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Negative regulation of hematopoiesis by the *fused in myeloproliferative disorders* gene product

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Abstract

The t(8;13) translocation, found in a rare and aggressive type of stem cell myeloproliferative disorder, leads to the generation of a fusion protein between the N-terminal gene product of fused in myeloproliferative disorders (FIM)/ZNF198 and the fibroblast growth factor receptor 1 (FGFR1) kinase domain. The chimeric protein was reported to have constitutively activated tyrosine kinase activity. However, little is known about a role of FIM in hematopoietic cell regulation. Here we show that FIM protein is ubiquitously expressed in mouse embryonic tissues but much less in hematopoietic cells. We also show that forced expression of FIM inhibits the emergence of hematopoietic cells in the cultured mouse aorta-gonad-mesonephros (AGM) region on embryonic day (E) 11.5, where definitive hematopoiesis is first found during embryogenesis. These results suggest that the expression level of FIM determines the development of hematopoiesis during mouse ontogeny.

Keywords: FIM; AGM; Hematopoiesis; Myeloproliferative disorder; Development

It has been shown that some leukemia-related genes play novel roles in developmental hematopoiesis. For example, the *RUNX1/AML1* gene, translocation of which has shown in acute myeloid leukemia [1], is required for the establishment of definitive hematopoiesis [2]. A stem cell myeloproliferative disorder with a multilineage involvement is associated with at least four different translocations with a breakpoint in region p11–12 of chromosome 8: t(6;8)(q27;p11), t(8;9)(p11;q33), t(8;13)(p12;q12), and t(8;19)(p12;q13.3), respectively. On the arm of chromosome 8, the *fibroblast growth factor receptor 1* (*FGFR1*) gene is involved in each case [3]. It has been shown that the partner genes of *FGFR1* on chromosomes 6q27, 9q33, 13q12, and 19q13.3 are *FGFR1 oncogene partner* (*FOP*) [4], *the centrosomal protein 110* (*CEP110*) [5], *fused in myeloproliferative disorders* (*FIM*) [6], and endogenous retrovirus HERV-Ks sequence [7], respectively. The 13q12 breakpoint gene has also been characterized as *ZNF198* [8] and *RAMP* [9].

The t(8;13)(p12;q12) rearrangement produces a fusion protein of FGFR1 and FIM protein which includes the five zinc finger motifs of FIM and the intracellular tyrosine kinase domain of FGFR1 [6,8]. Stable expression of the FIM–FGFR1 fusion protein in Ba/F3 cells resulted in IL-3 independent cell survival and proliferation, in which constitutive dimerization of the fusion protein was observed [10,11]. The localization of the FIM–FGFR1 fusion protein, which lacks the nuclear localization signals of FIM, was cytoplasmic, whereas that of FIM protein was nuclear [10]. Except for its distribution, the functions of FIM protein remain to be elucidated. In *Drosophila*, larvae for the recessive lethal allele *without children* (*woc*), the *Drosophila* homologue of *FIM*, are characterized by remarkable increase in the size of their hematopoietic organs and failure to pupariate [12]. Based on these results, we hypothesized that FIM negatively regulates hematopoiesis and controls the emergence of hematopoietic lineages during development.

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Materials and methods

Materials. Human FIM cDNA [6] was cloned into the pMY-IRES-GFP retrovirus vector which was designed to express both the inserted genes and GFP protein to ensure the detection of virus-infected cells.

Animals and cell culture. Timed-pregnant ICR mice were used to prepare cells from the aorta-gonad-mesonephros (AGM) regions. AGM cells were prepared from E11.5 mice and cultured as described previously [13].

Flow cytometry analysis and cell sorting. After 1 week of culture, nonadherent cells were collected and residual adherent cells were obtained by treatment with cell-dissociation buffer (Invitrogen, Carlsbad, CA). After washing in PBS containing 3% (v/v) fetal calf serum and 0.05% sodium azide, the cells were incubated for 20 min on ice with phycoerythrin-conjugated rat anti-mouse CD45 (30-F11) (Becton–Dickinson, Lincolin, NJ) and analyzed by FACScalibur (Becton–Dickinson). Sorting of GFP-positive and CD45-positive or Ter119 (TER119)-negative cells was performed by the FACScalibur sorting system (Becton–Dickinson).

