A) Constitutive phosphorylation of STAT5A in AML cell lines, including KG1a, NB4, Molm14 and U937, and CML cell line K562. They were immunoprecipitated with anti-STAT5A antibody and then were blotted with anti-p-Stat5 (Tyr 694/699) antibody. B) Constitutive phosphorylation of STAT5B in AML cell lines. Same experiment was performed with same cell lines using anti-STAT5B antibody for immunoprecipitation.

Transfection and siRNA knock-down

Transfection of siRNA was carried out by nucleofection using an AMAXA Nucleofector device (AMAXA, Gaithersburg, MD) under conditions suggested by the manufacturer. 2 x 10⁶ primary AML cells were suspended in 100 µl of cell line Nucleofector Kit V solution with 10 µg of siRNA and transferred to an AMAXA certified cuvette. The cuvette was inserted into the Nucleofector and processed with program U-15.

Quantitative real-time PCR

Target gene expression is investigated by quantitative real-time PCR (q-PCR). STAT5A primer sequence: sense strand 5'-GCCGGCTGTGTAT-GGTCTAT-3', sense strand 5'-AAG-TAGTGCCGGACCTCGAT-3'. STAT5B primer sequence: sense strand 5'-GTAAACCATGGCTGTGTG-GA-3', antisense strand 5'-AAATAAT-GCCGCACCTCAAT-3'. For Q-PCR, total RNA was isolated from AML cells using the Rneasy kit from Qiagen

(Venlo, the Netherlands) according to the manufacturer's recommendations. RNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad). For real time PCR, 1ul of cDNA was amplified in a total volume of 25ul using Applied Biosystems Power SYBR Green PCR Master Mix on an Applied Biosystems 7500 thermocycler. Data was quantified using ABI software.

Colony Forming Cells Assay/ Methylcellulose Assay

1x10⁴ transfected cells were plated into complete methylcellulose media supplemented with cytokines (MethoCult GF H4434 media, Stem Cell Technologies). Plates were incubated in 35-mm Petri dishes in triplicate for 3 weeks at 37°C in a humidified atmosphere of 5% CO₂. Number of colonies was counted at the end of the incubation period.

Results

Constitutive phosphorylation of STAT5A and STAT5B in AML cell lines

To evaluate the activation status of the STAT5A and STAT5B in AML

cells, we examined phosphorylation of STAT5A and STAT5B in the myeloid leukemia derived T cell lines using co-immunoprecipitation assay. The experiment was performed with four AML (KG1a, NB4, Molm14, and U937) and one CML (K562) cell lines. The STAT5A and STAT5B proteins from the cell lines were first immunoprecipitated with anti-STAT5A and STAT5B antibodies respectively and then p-STAT5 (Tyr 694/699) antibody was used to detect the immunoprecipitated phosphorylated STAT5A and STAT5B. As shown in Figure 1A, a strong band corresponding to phosphorylated Stst5a was detected in four of five cell lines except Molm14

line, whereas the phosphorylated STAT5B band was detected in all five cell lines (Fig. 1B). This result suggests that the AML cell lines constitutively express STAT5A as well as STAT5B.

Constitutive phosphorylation of STAT5A and STAT5B in primary AML cells

To determine if constitutive activation of the STAT5A and STAT5B observed in the AML-derived malignant cell line is also present in primary AML cells, we tested phosphorylation of STAT5A and STAT5B proteins in five primary AML cells. We can see from Figure 2 that peripheral blood sample

from 5 AML patients showed constitutive phosphorylation of both STAT5A and STAT5B. In addition, to exclude the induced phosphorylation of STAT5A and STAT5B in vivo, primary AML cells were kept in EGM2 media (Cellgro) overnight. Constitutive activation of STAT5A and STAT5B was still detectable. These findings indicate that phosphorylation of STAT5A and STAT5B proteins is constitutive in primary AML cells.

