One nonsense mutation, p.R578X, was identified in three individuals (Table 1). This mutation appears to be specific to the British population.[13] Notably, one patient reported by Kawasaki *et al.* was a compound heterozygote for a nonsense (p.E2857X) and missense (p.G2575R) mutation; this patient presented with moderate clinical severity RDEB-nHS, but developed very early-onset SCC of an aggressive nature.[14] In contrast, a second patient with RDEB-HS, who was a compound heterozygote for two different nonsense mutations (p.R525X and p.R578X) resulting in a lack of detectable type VII collagen expression in both skin and associated tumors, developed his first SCC at 35 years old.[12] There has been no evidence suggesting the correlation between clinical severity, *COL7A1* mutations, and tumor activity. Until these relationships are elucidated, all RDEB patients should be monitored closely for the development of SCC. As there are only scanty data from previous case reports documenting *COL7A1* mutations in SCC arising in RDEB in which SNB performed, it remains to be clarified if SNB in combination with gene diagnosis is beneficial for the management of associated SCC in RDEB patients. To answer this question, *COL7A1* mutation analysis should be performed in as many cases of RDEB as possible.

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## REFERENCES

1 Fine JD, Eady RA, Bauer EA *et al.* Revised classification system for inherited epidermolysis bullosa: Report of the Second International Consensus Meeting on diagnosis and classification of epidermolysis bullosa. *J Am Acad Dermatol* 2000; **42**: 1051–1066.
2 Yamada M, Hatta N, Sogo K, Komura K, Hamaguchi Y, Takehara K. Management of squamous cell carcinoma in a patient with recessive-type epidermolysis bullosa dystrophica. *Dermatol Surg* 2004; **30**: 1424–1429.
3 Weber F, Bauer JW, Sepp N *et al.* Squamous cell carcinoma in junctional and dystrophic epidermolysis bullosa. *Acta Derm Venereol* 2001; **81**: 189–192.
4 Perez-Naranjo L, Herrera-Saval A, Garcia-Bravo B, Perez-Bernal AM, Camacho F. Sentinel lymph node biopsy in recessive dystrophic epidermolysis bullosa and squamous cell carcinoma. *Arch Dermatol* 2005; **141**: 110–111.
5 Christiano AM, Amano S, Eichenfield LF, Burgeson RE, Uitto J. Premature termination codon mutations in the type VII collagen gene in recessive dystrophic epidermolysis bullosa result in nonsense-mediated mRNA decay and absence of functional protein. *J Invest Dermatol* 1997; **109**: 390–394.
6 Pulkkinen L, Uitto J. Mutation analysis and molecular genetics of epidermolysis bullosa. *Matrix Biol* 1999; **18**: 29–42.
7 Wagner JD, Evdokimow DZ, Weisberger E *et al.* Sentinel node biopsy for high-risk nonmelanoma cutaneous malignancy. *Arch Dermatol* 2004; **140**: 75–79.
8 Fine J-D, Johnson LB, Moshell A, Suchindran C. The risk of selected major extracutaneous outcomes in inherited epidermolysis bullosa. In: Fine J-D, Bauer EA, McGuire J, Moshell A (eds). *Epidermolysis Bullosa*. The Johns Hopkins University Press, Baltimore and London 1999; 193–205.
9 Bosch RJ, Gallardo MA, Ruiz del Portal G, Snchez P, Arce MF, Herrera E. Squamous cell carcinoma secondary to recessive dystrophic epidermolysis bullosa: report of eight tumours in four patients. *J Eur Acad Dermatol Venereol* 1999; **13**: 198–204.
10 Mallipeddi R. Epidermolysis bullosa and cancer. *Clin Exp Dermatol* 2002; **27**: 616–623.
11 Ortiz-Urda S, Garcia J, Green CL *et al.* Type VII collagen is required for Ras-driven human epidermal tumorigenesis. *Science* 2005; **307**: 1773–1776.
12 Pourreyron C, Cox G, Mao X *et al.* Patients with recessive dystrophic epidermolysis bullosa develop squamous-cell carcinoma regardless of type VII collagen expression. *J Invest Dermatol* 2007; **127**: 2438–2444.
13 Mellerio JE, Dunnill MG, Allison W *et al.* Recurrent mutations in the type VII collagen gene (COL7A1) in patients with recessive dystrophic epidermolysis bullosa. *J Invest Dermatol* 1997; **109**: 246–249.
14 Kawasaki H, Sawamura D, Iwao F *et al.* Squamous cell carcinoma developing in a 12-year-old boy with non-Hallopeau-Siemens recessive dystrophic epidermolysis bullosa. *Br J Dermatol* 2003; **148**: 1047–1050.

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SURGERY TODAY


*Case Reports*

# Combined Autologous Cellular Cardiomyoplasty Using Skeletal Myoblasts and Bone Marrow Cells for Human Ischemic Cardiomyopathy with Left Ventricular Assist System Implantation: Report of a Case

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**Abstract**
Myocardial regeneration therapy shows great promise as a treatment for heart failure. We recently introduced combined autologous cellular cardiomyoplasty with skeletal myoblasts and bone marrow cells as a treatment for human ischemic cardiomyopathy. We report the results of our first clinical trial of this technique, used to treat a patient with severe heart failure caused by ischemic cardiomyopathy who was being managed with a left ventricular assist system (LVAS). After combined cell transplantation, the patient showed signs of improved cardiac performance and angiogenesis, and reduced fibrosis.

**Key words** Cell transplantation · Heart failure · Left ventricular assist device

## Introduction

Recent evidence shows that cellular cardiomyoplasty has potential fundamental regenerative capability, and it has already been introduced in clinical trials using skeletal myoblasts[1] or bone marrow mononuclear cells (BM-MNCs).[2] Myoblast transplantation offers a potential therapeutic treatment for end-stage heart disease with no risk of arrhythmia.[1] In one clinical trial, BM-MNCs were found to effectively treat acute myocardial infarction by enhancing angiogenesis.[2] Furthermore, an adequate supply of angiogenic and growth factors has an important adjuvant effect in cellular cardiomyoplasty, because reduced regional blood flow restricts myoblast survival and growth in ischemic cardiomyopathy.[3] We previously reported that myoblast grafting combined with BM-MNC transplantation in dogs enhanced both myogenesis and angiogenesis, leading to the restoration of myocardium damaged by ischemia.[4] Based on this evidence, we decided to use this therapy to assess the safety of combined cell therapy in patients with severe ischemic cardiomyopathy managed with left ventricular assist system (LVAS). To the best of our knowledge, this is the first report of a patient treated with both BM-MNCs and myoblasts after LVAS implantation.

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## Case Report

A 53-year-old man with a history of two acute anterior myocardial infarctions suffered a third infarction of the left main trunk. The resulting cardiogenic shock was managed with percutaneous cardiopulmonary support (PCPS). He was weaned off PCPS in a few days but remained dependent on high-dose catecholamines. He had a large area of infarction in the left ventricular (LV) anterior and lateral wall and little viability was detected by Tl scintigraphy. Thus, combined cell therapy was approved by the Ethical Committee and Internal Review Board of Osaka University. After obtaining informed consent from the patient, a piece of skeletal muscle was excised from his medial vastus muscle and myoblasts were cultured according to published procedures.[1]

**Table 1.** Change in functional measures after cell transplantation (LVAS off test)

| | Pre Tx | 1 month after Tx | 3 months after Tx |
|---|---|---|---|
| EF (%) | 22 | 22.5 | 32 |
| Dd/Ds (mm) | 59/55 | 53/48 | 63/53 |
| ABP (mmHg) | 84/57 (69) | 87/52 (65) | 76/42 (51) |
| PAP (mmHg) | 57/29 (41) | 40/27 (34) | 32/14 (28) |
| CI (l/min/kg) | 2.09 | 1.76 | 1.7 |
| $SvO_2$ (%) | 55.5 | 60.6 | 60.7 |
| CVP (mmHg) | 13 | 11 | 7 |