RT-PCR. Total RNAs were isolated from E11.5 AGM regions or sorted cells that had been cultured for 7 days. cDNAs were synthesized from the 5 μg of the total RNAs as templates using Superscript II reverse transcriptase (Gibco-BRL, Rockville, MD). PCRs were performed using rTaq (Takara Biotechnology, Otsu, Japan). The primer sets used were as follows: 5′-gcttacgatgacagatatgatg-3′ and 5′-cctgatacagccttcctcttgg-3′ for FIM, 5′-ccttgtgaggccagagagtgt-3′ and 5′-tccctttgccagatgccttgc-3′ for GATA-1, 5′-accacccgatacccacctat-3′ and 5′-gccatggcagtcaccatgct-3′ for GATA-2, 5′-acgtctcactctcgaggcagcatg-3′ and 5′-gaagtcctccagcgcgtcatgcac-3′ for GATA-3, 5′-ccgtagatggcacgagc cgc-3′ and 5′-catgacggtgaccagagtgccatcc-3′ for AML1, 5′-atgctcaaacca agtgccca-3′ and 5′-gtacacgcagctgaaaatgc-3′ for c-Kit, and 5′-accacagtcc atgccatcac-3′ and 5′-tccaccaccctgttgctgta-3′ for G3PDH.

Immunohistochemistry. Mouse FIM was isolated from cDNAs of the AGM region at E11.5. The amino acid sequence of mouse FIM showed 96% homology with that of human FIM (data not shown) and we designed the antibodies using a peptide in the carboxyl terminal region as the antigen.

Mouse embryos were fixed in 4% paraformaldehyde/PBS, embedded in tissue-Tek, and frozen in liquid nitrogen. As much as 8–10 μm serial sections were stained with non-immune rabbit IgG, anti-FIM antibody, or anti-Ter119 antibody, respectively, followed by washing and detection with a Vectastain ABC-PO kit (Vector Laboratories, Burlingame, CA).

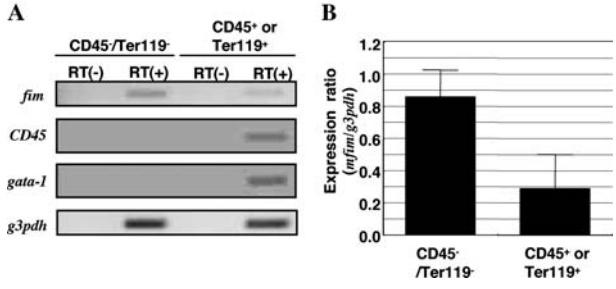

Fig. 1. The expression level of FIM. (A) RT-PCR was performed using RNA from sorted cells of mouse E11.5 AGM. cDNAs were used in PCRs using specific primers for FIM, CD45, and GATA-1, with G3PDH as a control. A representative experiment of three independent experiments is shown. (B) FIM expression was quantified using NIH Image. FIM was normalized against G3PDH expression. The relative FIM expressions (means ± SEM) of three independent experiments are shown.

Results and discussion

Hematopoiesis in the mouse embryo begins in the yolk sac, where blood islands of mesoderm origin develop at embryonic day (E) 8. Generation of the primitive erythroid lineage in the yolk sac is known as primitive hematopoiesis. In the developing embryo, mesoderm-derived precursor cells migrate into an environment permissive for hematopoiesis that is located in the AGM region [14]. Definitive hematopoiesis encompasses the development of all lineages, other than primitive erythroid, and includes definitive erythroid, myeloid, and lymphoid cells. Initially, we examined FIM expression in the AGM region at E11.5. Cells in the AGM region expressed mRNA for the *FIM* gene. The expression levels in hematopoietic cells were obviously lower than those in non-hematopoietic cells (Fig. 1). To further characterize the expression pattern of the *FIM* gene product, we also performed an immunohistochemical analysis of the AGM region with the anti-FIM antibody. The data demonstrated that FIM protein was ubiquitously expressed in the non-hematopoietic tissues, including endothelial cells, but much less in hematopoietic cells (Fig. 2). These results suggest that the expression level of the *FIM* gene may be negatively correlated with the progression of hematopoiesis.

