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Detection of lineage-specific STAT5A phosphorylation in peripheral blood cells from a myeloproliferative neoplasm model mouse using multicolor flow cytometry

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Introduction

Appropriate phosphorylation of signal transducer and activator of transcription (STAT) transcriptional factors, down-stream mediators of Janus kinases (JAKs), regulates expression of various genes during normal hematopoiesis. Reflecting dysregulation of the JAK-STAT pathway, constitutive activation of STAT3 and STAT5 is frequently detected in hematopoietic malignancies[1]. In particular, the JAK2 V617F hyperactive mutation, which leads to hyperphosphorylation of STAT5, was recently found to occur in most patients with myeloproliferative neoplasms (MPNs)[2] : mainly polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). Recent studies also indicate that the expression levels of the JAK2 V617F mutant may influence disease type selection[3]. We have therefore been investigating the possibility that monitoring the level of STAT5A phosphorylation could be helpful for the diagnosis, treatment and follow-up of MPNs.

Multicolor flow cytometry is a powerful diagnostic tool able to detect and separate abnormal cell populations from normal blood cells, even when the abnormal population is very small. And thanks to recent progress in intracellular staining methods, one can now use this method to detect phosphorylated proteins at the single cell level[4]. Standard detection protocols are generally provided by the manufacturer ; however, whole peripheral blood contains cells of different lineage that show differing degrees of membrane stability during fixation, and each lineage-specific antigen and phosphorylated protein has a different tolerance for fixation. Therefore, the protocol used to stain whole blood for detection of both intracellular phosphorylated proteins and surface lineage makers in the same cells should be optimized for each experiment.

Methods, results and discussion

To optimize staining of phosphorylated STAT5A along with a lineage marker, we used Jurkat cells transduced with a retroviral vector encoding STAT5A1*6[5] (**Fig. 1**). We first determined the optimal fixation, permeabilization and staining conditions for detecting FLAG on the intracellular STAT5A1*6 using flow cytometry

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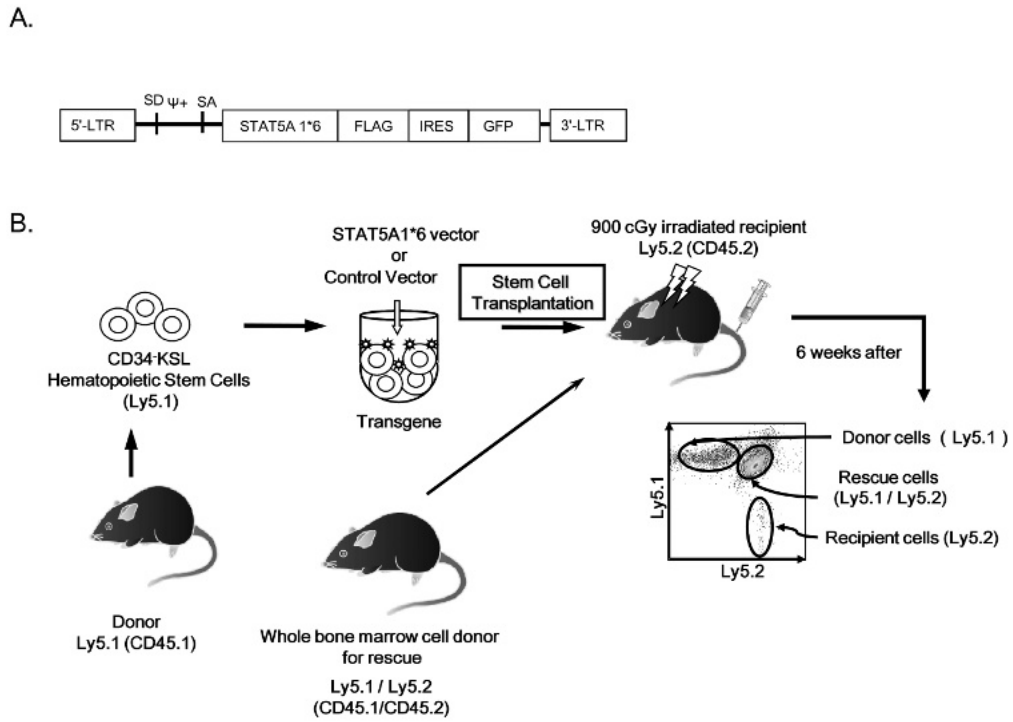

Fig. 1A Schematic presentation of the retroviral vector encoding STAT5A1*6 (GCDNsamSTAT5A1*6 I/G). Expression of STAT5A1*6 is driven by the retroviral promoter long terminal repeat (LTR). SD, splicing donor; SA, splicing acceptor; ψ^+, packaging signal; IRES, internal ribosomal entry site; GFP, green fluorescent protein.