LVAS, left ventricular assist system; Tx, cell transplantation; EF, ejection fraction; Dd/Ds, end-diastolic/end-systolic dimension; ABP, arterial blood pressure; PAP, pulmonary artery pressure; CI, cardiac index; $SvO_2$, venous oxygen saturation; CVP, central venous pressure

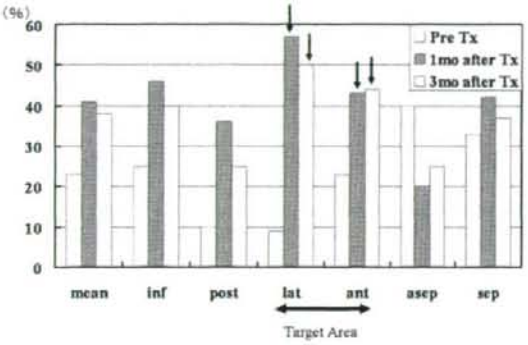


**Fig. 1.** The Color Kinesis index revealed restoration of regional diastolic function in the transplanted lesion in the anterior and lateral wall. *Arrows* indicate the improvement in regional diastolic function in the lesion post transplantation compared with the same lesion before transplantation. *Tx*, cell transplantation; *mo*, month; *inf*, inferior; *post*, posterior; *lat*, lateral; *ant*, anterior; *asep*, anteroseptal; *sep*, septal

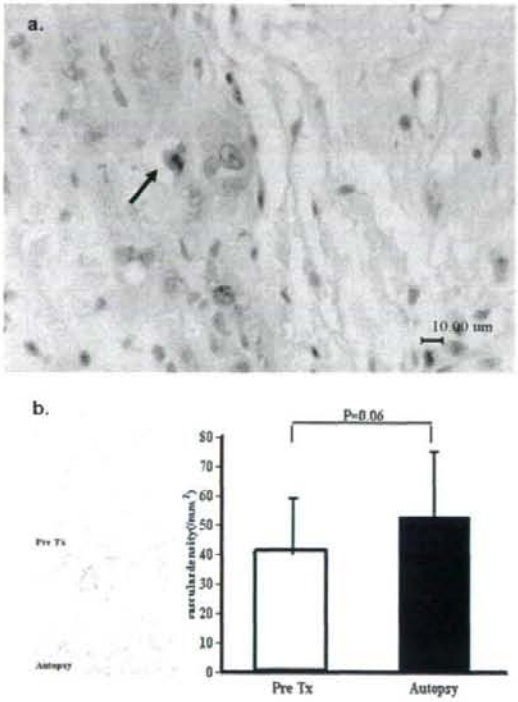


**Fig. 2. a** Detection of transplanted myoblasts. Fast-isoform myosin heavy chain-positive cells were detected in the transplantation site (*arrow*). **b** Induction of angiogenesis after cell transplantation. The number of vessels in the lesion was higher after cell transplantation than in the pre-cell-transplantation sample

Fluorescence-activated cell sorter (FACS) analysis with CD 56 antibody (BD, Rockville, MD, USA) revealed 46.6% purity of myoblasts. During the culture his heart failure worsened, so we performed left ventriculoplasty, as overwrapping, and mitral annuloplasty combined with extracorporeal pneumatic LVAS (Toyobo, Tokyo, Japan) implantation. About 3 months after this operation, we performed a left thoracotomy and, using a syringe, injected myoblasts ($3 \times 10^8$ cells/3 ml) and BM-MNCs ($1 \times 10^8$ cells/3 ml) separately into 30 points each, respectively, in the area of the transmural anterior and lateral myocardial infarction. We aspirated bone marrow from the ileum and sorted MNCs on a CS3000-Plus blood-cell separator (Baxter, Deerfield, IL, USA) according to published procedures.[5] The follow-up examinations showed a significant decrease in BNP, from $825 \pm 565$ pg/ml before to $275 \pm 167$ pg/ml after cell transplantation ($P < 0.05$), and an increase in the ejection fraction, from 22% before to 32% 3 months after cell transplantation in serial LVAS-off tests (Table 1). There was also an improvement in regional diastolic function, as assessed by the Color Kinesis index according to published procedures[6] (Fig. 1). No detrimental effects such as life-threatening arrhythmias were observed during follow-up. Unfortunately, the patient died of bacterial sepsis 466 days after surgery.

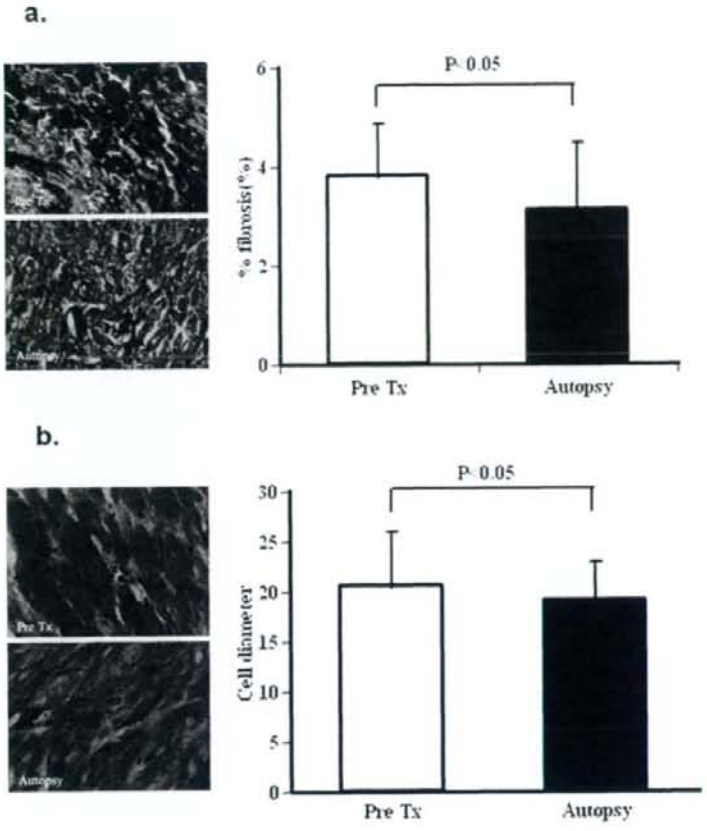


**Fig. 3.** **a** Reduction of percent fibrosis after cell transplantation. The percent fibrosis after cell transplantation was significantly lower than in the pre-cell-transplantation sample. **b** Shortening of the minor axis of host cardiomyocytes after cell transplantation. The hypertrophy of the host cardiomyocytes recovered remarkably compared with the pre-cell-transplantation sample

Immunostaining of fast-isoform myosin heavy chain (Sigma, St. Louis, MO, USA) of the autopsy sample revealed transplanted myoblasts in the site of transplantation (Fig. 2). Immunohistochemical staining of factor VIII-related antigen (DAKO EPOS Anti-Human Von Willebrand Factor/HRP; DAKO, Glostrup, Denmark) revealed that vascular density was higher (LVAS implantation vs autopsy = 40.53 ± 22.09/mm$^2$ vs 52.82 ± 18.72/mm$^2$; $P = 0.06$), the percent fibrosis (Masson's trichrome stain) was significantly lower (LVAS implantation vs autopsy = 3.78% ± 1.08% vs 3.11% ± 1.35%; $P < 0.05$) (Fig. 3) and the cell diameter was significantly shorter (LVAS implantation vs autopsy = 20.55 μm ± 5.37 μm vs 18.89 μm ± 3.98 μm; $P < 0.05$) than in samples obtained at the time of LVAS implantation.