Depletion of STAT5A/B suppresses growth of the AML cells

We examined next the effects of STAT5A/B depletion on proliferation of AML cells using colony forming cells (CFCs) assay. U937 cell line was used to evaluate the specificity of STAT5A/B knockdown. The efficiency of STAT5A/B siRNA transfection was evaluated by performing quantitative real-time RT-PCR. As shown in Figure 3A, STAT5A/B expression was significantly diminished from the siRNA knockdown. After determining the specificity of STAT5 siRNA, four

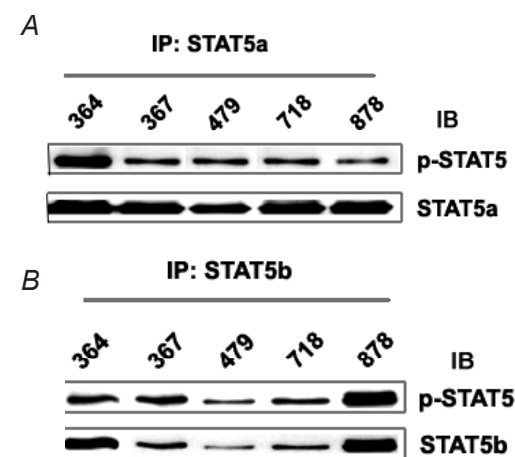


Figure 2. Constitutive phosphorylation of STAT5A and STAT5B in primary AML cells. A) Constitutive phosphorylation of STAT5A in AML primary cells. The primary cells from five AML patients were studied using the same experimental setup as described in Figure 1A. B) Constitutive phosphorylation of STAT5B in AML primary cells. Same experiment was performed with same primary cells using anti-STAT5B antibody for immunoprecipitation.

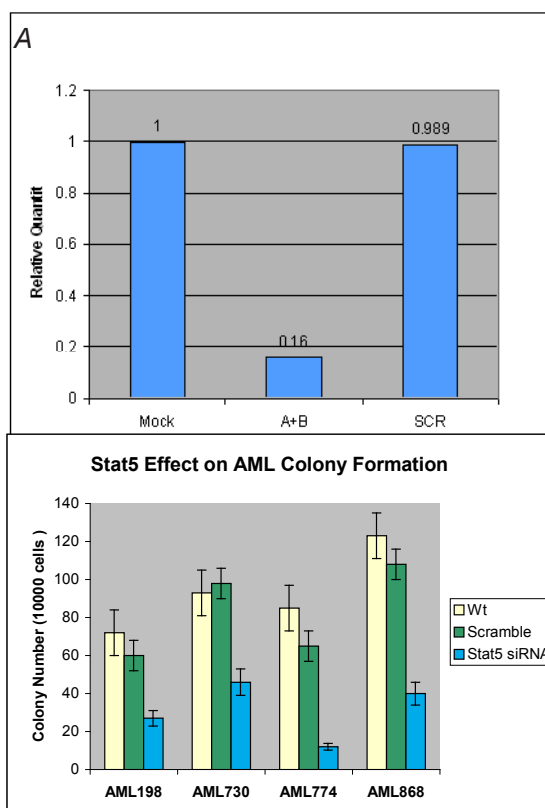


Figure 3. Depletion of STAT5A/B suppresses growth of the AML cells. A) Knockdown of STAT5A/B. U937 cells were transfected with STAT5A/B siRNA- or control, non-targeting scramble (SCR) siRNA and analyzed at 72 hr for post-transfection efficiency. B) Colony Forming Cells (CFCs) assay. The primary cells from five AML patients were transfected with STAT5A/B siRNA or control, non-targeting scramble (SCR) siRNA and analyzed in the colony formation assay after 3 weeks. The results are depicted as percentage with reference to the number of colonies formed by untreated cells.

AML primary cells were transfected with STAT5A/B siRNA and evaluate the effects of STAT5A/B depletion on the proliferation of the primary AML cells. As shown Figure 3B, all four representative AML primary samples show that STAT5A/B siRNA treatment reduced colony number about 50% - 90% compared with both the control-transfected and wild type cultures. This result indicates that constitutive phosphorylation of STAT5A/B contributes to AML cell transformation.

Discussion

STAT5 is well recognized as oncoprotein and its persistent activation has been identified in a large spectrum of lymphoid and nonlymphoid malignancies (9). It promotes oncogenesis by modulating several key functions of the malignant cells such as survival, proliferation, migration, invasion, induction of angiogenesis, and evasion of the immune response. Whereas previous studies from numerous laboratories including ours detected STAT5 activation by using a phospho-STAT5 antibody that reacts with both STAT5A and STAT5B, in this study we examined the functional status of STAT5A and STAT5B involved in malignant AML cells by using co-immunoprecipitation method. Four of AML cell lines and one CML express activated STAT5A and all five lines express activated STAT5B. In addition, all primary cells from 5 AML patients showed constitutive phosphorylation of both STAT5A and STAT5B. We also observed that the level of STAT5A and STAT5B phosphorylation among the cell lines and primary cells varies. This discrepancy may be grade-dependent and may represent biological differences. Moreover, we found that the depletion of STAT5A/B through siRNA knockdown affects the AML cell proliferation and survival. This indicated that both STAT5A and STAT5B contribute to the AML transformation by promoting cell growth and survival. This is suggesting that STAT5A/B targeted genes and their down stream

effectors possibly play roles in cell cycle or cell division. These possible target genes could also contribute significantly to unknown kinases or phosphatases that can become important therapeutic targets in AML patients.