Fig. 1B Schematic presentation of the experimental design. Highly purified hematopoietic stem cells (CD 34-KSL HSC) were obtained from C57B/6 (ly5.1) bone marrow and transduced with GCDNsamSTAT5A1*6 I/G or control vector. The genetically modified HSCs were then transplanted to 950 cGy-irradiated C57B/6 (Ly5.2) recipient mice along with 7.5×10^5 whole bone marrow cells from a C57B/6 (Ly5.1/Ly5.2) mouse as rescue cells. Peripheral blood from the recipient mouse was analysed 6 weeks after transplantation. The genetically modified donor cells, recipient cells and rescue cells could be identified based on their expression of CD45.1 (Ly5.1) and /or CD45.2 (Ly5.2).

(MoFlo, Beckman-Coulter, Brea, CA) with an anti-FLAG antibody (AD0059F, Perkin Elmer Japan, Kanagawa, Japan). By comparing several conventional methods, we determined the best conditions for detecting intracellular molecules to be fixation with prewarmed BD Phosflow Fix Buffer I (BD Japan, Tokyo, Japan) for 10 min at 37℃, followed by permeabilization with BD Phosflow Perm Buffer III (BD Japan) for 30 min at 4℃.

We confirmed our ability to detect intracellular STAT5A1*6 under the selected conditions using peripheral blood cells from a progressive MPN model mouse[6]. Establishment of the MPN mouse was described previously. Briefly, highly purified HSCs (CD34-KSL)[7] from C57BL/6J mouse (B6, CREA Japan, Tokyo, Japan) bone marrow were transduced using a retroviral vector encoding STAT5A1*6, and then transplanted into a lethally irradiated B6 mouse. Six weeks later,

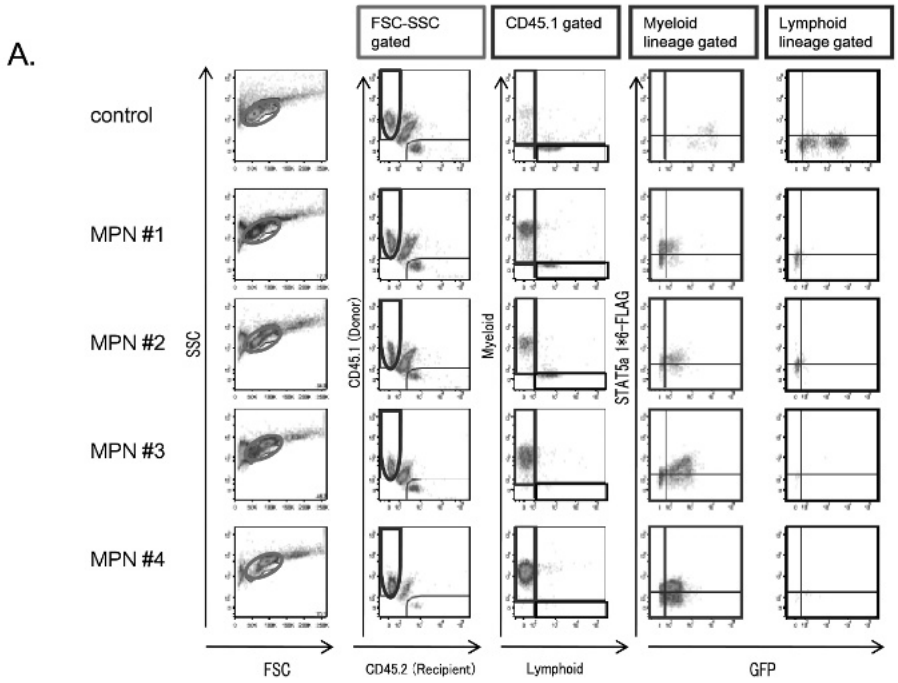

Fig. 2A Representative result of intracellular STAT5A1*6 and surface lineage-antigen staining in peripheral blood cells from a MPN mouse. Samples of whole blood from a MPN mouse were processed as summarized in Table 1. Intracellular STAT5A1*6 was detected only in myeloid cells from the MPN mouse, which is indicative of the myeloid origin of the disease.

Fig. 2B Human ESC-derived hematopoietic cells[8] were transduced using control vector or GCDNsam STAT5 A1*6 I/G, then cultured in the presence of EPO, SCF and TPO. Cells were harvested on day 10 of culture and processed for intracellular phosphoSTAT5A and glycophorin A double staining using anti-phospho STAT5A (Y694), anti-glycophorin A and anti-CD45 antibodies. Forced EPO-induced STAT5A phosphorylation has been detected in glycophorin A-positive cells. The numbers in the plot indicate the percentages of cells in the quadrants.

peripheral blood cells were collected for flow cytometric analysis (**Fig. 2**). After lysing the erythrocytes by incubating the cells in 1 ml of 140 mM NH_4CL for 15 min at room temperature, the remaining white blood cells (~$1x10^6$ cells) were fixed and permeabilized under the aforementioned conditions. To block nonspecific staining of intracellular molecules by the specific antibodies, 50 μl of 10x diluted affinity-purified mouse IgG1, k (eBioscience, San Diego, CA) was added to the

test cells and incubated for 30 min at 4℃ prior to addition of the specific antibodies. This enabled efficient staining of intracellular STAT5A1*6.