## Discussion

We decided to perform combined cell therapy instead of single cell therapy for this patient based on our finding that combined cell therapy has a more synergistic effect on severely damaged myocardium. To our knowledge, this is the first case in which both myoblasts and BM-MNCs were transplanted into human ischemic myocardium in a patient managed with LVAS, and recovery of cardiac function and histological changes were observed. However, we could not establish if the dramatic functional improvement was attributable to decreased LV distension by LVAS or to the cell transplantation because of the lack of an appropriate control. The BNP levels were significantly lower after cell transplantation than under LVAS before cell transplantation. For patients with ischemic cardiomyopathy, LVAS implantation does not achieve sufficient recovery of myocardial function.[7] Thus, we assume that the increased cardiac function was due to cell transplantation, although further clinical studies must be done to analyze the effects of cell transplantation. After LVAS implantation, decreased LV distension contributed to the reduced cell diameter and lower BNP, in accordance with previous reports. We detected other changes such

as improved regional diastolic function and vascular density in the targeted region in addition to the changes evoked by LVAS implantation. This indicates that cell transplantation had a positive effect on the distressed ischemic myocardium.

Enhanced expression of angiogenic factors by the transferred cells might induce angiogenesis and reduce fibrosis. We speculated that the transplanted cells, which have high elasticity, survived in the ischemic myocardium and may have contributed to regional diastolic improvement at the site of transplantation. Thus, combined therapy with myoblast and BM-MNCs transplantation could become part of the armamentarium of regenerative therapies for severely damaged ischemic myocardium.

## References

1. Dib N, Michler RE, Pagani FD, Wright S, Kereiakes DJ, Lengerich R, et al. Safety and feasibility of autologous myoblast transplantation in patients with ischemic cardiomyopathy: four-year follow-up. Circulation 2005;112(12):1748–55.
2. Perin EC, Dohmann HF, Borojevic R, Silva SA, Sousa AL, Mesquita CT, et al. Transendocardial, autologous bone marrow cell transplantation for severe, chronic ischemic heart failure. Circulation 2003;107(18):2294–302.
3. Miyagawa S, Sawa Y, Taketani S, Kawaguchi N, Nakamura T, Matsuura N, et al. Myocardial regeneration therapy for heart failure: hepatocyte growth factor enhances the effect of cellular cardiomyoplasty. Circulation 2002;105(21):2556–61.
4. Memon IA, Sawa Y, Miyagawa S, Taketani S, Matsuda H. Combined autologous cellular cardiomyoplasty with skeletal myoblasts and bone marrow cells in canine hearts for ischemic cardiomyopathy. J Thorac Cardiovasc Surg 2005;130(3):646–53.
5. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, et al. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 2002;10; 360(9331):427–35.
6. Ishii K, Miwa K, Makita T, Okuda N. Prolonged postischemic regional left ventricular delayed relaxation or diastolic asynchrony detected by color kinesis following coronary vasospasm. Am J Cardiol 2003;91(11):1366–69.
7. Mancini DM, Beniaminovitz A, Levin H, Catanese K, Flannery M, DiTullio M, et al. Low incidence of myocardial recovery after left ventricular assist device implantation in patients with chronic heart failure. Circulation 1998;98:2383–9.

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# FIP1L1-PDGFRα imposes eosinophil lineage commitment on hematopoietic stem/progenitor cells

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Running title: Enforced eosinophil development by FIP1L1-PDGFRα.

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**Although leukemogenic tyrosine kinases (LTKs) activate a common set of downstream molecules, the phenotypes of leukemia caused by LTKs are rather distinct. Here we report the molecular mechanism underlying the development of hypereosinophilic syndrome/chronic eosinophilic leukemia by FIP1L1-PDGFRα. When introduced into c-Kit$^{high}$Sca-1$^{+}$Lineage$^{-}$ cells (KSLs), FIP1L1-PDGFRα conferred cytokine-independent growth on these cells and enhanced their self-renewal, while it did not immortalize common myeloid progenitors (CMPs) in *in vitro* replating assays and transplantation assays. Importantly, FIP1L1-PDGFRα but not TEL-PDGFRβ enhanced the development of Gr-1$^{+}$IL-5Rα$^{+}$ eosinophil progenitors from KSLs. FIP1L1-PDGFRα also promoted eosinophil development from CMPs. Furthermore, when expressed in megakaryocyte/erythrocyte progenitors (MEPs) and common lymphoid progenitors (CLPs), FIP1L1-PDGFRα not only inhibited differentiation towards erythroid cells, megakaryocytes, and B-lymphocytes, but aberrantly developed eosinophil progenitors from MEPs and CLPs. As for the mechanism of FIP1L1-PDGFRα-induced eosinophil development, FIP1L1-PDGFRα was found to more intensely activate MEK1/2 and p38MAPK than TEL-PDGFRβ. In addition, a MEK1/2 inhibitor and a p38MAPK inhibitor suppressed FIP1L1-PDGFRα-promoted eosinophil development. Also, RT-PCR analysis revealed that FIP1L1-PDGFRα augmented the expression of C/EBPα, GATA-1 and GATA-2, while it hardly affected PU.1 expression. In addition, shRNAs against C/EBPα and GATA-2, and GATA-3KRR that can act as a dominant-negative form over all GATA members inhibited FIP1L1-PDGFRα-induced eosinophil development. Furthermore, FIP1L1-PDGFRα and its downstream Ras were inhibited PU.1 activity in luciferase assays. Together, these results indicate that FIP1L1-PDGFRα enhances eosinophil development by modifying the expression and activity of lineage-specific transcription factors through Ras/MEK and p38MAPK cascades.**

During the last decade, it has become clear that hematopoietic growth factors regulate only growth and survival of hematopoietic cells, while lineage-specific transcription factors such as GATA-1, GATA-3, PU.1, Pax-5, C/EBPα and C/EBPε crucially control the lineage commitment and lineage-specific differentiation. For example, G-CSF signaling induced megakaryopoiesis in G-CSF receptor(R)-transgenic mice (1). Also, erythropoietin (EPO) was found to promote terminal granulocytic differentiation in EPO-R-transgenic mice. These data led us to consider that signal transduction molecules activated by hematopoietic growth factors would not influence the lineage commitment of hematopoietic stem cells/progenitor cells (HSCs/HPCs) or subsequent lineage-specific differentiation (2). However, it has very recently been shown that the MEK/ERK pathway is involved in myeloid lineage commitment (3). Also, PKB (c-Akt) was shown to be involved in

lineage decision during myelopoiesis (4). In addition, FLT3 activating mutations were proved to inhibit C/EBPα activity through ERK1/2-mediated phosphorylation (3,5). These results suggest that signal transduction molecules activated by hematopoietic growth factors or their genetic mutations would not only promote growth and survival but also influence lineage commitment and subsequent differentiation of hematopoietic cells.

Activating mutations of the tyrosine kinases (TKs) such as c-Kit, platelet-derived growth factor receptor (PDGFR), FLT3, and c-ABL are provoked by several mechanisms, including chromosomal translocations and various mutations involving their self-regulatory regions. These mutations are often involved in the pathogenesis of various types of hematologic malignancies. BCR-ABL is known to cause chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia, Most of patients with PDGFRβ rearrangement reveal common clinical features resembling CML or choronic myelomonocytic leukemia (CMML). In contrast, FLT3 mutations (ITD and point mutations in the TK domain) are primarily detectable in acute myeloid leukemia (AML) or myelodysplastic syndromes (MDS) (6-8). Also, c-Kit mutations in the TK domain (Asp816 to Val, Tyr, Phe, His) are found in patients with aggressive mastocytosis, MDS, and AML (9-15). Although these leukemogenic TKs (LTKs) activate a common set of downstream signaling molecules such as Ras/MAPK, PI3-K/Akt/mTOR, and STATs, the mechanisms by which LTKs cause different disease phenotypes remain to be clarified.