Our findings also have potential therapeutic implications for AML, CML and, possibly, other malignancies that express constitutive phosphorylation of STAT5A/B. Future studies will include the identification of STAT5A/B target genes and their biological effects in AML cells. In conclusion, our data indicate that constitutive phosphorylation of STAT5A/B expression is required for the growth and proliferation of AML cells.

Acknowledgements

I would like to thank everyone in the Carroll lab for their help and support. Also, thanks to Dr. Carroll's grant (NIH) and CURF for funding this research. Finally, I would like to thank Dr. Harriet Joseph, Dr. Gregory Guild, and everyone from CURF. Without their invaluable help, this research would not be possible.

References

1. Kenneth A. Foon, Robert F. Todd, III. Immunologic classification of Leukemia and Lymphoma. *Blood*. 1986; 68(1): 1-31.
2. Dexter TM, Lajtha LG. Factors controlling the proliferation of Hematopoietic stem cells in vitro. *Br J Haematol*. 1974;28:525-530.
3. Lowenberg B, Touw IP. Hematopoietic growth factors and their receptors in acute leukemia. *Blood*. 1993;81:281-292.
4. William L. Carroll, Deepa Bhojwani, Dong-Joon Min, Elizabeth Raetz, Mary Relling, Stella Davies, James R. Downing, Cheryl L. Willman and John C. Reed. *Pediatric Acute Lymphoblastic Leukemia*. American Society of Hematology. 2003; (1) 102-131.
5. Charles L. Sawyers, Andreas Hochhaus, Eric Feldman, John M. Goldman, Carole B. Miller, Oliver G. Ottmann, Charles A. Schiffer, Moshe Talpaz, Francois Guilhot, Michael W. N. Deininger, Thomas Fischer, Steve G. O'Brien, Richard M. Stone, Carlo B. Gambacorti-Passerini, Nigel H. Russell, Jose J. Reiffers, Thomas C. Shea, Bernard Chapuis, Steven Coutre, Sante Tura,

Enrica Morra, Richard A. Larson, Alan Saven, Christian Peschel, Alois Gratwohl, Franco Mandelli, Monique Ben-Am, Insa Gathmann, Renaud Capdeville, Ronald L. Paquette, and Brian J. Druker. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. *Blood*. 2002, 99 (10), 3530-3539.

6. Y Ravindranath, M Chang, C P Steuber, D Becton, G Dahl, C Civin, B Camitta, A Carroll, S C Raimondi and H J Weinstein for the Pediatric Oncology Group. Pediatric Oncology Group (POG) studies of acute myeloid leukemia (AML): a review of four consecutive childhood AML trials conducted between 1981 and 2000. *Leukemia* 2005. 19, 2101-2116.
7. Calo V, Migliavacca M, Bazan V, et al. (2003). STAT proteins: from normal control of cellular events to tumorigenesis. *J Cell Physiol.*, 197:157-168.
8. Alvarez JV, Frank DA. (2004). Genome-wide analysis of STAT target genes: elucidating the mechanism of STAT-mediated oncogenesis. *Cancer Biol Ther.*, 3:1045-1050.
9. Yu H, Jove R. (2004). The STATs of cancer--new molecular targets come of age. *Nat Rev Cancer*, 4:97-105.
10. Darnell JE. (2005). Validating STAT53 in cancer therapy. *Nature Med.*, 11:595-596.
11. Ambrosio, R., Fimiani, G., Monfregola, J., Sanzari, E., De Felice, N., Salerno, M.C., Pignata, C., D'Urso, M., Ursini, M.V. (2002). The structure of human STAT5A and B genes reveals two regions of nearly identical sequence and an alternative tissue specific STAT5B promoter. *Gene*. 285(1-2), 311-8.
12. Buitenhuis, M., Coffey, P.J., and Koenderman, L. (2004). Signal transducer and activator of transcription 5 (STAT5). *Int. J. Biochem. Cell. Biol.* 36, 2120-2124.
13. K Spiekermann, S Biethahn, S Wilde, W Hiddemann, F Alves. Constitutive activation of STAT transcription factors in acute myelogenous leukemia. *European Journal of Haematology* 2001; 67 (2) , 63-71