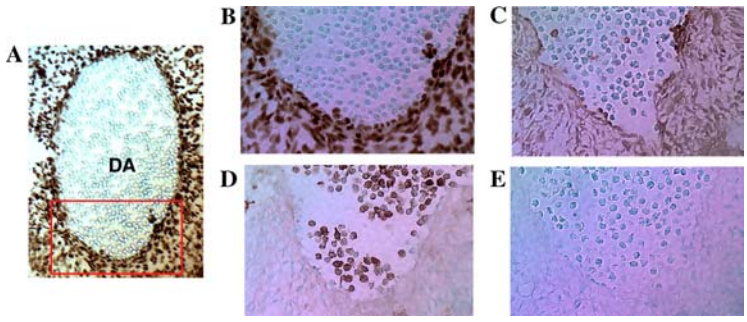

Fig. 2. FIM expression remarkably decreases in hematopoietic cells in the AGM region. (A–E) FIM and Ter119 immunostaining in the dorsal aorta using an avidin–biotin complex method. (A) HRP detection of FIM (brown). (B) High magnification of the red square region in (A). (C) Blocking by the FIM peptide antigen. (D) HRP detection of Ter119 (brown). (E) Staining with pre-immuned rabbit IgG as a negative control.

To test this hypothesis, we examined the effect of the FIM products on hematopoietic development using an in vitro culture of the AGM region and a retroviral gene transfer system. Recent studies have established the in vitro culture in which hematopoietic progenitors and differentiated cells develop from the AGM region of mouse embryos at E11.5 [13]. Three days after infection, expression of GFP in vehicle- and FIM-infected cases was observed in the adherent cells (Fig. 3A). The percentage of GFP positive cells in the adherent cells on day 5 of infection (Fig. 3B) was similar to that on day 3 (Fig. 3A). These results indicated that retrovirus-mediated forced expression of FIM has no significant influence on the survival of the adherent cells in the AGM culture. However, forced expression of FIM almost completely inhibited the emergence of CD45-positive nonadherent cells (Fig. 3C). The percentage of the $CD45^+GFP^+$ nonadherent cells to the total $CD45^+$ nonadherent cells in the FIM-infected culture was obviously lower (0.7%) than that in the vector-infected culture (32.2%). These data suggest that FIM inhibits the emergence of hematopoietic cells from the AGM culture presumably from adherent cells. Cultured AGM cells overexpressing FIM showed limited expression of mRNAs for essential hematopoietic transcription factors, GATA-2, and AML1 (Fig. 4). These data imply that FIM inhibits hematopoietic cell development in the AGM region at least in vitro.