FIP1L1-PDGFRα is a fusion gene, which was originally identified in the patients with hypereosinophilic syndrome (HES)/chronic eosinophilic leukemia (CEL) (16,17). FIP1L1-PDGFRα fusion protein supports cytokine-independent growth and survival of hematopoietic cells as a constitutively active TK (16,18-21). As for the downstream signaling molecules, FIP1L1-PDGFRα was shown to activate STAT5, PI-3K, and Ras/ERK pathways like other LTKs such as BCR-ABL, TEL-ABL, TEL-JAK2, and TEL-PDGFRβ (18). In addition, Buitenhuis *et al.* recently reported that activation of PI-3K, ERK1/2, and STAT5 is pivotal for FIP1L1/PDGFRα-induced myeloproliferation (22).

The concept of "cancer stem cell" has widely been recognized and validated in various types of cancers, including breast cancer, brain tumors, colon cancer, lung cancer, and malignant melanoma. This concept was originally established in AML as "leukemic stem cell (LSC)" (23,24). In this concept, LSCs are defined as specific leukemic cells that can cause leukemia when transplanted into NOD/SCID mice. In AML, although leukemic blasts often display relatively homogenous features, they are organized in a hierarchy. Among them, LSCs reveal the most immature $CD34^+CD38^-$ phenotype similar to normal HSCs, whereas several antigen expressions are different. LSCs, which account for only 0.2-1.0% of AML cells in the bone marrow (BM), have both abilities to self-renew and to produce restrictedly differentiated leukemia cells, thereby keeping themselves and yielding leukemia cells composing the majority (23,25,26). It is still unclear whether LSCs originate solely from HSCs or they are generated from non-stem immature cells that acquired *de novo* self-renewal ability. It has been shown that, although common myeloid progenitors (CMPs) and granulocyte/monocyte progenitors (GMPs) have very limited life spans, several leukemogenic oncogenes, such as MLL-ENL, MOZ-TIF2, and MLL-AF9, have an ability to immortalize these cells, thereby enabling them to act as LSCs (27,28). On the other hand, although LSCs in chronic phase of CML are at a HSC level, CML cells at a CMP/GMP level can act as LSCs in accelerated phase, suggesting that additional gene mutations can change the main LSC population during disease progression. From these findings, it is now speculated that the leukemia phenotype is determined by the biologic property of mutated gene and/or the lineage and differentiation state of LSCs.

In an attempt to analyze the molecular mechanisms by which each LTK causes leukemia with the specific phenotype, we introduced FIP1L1-PDGFRα, which plays a causal role in HES/CEL, into murine HSCs and various types of HPCs. As a result, we found that FIP1L1-PDGFRα specifically enhanced eosinophil development from HSCs/HPCs, and imposed the lineage conversion to eosinophil lineage on megakaryocyte/erythroid progenitors (MEP) and common lymphoid progenitors (CLPs) through Ras/MEK and p38MAPK cascades by modifying the expression and activity of lineage-specific transcription factors.

**Experimental Procedures**

*Reagents and antibodies*----- Recombinant human(h) TPO was provided by Kirin Brewery (Tokyo, Japan). Recombinant hFLT3L, hIL-6, murine(m)SCF, mIL-5, mIL-7, mGM-CSF, and hEPO were purchased from Peprotech (Hamburg, Deutschland).

*Antibodies, cell staining, and sorting*----- To isolate KSLs and CLPs, murine BM cells were stained with phycoerythrin (PE)-conjugated anti-IL-7Rα chain (SB/199) (eBioscience, San Diego, CA, USA), FITC-cojugated anti-Sca-1 (E13-161-7), APC-conjugated anti-c-Kit (2B8) monoclonal antibodies (mAbs), and biotinylated rat Abs specific for the following lineage markers: Ter119 , CD3ε (145-2C11), B220 (RA3-6B2), and Gr-1 (RB6-8C5), followed by the staining with streptavidin-PerCP/Cy5.5 (BD Bioscience Phermingen, San Diego, CA, USA). Then, KSLs and CLPs were sorted as IL-7Rα$^-$Lin$^-$Sca-1$^{hi}$c-Kit$^{hi}$ and IL-7Rα$^+$Lin$^-$Sca-1$^{lo}$c-Kit$^{lo}$ populations, respectively. For myeloid progenitor sorting, murine BM cells were stained with PE-conjugated anti-FcγRII/III (2.4G2), FITC-conjugated anti-CD34 (RAM34) (BD Bioscience Pharmingen), APC-conjugated anti-c-Kit, biotinylated anti-Sca-1, anti-IL-7Rα (SB/199) (Serotec, Raleigh, NC, USA) mAbs, and the above described lineage cocktail mAbs (BD Bioscience Pharmingen), followed by the staining with avidin-APC/Cy7 (BD Bioscience Pharmingen). After the staining, IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRII/III$^{lo}$ were sorted as CMPs, IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^+$FcγRII/III$^{hi}$ as GMPs , IL-7Rα$^-$Lin$^-$Sca-1$^-$c-Kit$^+$CD34$^-$FcγRII/III$^{lo}$ as MEPs as described previously (29). All of these HSCs and HPCs were isolated using FACS Aria (BD bioscience, San Jose, CA USA). In all analyses and sorting, dead cells were excluded by the staining with 7-Amino-Actinomycin D (7AAD) (CALBIOCHEM, La Jolla, CA, USA). Cells were stained with PE-conjugated CD125(IL-5 Receptor α-subunit, T21) and APC-conjugated Gr-1(RB6-8C5) (BD Bioscience Pharmingen) for detection of eosinophil lineage.

*Plasmids*---- Expression vectors for FIP1L1-PDGFRα and TEL-PDGFRβ were kindly provided by Dr. D.Gary Gilliland (Harvard Medical School, Boston, MA). Expression vectors for PDGFRα V561D and D842V were kindly provided by Dr. Hirota S (Hyogo Medical School, Hyogo, Japan). FIP1L1-PDGFRα, TEL-PDGFRβ, PDGFRαV561D, and PDGFRαD842V were cloned into the murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-EGFP (pMie) vector. Also, we constructed FIP1L1-PDGFRβ and TEL-PDGFRα by the PCR method and subcloned them into pMie.

Short-hairpin RNA (shRNA) interference oligonucleotides against GATA-2 and C/EBPα described in the previous papers (30,31) were cloned into a shRNA expression vector, pCS-RfA-CG, which was kindly provided by Dr. Miyoshi H (RIKEN BioResource center, Tsukuba, Japan). The retrovirus expression vector for dominant-negative GATA was constructed by cloning human GATA-3KRR cDNA that can inhibit GATA-1, GATA-2, and GATA-3 (32)into pMie.

*Cell culture and preparation*---Murine BM cells were obtained from 6–8-week old C57BL/6J mice, which were purchased from CLEA, Japan (Tokyo, Japan). After sedimentation of the red blood cells with 6% hydroxyethyl starch (HES), mononuclear cells (MNCs) were separated by density gradient centrifugation, using HISTOPAQUE 1083 (SIGMA DIAGNOSIS St.Louis,MD, USA). KSLs, CMPs, GMPs and MEPs were purified from MNCs, and cultured in RPMI1641 medium (Nacalai tesque, Kyoto, Japan) supplemented with 10% FCS (EQUITECH-BIO, Kerrville, TX, USA) in the presence of mSCF (50 ng/ml), hFLT3L (10 ng/ml), and hIL-6 (50 ng/ml), and hTPO (50 ng/ml) for 48 h, and the cells were subjected to the retrovirus infection.