Because each surface antigen and antibody had a different tolerance for the fixation and permeabilization protocol, we next identified the optimal staining time for surface antigens. Antibodies for myeloid (anti-mouse Gr-1 Pacific Blue, 108430, Biolegend Japan, Tokyo, Japan ; anti-mouse Mac-1 Pacific Blue, 101224, Biolegend Japan) and lymphoid (anti-mouse B220 APC-Cy7, 25-0452-82, eBioscience ; anti-mouse CD4 APC-Cy7, 100414, Biolegend Japan ; anti-mouse CD8a APC-Alexa Fluor 780, 47-0081-82, eBioscience) antigens showed measurable intensity, even when added before fixation and permeabilization. By contrast, anti-CD45 antibodies (anti-mouse CD45.1 PE-Cy7, 25-0453-82, eBioscience ; anti-mouse CD45.2 PE, 12-0454-83, eBioscience) lost their affinity for CD 45 antigen under the same conditions. In that context, we labeled using a double staining method in which myeloid and lymphoid antigens were labeled before fixation and permeabilization, while CD45 and intracellular molecules were labeled afterward. The optimized staining protocol is summarized in **Table 1**. Using this approach, we have been able to detect myeloid cell-specific expression of STAT5A1*6 in MPN mice (**Fig. 2A**), which is indicative of the myeloid cell origin of MPN.

To determine whether this staining protocol can be applied to human cells, we checked for phosphorylated STAT5A in STAT5A1*6-transduced human ESC (khESC3, kindly provided from Dr. Suemori of Kyoto Univ.) -derived blood cells (anti-phospho STAT5 Alexa Fluor® 647 antibody, BD Japan ; anti-human CD45 PE-Cy7 antibody, BD Japan ; anti-human Glycophorin A Pacific Blue antibody, eBioscience). As indicated in **Fig. 2B**, among ESC-derived cells transduced with STAT5A1*6, we were able to detect aberrantly strong phosphorylation of STAT5A in Glycophorin A-expressing erythroid cells using multicolor staining (Shimizu T et al., in submission).

In conclusion, we have optimized a method

Table 1 Detection procedure for lineage-specific STAT5A phosphorylation by multi-color flow cytometry using peripheral blood cells

Step	Procedure
1	Sampling peripheral blood
2	Count WBC number
3	Adjust $1x10^6$ cells/ sample tube
4	Lyse RBCs with 1ml of 140mM NH_4CL for 20 min at RT
5	Staining with anti-myeloid and lymphoid marker antibodies
6	Fix with pre-warmed($37C^o$) BD Phosflow Fix Buffer for 10 min at $37C^o$
7	Wash cells once
8	Permerbilize cells with BD Perm Buffer III for 30 min at $4C^o$
9	Blocking with Affinity Purified Mouse IgG1, k, for 30 min at RT
10	Staining with anti-CD45 and anti-phospho STAT5 (or anti-FLAG, if needed)
11	Analyze by flow cytometry

for intracellular molecular staining together with multicolor surface antigen staining of peripheral blood cells from MPN mice. Further analysis of MPN patients using this method would be expected to clarify the relationship between STAT5A phosphorylation and MPN disease type selection and progression.

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骨髄増殖性疾患への診療応用を目標としたマルチカラーフローサイトメトリーによる細胞内リン酸化 STAT5A 分子と表面分化抗原との同時染色法の開発

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古典的な骨髄増殖性疾患（classical myeloproliferative neoplasm；classical MPN）である真性多血症（polycythemia vera；PV）の大多数や本態性血小板血症（Essential thrombocythemia；ET）ならびに原発性骨髄線維症（Primary myelofibrosis；PMF）の一部において JAK2 遺伝子変異が報告されている．当研究室では JAK2 下流因子の一つである STAT5A の活性型（STAT5A1*6）を造血幹細胞に遺伝子導入して骨髄移植を行い，MPN モデルマウスの作製に成功している（Kato Y et al., 2005, *JEM*）．本研究ではフローサイトメトリー（FCM）を用いて MPN モデルマウス末梢血中の各種細胞群における STAT5A1*6 発現解析ならびにヒト血液細胞内におけるリン酸化 STAT5A 発現解析を試みた．

細胞内蛋白質である STAT5A の解析は，細胞固定，膜透過処理後に染色する必要がある．我々は Phosflow 用試薬（BD 社）を用い，細胞固定，膜透過処理及び染色条件の最適化を行った結果，MPN モデルマウス末梢血において，種々の分化抗原マーカーを同時染色しつつ，STAT5A1*6 を定量的に解析することに成功し，同モデルマウスでは末梢血顆粒球分画特異的に活性化 STAT5A の強発現が見られることが明らかになった．また，本法を用いてヒト末梢血単核球やヒト胚性幹細胞由来血液細胞を解析した結果，活性化 STAT5A を表面抗原と同時染色することが可能であった．末梢血における分画別活性化 STAT5A 定量が，MPN 診療に貢献できる可能性を示唆している．

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