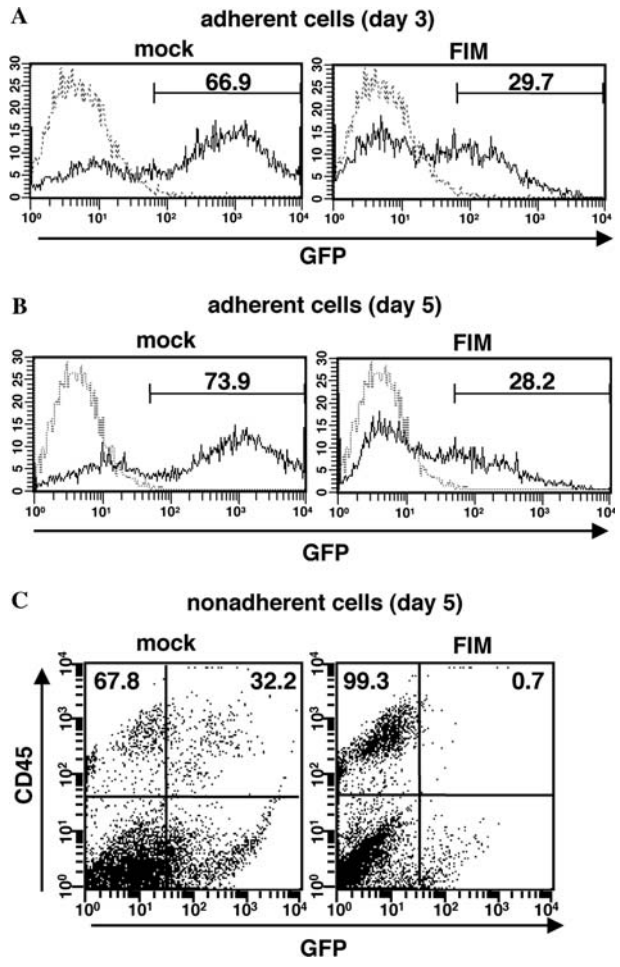

Fig. 3. Forced expression of FIM inhibits hematopoiesis in the AGM culture. E11.5 AGM cells were cultured with SCF (100 ng/ml), bFGF (1 ng/ml), and OSM (10 ng/ml). On day 2 of culture, cells were infected with retrovirus encoding IRES-GFP alone (left) or human FIM-IRES-GFP (right). (A) Three days after infection, adherent cells were treated with cell-dissociation buffer and expression of GFP was analyzed by flow cytometry. Dotted line indicates non-infected-adherent cells and the number shows the percentage of GFP positive cells. (B) Five days after infection, adherent cells were analyzed by flow cytometry. The number shows the percentage of GFP positive cells. Dotted line indicates no infected-adherent cells and the number shows the percentage of GFP positive cells. (C) Five days after infection, nonadherent cells were stained with anti-CD45 antibody. The number shows the percentage of the $CD45^+GFP^-$ or $CD45^+GFP^+$ nonadherent cells to the total $CD45^+$ nonadherent cells.

It has been shown that development of hematopoiesis requires the expression of several transcription factors, for example, GATA-1 [15], GATA-2 [16], AML1 [2], LMO2 [17], SCL [18], and c-myb [19,20]. However, little is known about negative regulatory factors involved in hematopoietic cell development. In *Drosophila*, larvae homozygous for the recessive lethal allele *woc*, the *Drosophila* homologue of *FIM*, are characterized by an up to 100-fold increase in the size of their hematopoietic organs, in which there is an obvious increase in the number of hemocyte precursors [12]. In this report, we showed that retrovirus-mediated expression of FIM inhibited hematopoiesis in the AGM culture. FIM-expressing cells showed no detectable transcripts for either the *GATA-2* or *AML1* gene (Fig. 4). These results suggest that FIM may negatively control differentiation from hemangioblasts to hematopoietic cells by inhibiting the expression of hematopoietic transcriptional factors. Our present results raise a possibility that chromosomal translocation involving the FIM gene may attenuate the negative regulatory function of FIM. In addition to constitutive activation of the tyrosine kinase domain of FGFR1 in the

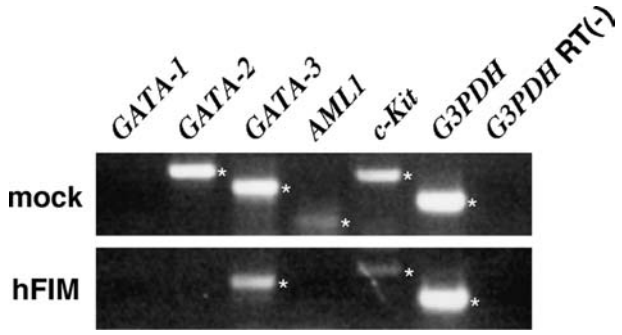

Fig. 4. Loss of GATA2 and AML-1 expression in FIM expressing cultured AGM cells. Retrovirus-infected AGM cells were cultured for 6 days and then sorted by GFP. Total RNA was isolated from GFP^+ cells and reverse transcribed. The cDNA was used in PCRs with specific primers for GATA-1, GATA-2, GATA-3, AML1, c-Kit, and G3PDH. Asterisks indicate specific bands.

FIM–FGFR1 fusion protein [6], loss of a presumable negative regulatory domain of FIM in this fusion protein (current study) might contribute to pathogenesis of myeloproliferative disorders. It has been reported that several extracellular factors like bone morphogenetic protein-4 (BMP4) [21] and oncostatin M regulate hematopoietic development [22]. To examine the effect of FIM on signaling pathways initiated by ligands, we have tried transactivation assays for their respective downstream transcription factors, Smads, and STATs [23–25]. However, FIM had no effect on the signaling pathways involving these transcription factors (data not shown). Further biochemical studies will reveal the role of FIM in hematopoietic cell regulation during mouse development or in leukemogenesis in more detail.

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