*Retrovirus transduction*---The conditioned media containing high titer retrovirus particles were prepared as described previously (33). Briefly, an ecotropic packaging cell line, 293gp kindly provided by Dr. Miyoshi H (RIKEN BioResource center, Tsukuba, Japan), was transfected with each retrovirus vector by calcium phosphate coprecipitation method. After 12 h, the cells were washed and cultured for 48 h. To produce lentivirus, 293T cells were transfected with each shRNA expression vector together with a packaging vector (pCAG-HIVgp) and a lentivirus envelope and Rev construct (pCMV-VSV-G-RSV-Rev), both

of which were provided by Dr. Miyoshi H. Then, the supernatant containing virus particles was collected, centrifuged, and concentrated by 50-fold in volume. The precultured murine BM cells were infected with each retrovirus in the RPMI1641 medium supplemented with the same medium containing protamine sulphate for 48 h in 6-well dishes coated with RetroNectin (Takara Bio Inc., Shiga, Japan).

*Colony assays*--- Cells were seeded into methylcellulose medium (MethoCult GF M3434; Stem Cell Technologies, Vancouver, BC, Canada) at a density $2.5 \times 10^2$ cells per 35-mm dish and were cultured with 5% $CO_2$ at 37°C. All cultures were performed in triplicate, and the numbers of colonies were counted after 10 days.

*In vitro immortalization assays for HPCs*. --- Immortalization assays of HPCs in vitro were performed as previously described (34). In brief, $10^4$ cells were plated in 1.1 ml of methylcellulose medium (methocult M3434). After the cultures for 1 week, colony numbers were counted, and single-cell suspensions of colonies ($10^4$ cells) were subsequently replated under identical conditions. Replating was repeated every week in the same way.

*Luciferase assays*. Luciferase assays were performed with a Dual-Luciferase Reporter System (Promega, Madison, WI) as previously described (35). Briefly, 293T or NIH3T3 cells ($2 \times 10^5$ cells) were seeded in a 60-mm dish and cultured for 24 h. Using the calcium phosphate coprecipitation method, cells were transfected with 2 $\mu$g reporter gene (pGL3-3×MαP-luciferase, 3×MHC-luciferase or 1×MPO-luciferase) in combination with 2 $\mu$g pcDNA3-GATA1, pcDNA3-PU.1(36), or pcDNA3-C/EBPα together with 6 $\mu$g an empty vector or an effector vector for FIP1L1-PDGFRα, H-RasG12V, 1*6-STAT5A(37), or CAAX-p110(38) and 10 ng pRL-CMV, a *Renilla* luciferase expression vector. After 12 h, cells were washed, serum-starved for 24 h, and subjected to luciferase assays. After 36 h, the cells were lysed and subjected to the measurement for luciferase activity. The relative firefly luciferase activity was calculated by normalizing transfection efficiency according to the *Renilla* luciferase activity.

*Semiquantitative RT-PCR Analysis*---Total RNA was isolated from FACS-sorted GFP-positive $5 \times 10^4$ cells using Trizol Reagent (Invitrogen, Carlsbad, CA). RT-PCR was performed using a SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. The cDNA product (1 μL) was resuspended in 20 μL of the PCR reaction buffer containing 0.5 U *Taq*Gold DNA polymerase (Perkin Elmer, Foster City, CA), 2 mM $MgCl_2$, and 15 pmol forward and reverse primers. The sequences of forward/reverse primer sets as follows: :
C/EBPα, 5'-GCC TGG CCT TGA CCA AGG AG -3' and 5' –CAC AGG ACT AGA ACA CCT GC-3',
GATA-1, 5'- GGA ATT CGG GCC CCT TGT GAG GCC AGA GAG -3' and 5'- CGG GGT ACC TCA CGC TCC AGC CAG ATT CGA CCC-3'
GATA-2, 5'- CGG AAT TCG ACA CAC CAC CCG ATA CCC ACC TAT-3'
and 5'-CGG AAT TCG CCT ACG CCA TGG CAG TCA CCA TGC T-3',
IL5-Rα, 5'- GCC CTT TGA TCA GCT GTT CAG TCC AC -3'
and 5'- CGG AAC CGG TGG AAA CAA CCT GGT C-3'
MBP, 5'- ACC TGT CGC TAC CTC CTA -3' and 5'- GTG GTG GCA GAT GTG TGA-3'
PU.1, 5'- GAT GGA GAA AGC CAT AGC GA-3' and 5'- TTG TGC TTG GAC GAG AAC TG-3'
HPRT, 5'- CAC AGG ACT AGA ACA CCT GC-3' and 5'- GCT GGT GAA AAG GAC CTC T-3'.
The PCR products were electrophoresed in agarose gels containing ethidium bromide and their amounts were analyzed with Fluor Imager595 and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

*Transplantation assays*---Transplantation assays were performed according to procedures described previously (39). Briefly, 8-12 week-old CD45.2 mice were lethally irradiated (900 rad) 24 h before the transplantation. BM cells isolated from congenic C57BL/6 (B6-Ly5.1) mice were transduced with FIP1L1-PDGFRα, and 10000 GFP-positive cells were injected intravenously in combination with $2 \times 10^5$ normal BM cells with CD45.2 phenotype. Chimeric analyses were performed at 4 weeks, 8 weeks and mice were sacrified 16 week after transplantation. Animal care was performed according to institutional guidelines.

*Measurement of phosphorylation of intracellular signaling molecule*---

Phosphorylation of intracellular moleclules was assessed using Phosflow technology (BD Bioscience) according to the manufacturer's recommendation. Briefly, cells were fixed with Phosflow Fix Buffer and incubated at 37°C for 10-15 minutes. After permeabilization at room temperature for 10 minutes, cells were washed twice with Phosflow Perm/Wash Buffer and incubated at room temperature for 10 minutes. After the binding reaction to each Ab, cells were washed once with Phosflow Perm/Wash buffer, resuspended in 500 μl of BD Pharmingen Stain Buffer (BD bioscience), then subjected to flow cytometric analysis. All experiments were repeated independently at least three times and confirmed reproducibility.

*Statistical Analyses*---Statistical analyses were performed using Student t-test.

## RESULTS

*Effects of FIP1L1-PDGFRα on the growth and survival of murine KSL cells*. To investigate the effects of LTKs on the growth, differentiation, and survival of HSCs/HPCs, we constructed bicistronic retrovirus vectors for FIP1L1-PDGFRα and TEL-PDGFRβ, which express these cDNAs together with EGFP through the IRES in the infected cells. At first, we introduced these retrovirus vectors into KSL cells. After 48-h infection, 55% to 65% of KSLs were found to be GFP-positive in all of transfectants by flow cytometric analysis (data not shown). Next, we isolated retrovirus-infected cells as GFP-positive cells and cultured them in the medium with or without SCF, TPO, FLT3L and IL-6. As shown in Fig. 1*A*, *left panel*, neither FIP1L1-PDGFRα nor TEL-PDGFRβ further augmented cytokine-dependent growth of KSLs. However, these LTKs enabled KSLs to survive and proliferate under cytokine-deprived condition at least for 96 h, while Mock (an empty retrovirus)-infected KSLs rapidly led to apoptosis in this condition (Fig. 1*A*, *right panel*).

Next, we performed colony assays using these retrovirus-infected KSLs. After 2-day retrovirus infection, GFP-positive cells were sorted and plated into methylcellulose medium containing the cytokine cocktail (EPO, TPO, SCF, G-CSF, and IL-3), and numbers of colonies were counted after 10 days. As shown in Fig. 1*B*, total number of colonies that developed from FIP1L1-PDGFRα- or TEL-PDGFRβ–infected KSLs was increased by 40-50% as compared with that from Mock-infected KSLs. Also, these colonies were larger than those yielded from Mock-infected KSLs (data not shown). However, the proportion of CFU-GEMM, CFU-GM, CFU-G, CFU-M, and BFU-E were roughly the same among 3 transfectants, indicating that these LTKs scarcely influence the lineage commitment and differentiation of KSLs in colony assays performed in this cytokine combination (Fig. 1*B*).

We next performed an *in vitro* immortalization assay using FIP1L1-PDGFRα-, TEL-PDGFRβ- or Mock-transduced KSLs and CMPs. After the 1st and 2nd plating, both FIP1L1-PDGFRα- and TEL-PDGFRβ-transduced KSLs yielded slightly increased number of colonies relative to Mock-transduced KSLs, whereas these differences were not significant (Fig. 1*C*, *left panel*). Also, in contrast to Mock-transduced KSLs, FIP1L1-PDGFRα- or TEL-PDGFRβ-transduced KSLs still kept colony-forming activities even after the 3rd and 4th plating (FIP1L1-PDGFRα vs Mock at the 3rd plating, $p<0.01$; at the 4th plating, $p<0.01$), although these activities were rather reduced (Fig. 1*C*, *left panel*). On the other hand, even if FIP1L1-PDGFRα or TEL-PDGFRβ was introduced, CMPs could not form any colony at the 3rd plating as was the case with Mock-infected CMPs (Fig. 1*C*, *right panel*). To evaluate leukemogenic potential of FIP1L1-PDGFRα-transduced KSLs *in vivo*, we transplanted these cells into lethally irradiated mice in combination with freshly prepared competetitor KSLs. As a result, although none of mice transplanted with Mock-transduced KSLs developed leukemia or MPD, FIP1L1-PDGFRα-transduced KSLs developed MPD in 3 mice and acute leukemia in 1 mouse out of 5 recipient mice within 15 weeks after transplantation (Table 1). However, in agreement with the prevous report (16), none of 5 recipient mice developed eosinophilic disorders. In addition, none of 10 mice transplanted with FIP1L1-PDGFRα-transduced CMPs developed MPD or leukemia (data not shown). Together, these results indicate that FIP1L1-PDGFRα can confer the ability of cytokine-independent gorwth/surival on KSLs and enhance their self-renewal, while it cannot immortalize CMPs *in vitro* or *in vivo*.

*Effects of FIP1L1-PDGFRα and TEL-PDGFRβ on differentiation from KSLs*. We next

investigated whether FIP1L1-PDGFRα or TEL-PDGFRβ influences the lineage commitment and subsequent differentiation of KSLs. For this purpose, we infected retrovirus harboring FIP1L1-PDGFRα or TEL-PDGFRβ into KSLs, cultured them with SCF, TPO, FLT3L and IL-6, and examined the expression of a granulocyte marker (Gr-1) and an eosinophil marker (IL-5 receptor α, CD125) in GFP-positive cells by flow cytometry. After 4-day cultures, there was not an apparent difference in the expression pattern of these markers among FIP1L1-PDGFRα-, TEL-PDGFRβ-, and Mock-transduced KSLs (Fig. 2*A*, *upper panel*). However, after 6-day cultures, TEL-PDGFRβ- or FIP1L1-PDGFRα-transduced KSLs yielded siginificantly increased Gr-1$^+$ fraction (66.8% and 77.5%, respectively) compared with Mock-transduced KSLs (49.6%). In addition, it was of paticular interest that 51.8% of FIP1L1-PDGFRα-tranduced KSLs became to express CD125 and Gr-1 simultaneously, whereas only 6.0% of Mock- and 14.0% of TEL-PDGFRβ-transduced KSLs revealed this phenotype (FIP1L1-PDGFRα vs Mock, $p<0.01$, Fig. 2*A*, *lower panel*). These results imply that FIP1L1-PDGFRα but not TEL-PDGFRβ preferentially imposes the commitment and differentiation to the eosinophilic lineage.

To examine whether Gr-1$^+$CD125$^+$ cells that developed from FIP1L1-PDGFRα-transduced KSLs are actually eosinophil precursors, we further cultured these KSLs with a cytokine cocktail containing IL-5 for additional 5 days. As a result, most of FIP1L1-PDGFRα- but not Mock- or TEL-PDGFRβ-transduced KSLs became to possess large granules characteristics of mature eosinophil in the MG staining, which were positive for the eosinostain (Fig. 2*B*). Furthermore, after 10-day cultures, we examined the mRNA expression of eosinophil-related genes, GATA-1, IL-5Rα, and C/EBPε by RT-PCR analysis using sorted GFP-positive cells. As shown in Fig. 2*C*, IL-5Rα and C/EBPε mRNAs were detected only in FIP1L1-PDGFRα-transduced KSLs. Also, GATA-1 mRNA was more intensively expressed in FIP1L1-PDGFRα-transduced KSLs than in Mock- or TEL-PDGFRβ-transduced KSLs. These data indicate that Gr-1$^+$CD125$^+$ cells that developed from FIP1L1-PDGFRα-transduced KSLs can indeed differentiate into mature eosinophils.

*Effects of FIP1L1-PDGFRα on differentiation of CMPs, MEPs, and CLPs.* It was previously shown that eosinophil precursors stochastically develop from HSCs through MMP, CMP, and GMP (40,41). So, at first, we examined whether FIP1L1-PDGFRα can enhance the development of eosionophils from CMPs. For this purpose, we isolated CMPs from murine BM mononuclear cells by FACS sorting using several markers (Fig. 3*A*). Then, we introduced FIP1L1-PDGFRα into these cells and cultured them with SCF, IL-6, FLT3L, and TPO for 6 days. As was the case with KSLs, FIP1L1-PDGFRα remarkably enhanced the development of Gr-1$^+$CD125$^+$ cells from CMPs compared with Mock cultures (57% vs. 6%, $p<0.01$, Fig. 3*B*).

Our next question was whether FIP1L1-PDGFRα could convert the lineages of MEPs and CLPs, which were already committed to the other lineages, into the eosinophil lineage. To address this issue, we introduced FIP1L1-PDGFRα or TEL-PDGFRβ into MEPs. When cocultured with a stroma cell line OP-9 in the presence of SCF and EPO for 9 days, 58% of Mock-infected and 41% of TEL-PDGFRβ-infected MEPs became to reveal Ter119$^+$CD125$^-$ erythroid phenotype. In contrast, only 26% of FIP1L1-PDGFRα-infected MEPs revealed this phenotype (FIP1L1-PDGFRα vs Mock, $p<0.05$, Fig. 3*C*, *upper panel*). Moreover, 50% of FIP1L1-PDGFRα-transduced MEPs differentiated into CD125$^+$Gr-1$^+$ cells, while only 16% of Mock- and 14% of TEL-PDGFRβ-infected MEPs revealed this phenotype (FIP1L1-PDGFRα vs Mock, $p<0.01$, Fig. 3*C*, *lower panel*). Similarly, after 9-day cultures in serum-free medium supplemented with TPO and IL-11, although Mock-transduced MEPs effectively gave rise to CD41$^+$Gr-1$^-$ cells (17%), only 2% of FIP1L1-PDGFRα-infected MEPs revealed this phenotype (FIP1L1-PDGFRα vs Mock, $p<0.01$, Fig. 3*D*). Also, Mock-transduced MEPs were found to become large polyploid megakaryocytes in morphological analysis, while most of FIP1L1-PDGFRα-transduced MEPs remained small and mononuclear (Fig. 3*E*). Together, these results indicate that FIPIL1-PDGFRα inhibits erythroid and megakaryocytic differentiation from MEPs and imposes lineage conversion to the eosinophil lineage.

Next, we introduced FIP1L1-PDGFRα into CLPs, and cocultured them with OP-9 cells in

the presence of SCF, IL-7 and FLT3L. After 10-day cultures, 97% of Mock- and 95% of TEL-PDGFRβ-transduced CLPs became to have B220$^+$CD125$^-$ B-lymphoid phenotype, while only 38% of FIP1L1-PDGFRα–transduced CLPs had this phenotype (FIP1L1-PDGFRα vs Mock, $p<0.01$). Furthermore, a considerable proportion of FIP1L1-PDGFRα-transduced CLPs but not Mock- or TEL-PDGFRβ-transduced CLPs aberrantly differentiate into Gr-1$^+$CD125$^+$ cells [% of Gr-1$^+$CD125$^+$ cells: FIP1L1-PDGFRα 57% vs. Mock 1% ($p<0.01$), TEL-PDGFRβ 0%] (Fig. 3*F*). We further cultured Gr-1$^+$CD125$^+$ cells that developed from FIP1L1-PDGFRα-transduced CLPs with a cytokine cocktail containing IL-5, and confirmed that these cells became to be positive for eosinostain (data not shown). These results indicate that FIP1L1-PDGFRα inhibits B-lymphoid differentiation from CLPs and instructs them to differentiate into the eosilonophil lineage.

*Function of FIP1L1 and PDGFRα in the fusion protein*. It was previously shown the FIP1L1 moiety is dispensable for kinase activation and for transforming properties of FIP1L1-PDGFRα (42). To determine the role of FIP1L1 in FIP1L1-PDGFRα-enhanced eosinophil development, we generated two artificial chimeric constructs, FIP1L1-PDGFRβ and TEL-PDGFRα, in which FIP1L1 in FIP1L1-PDGFRα and TEL in TEL-PDGFRβ were completely replaced (Fig. 4*A*). In addition, we generated retrovirus vectors for constitutively active PDGFRα (PDGFRαV561D and PDGFRαD842V), which are considered to be causative mutations of gastrointestinal stromal tumors (GIST) (43) (Fig. 4*A*). When expressed in a murine IL-3-dependent cell line, Ba/F3, all of 4 PDGFR mutants conferred IL-3-independent growth on these cells (data not shown). Also, Western blot analysis demonstrated that these PDGFR mutants phosphorylated various cellular proteins including themselves (data not shown), indicating that these proteins act as constitutively active tyrosine kinases.

We transduced these retrovirus expression vectors into KSLs and cultured them with SCF, TPO, FLT3L and IL-6 for 6 days. As shown in Fig. 4*B*, only TEL-PDGFRα but not FIP1L1-PDGFRβ, PDGFRαV561D or PDGFRαD842V promoted eosinophil development from KSLs (% of Gr-1$^+$CD125$^+$ fraction: TEL-PDGFRα 74%; FIP1L1-PDGFRβ 11%; PDGFRαV561D 11%; PDGFRαD842V 16%) (TEL-PDGFRα vs Mock, $p<0.01$) (Fig. 4*B*), indicating that FIP1L1 is dispensable for FIP1L1-PDGFRα-mediated eosinophil development and that PDGFRα-mediated signaling but not PDGFRβ-mediated signaling is required for inducing eosinophil development. However, because neither PDGFRαV561D nor PDGFRαD842V promoted eosinophil development, specific kinase activity transmitted from chimeric PDGFRα was supposed to be necessary to enhance eosinophil development.

*Both a MEK1/2 inhibitor and a p38MAPK inhibitor blocked FIP1L1/PDGFRα-induced eosinophil development from KSLs*.

PDGFRα activates various downstream cascades, thereby exerting its biologic activity (44). To seek out the mechanism underlying instructive eosinophil differentiation induced by FIP1L1-PDGFRα, FIP1L1-PDGFRα- or Mock-transduced KSLs were cultured with or without several kinase inhibitors as indicated (Fig. 5*A*).

As shown in Fig. 5*A*, *upper panel*, none of a JNK inhibitor, a PI3K inhibitor (LY294002), a Src inhibitor (PPI), or a JAK2/STAT inhibitor (AG490) influenced FIP1L1-PDGFRα-enhanced eosinophil development, since about 20% of cells became to be CD125$^+$Gr1$^+$ after 5-day cultures as was seen after the culture without an inhibitor (Fig. 2*A*). In contrast, a MEK inhibitor (PD98059) and a p38MAPK inhibitor (SB202190) reduced CD125$^+$Gr1$^+$ fraction to 3.4% ($p<0.05$) and 1.7% ($p<0.01$), respectively (Fig. 5*A*, *lower panel*). We also analyzed the phosphorylation states of ERK, STAT5, and p38MAPK in FIP1L1-PDGFRα- or TEL-PDGFRβ-transduced KSLs by flow cytometry. As shown in Fig. 5*B*, ERK1/2, and p38MAPK but not STAT5 were phosphorylated more intensely in FIP1L1-PDGFRα-transduced KSLs than in Mock- or TEL-PDGFRβ-transduced KSLs. These data suggest that FIP1L1-PDGFRα instructs HSCs/HPCs to differentiate into eosinophil progenitors through the activation of MEK1/2-ERK1/2 and p38 pathways.

*Effects of FIP1L1-PDGFRα on the expression and activity of lineage-specific transcription factors in KSLs*. To further clarify the mechanism through which FIP1L1-PDGFRα enhanced eosinophil development, we analyzed

the effects of FIP1L1-PDGFRα on the expression of GATA-1, GATA-2, C/EBPα, and PU.1, all of which have been reported to be key transcription factors for eosinophil development (45-47). To detect the changes in the expression of these factors that precede the phenotyptic change, we isolated mRNA from sorted GFP-positive KSLs after 48-h retrovirus infection and performed semiquantitative RT-PCR analysis, since an apparent phenotyptic change was not observed until 4 days (Fig. 2*A*, *upper panel*). As shown in Fig. 6*A*, though the expression of PU.1 was not so different among three transfectants, FIP1L1-PDGFRα augmented the expression of C/EBPα ($p<0.01$) and GATA-1 ($p<0.01$) compared to Mock-transduced KSLs. Furthermore, the expression of GATA-2 was significantly higher in FIP1L1-PDGFRα-transduced KSLs than in Mock- or TEL-PDGFRβ-transduced KSLs (FIP1L1-PDGFRα vs Mock, $p<0.01$).

To evaluate the roles for these transcription factors in FIP1L1-PDGFRα-induced eosinophil development, we inhibited the expression or function of these transcription factors using shRNAs or a dominant-negative mutant. At first, we confirmed that these shRNAs suppressed the expression of C/EBPα and GATA-2 considerably (Fig. 6*B*, *upper panel*). When coexpressed with FIP1L1-PDGFRα in this condition, shRNA against C/EBPα reduced FIP1L1-PDGFRα-induced $CD125^+Gr1^+$ fraction from 11.7% to 3.9% ($p<0.01$) (Fig. 6*B*, *lower panel*). Similarly, shRNA against GATA-2 suppressed this fraction to 6.8% ($p<0.05$). Also, GATA3KRR, which can inhibit both GATA-1 and GATA-2, reduced FIP1L1-PDGFRα-induced $CD125^+Gr1^+$ fraction from 13.9% to 3.5% ($p<0.01$) (Fig. 6*C*). These results indicate that both GATA-2 and C/EBPα are required for FIP1L1-PDGFRα-induced eosilophil development.

We also examined the effects of FIP1L1-PDGFRα, and its downsteram signaling molecules (i.e., Ras, STAT5 and PI3-K) on transcription activities of these factors with luciferase assays using reporter genes and effector genes in combinations as indicated in Fig 7. In NIH3T3 cells, transiently transduced reporter genes for GATAs (3×MαP-luciferase), PU.1 (3×MHC-luciferase), and C/EBPα (1×MPO-luciferase) were activated by cotransfected GATA-1, PU.1, and C/EBPα by 7 fold, 7 fold, and 5 fold, respectively (Fig. 7). Also, the estradiol treatment activated 3×MαP-luciferase in GATA-2/ER-transfected cells. When FIP1L1-PDGFRα or a constitutively active form of H-Ras (H-RasG12V), STAT5 (1*6-STAT5A), or PI3-K (CAAX-p110) was further cotransfected, 1*6-STAT5A and CAAX-p110 scarcely affected transcription activities of GATA-1, GATA-2, PU.1 and C/EBPα. In contrast, both FIP1L1-PDGFRα and H-RasG12V reduced PU.1 activities to 30-40% ($p<0.01$). Similar results were also obtained from 293T cells (data not shown). These results indicate that FIP1L1-PDGFRα regulates the expression and activities of various transcription factors, thereby promoting eosinophil development, and suggest that Ras may be a pivotal downstream mediator of FIP1L1-PDGFRα in this process.

## DISCUSSION

In this study, we found that TEL-PDGFRα, but not FIP1L1-PDGFRβ, PDGFRαD562V or PDGFRαD842V promoted eosinophil development from KSLs as efficiently as FIP1L1-PDGFRα. This result indicates that constitutive TK activity transmitted from chimeric structure of PDGFRα is necessary to augment eosinophil development. In agreement with our finding, novel mutations identified in CEL were restricted to the chimeric form of PDGFRα, that is, KIF5B-PDGFRα formed by t(4;10)(q12;p11), STRN-PDGFRα by t(2;4)(p24;q12), and ETV6-PDGFRα by t(4;12)(q2?3;p1?2). As for the roles for downstream signaling molecules, the current results indicate that Ras/MEK and p38MAPK play essential roles in FIP1L1-PDGFRα-induced eosinophil development. However, this finding seems to be inconsistent with the fact that Ras/MEK is activated by various LTKs and normal hematpoietic growth factors. As for this reason, because FIP1L1-PDGFRα more intensely activated MEK/ERK and p38MAPK than TEL-PDGFRβ, we speculated that leukemogenic signals transmitted from chimeric PDGFRα would be quantitatively and qualitatively different from those from wild type TKs or other LTKs, thereby specifically promoting eosinophil development. In addition to the regulation of neoplastic cell proliferation, ERK has also been implicated in the control of

signaling cascades associated with eosinophilia in asthma. Duan *et al.* reported that an MEK inhibitor dramatically inhibited OVA-induced lung tissue eosinophilia and airway hyperresponsiveness(48). Also, p38MAPK is important for the induction of eosinophilia and function of terminal differentiated eosinophils in allergic airway inflammation(49,50). In addition, our data suggest that p38MAPK would regulate eosinophil development at the early stage of hematopoiesis. Further studies to elucidate the crucial signal transduction mechanisms that control eosinophil develpment will provide better rationale for the design of drug therapy not only for FIP1L1-PDGFRα-associated HES/CEL but also for allergic inflammation.

Our *in vitro* studies showed that FIP1L1-PDGFRα confers cytokine-independence on KSLs and enhances their self-renewal activity, while it did not immortalize CMPs. In addition, although FIP1L1-PDGFRα-transduced KSLs caused MPD in recipient mice, FIP1L1-PDGFRα-transduced CMPs did not. These results indicate that FIP1L1-PDGFRα cannot confer self-renewal activity on CMPs, and that the genetic alternation of FIP1L1-PDGFRα that causes CEL/HES occurs at a HSC level but not at a CMP level. In addition, we confirmed that mature eosinophils were generated from FIP1L1-PDGFRα-tranduced KSLs in the presence of IL-5, indicating that FIP1L1-PDGFRα does not impair terminal differentiation of eosinophils. Also, when expressed in MEPs or CLPs, FIP1L1-PDGFRα brought about lineage conversion to eosinophil lineage. Together, these results suggest that, although LSCs harboring FIP1L1-PDGFRα derived from HSCs would continuously produce an excess number of mature eosinophils, a part of eosinophils might be derived from FIP1L1-PDGFRα-harboring MEPs or CLPs.

In a previous report, FIP1L1-PDGFRα-transduced HSCs/HPCs caused myeloproliferative disorder in the recipient mice like BCR-ABL- or TEL-PDGFRβ-transduced KSLs (16,51,52), which was rather different from simple eosinophilia observed in human HES/CEL. Also in our transplantation experiment, none of 5 mice transplanted with FIP1L1-PDGFRα-expressing KSLs developed eosinophilic disorders. However, we also observed that, whereas FIP1L1-PDGFRα-introduced KSLs differentiated up to IL-5Rα$^+$ eosinophil precursors under the cultures without IL-5, supplement of IL-5 rendered these IL-5Rα$^+$ cells to undergo eosinophilic terminal differentiation. In accord with this hypothesis, Yamada et al. reported that transplantation of FIP1L1-PDGFRα-transduced HSCs/HPCs obtained from IL-5 transgenic mice resulted in marked eosinophilia resembling HES/CEL in the recipient mice (52). Since p210BCR-ABL-transduced HSCs/HPCs did not cause eosinophilia even in the presence of IL-5 overexpression in the recipient mice, the induction of eosinophilia was attributable to FIP1L1-PDGFRα, Together with our results, these lines of evidence suggest that, although FIP1L1-PDGFRα is a major etiologic factor causing eosinophilia, it is not sufficient to induce HES/CEL but requires additional events such as IL-5 overexpression. In fact, some patients with FIP1L1-PDGFRα-associated HES were complicated with T-cell lymphoma (53-55). The frequency of FIP1L1-PDGFRα-induced HES/CEL was not so high (about 10%) as initially reported. However, similar LTK is supposed to be involved in the pathogenesis of HES/CEL because imatinib is effective in some patients who do not have a FIP1L1-PDGFRα mutation (56). Also, a significant proportion of patients with HES/CEL have abnormal T-lymphocyte populations, such as such as CD3$^+$CD4$^-$CD8$^-$ and CD3$^-$CD8$^+$ T cells, which secret high levels of IL-5 (57). Currently, HES is categorized into two groups, "myeloproliferative variant " and "T-cell-mediated HES", and these groups are thought to be independent of each other (58,59). However, because T-cell differentiation might be perturbed by FIP1L1-PDGFRα, it may be meaningful for the better understanding of the pathogenesis of HES/CEL to clarify the relationship between these two groups.

Iwasaki *et al.* isolated eosinophil progenitors from murine BM, and they concluded that eosinophil developmental pathway would diverge from neutrophils and monocytes at the GMP stage (60). The lineage commitment of HSCs/HPCs and subsequent linegage-specific differentiation are crucially regulated by lineage-specific transcription factors such as GATA-1, GATA-3, PU.1, C/EBPα, and C/EBPε.

Among them, GATA-1 and PU.1 are known to antagonize each other, and induces differentiation to erythroid/megakalyocyte or myeloid lineage, respectively (61-63). CEBP family (CEBPα and CEBPε) is essential for the differentiation to myeloid lineage (64-66). Friend of GATA (FOG) and C/EBPβ regulate the eosinophil lineage induction antagonistically (67). Furthermore, enforced expression of C/EBPα converts MEPs to eosinophils (68) and expression of PU.1 converts them to GMPs (61,67). Also, forced expression of GATA-1 in myeloid cells induces the formation of either MEPs or eosinophils, depending on the concentration of the factor (69). In addition, it was recently reported that C/EBPα expression followed by GATA-2 expression in GMPs is critical for eosinophil lineage specification (46). However, it is plausible that the mechanism of lineage commitment in leukemic cells is somewhat different from that in normal hematopoietic cells. In this study, we found that FIP1L1-PDGFRα enhanced the expression of GATA-1, GATA-2 and C/EBPα, and suppressed PU.1 expression. Also, FIP1L1-PDGFRα suppressed transcription activities of PU.1. These results suggest that LTKs can influence the lineage commitment of HSCs/HPCs and subsequent differentiation by modifying the expression and activity of lineage-specific transcription factors.

In conclusion, we here found that FIP1L1-PDGFRα can enhance eosinohhil development from HSCs/HPCs through the MEK/ERK and p38MAPK cascades by controlling the expression and activity of lineage-specific transcription factors. Furthermore, as far as we explored, this is the first report providing evidence that LTK has an ability to convert the lineage of committed progenitor cells. Further studies based on these findings would undoubtedly provide more useful information to understand the pathophysioloy of various hematologic malignancies caused by LTKs.

## REFERENCES


1. Semerad, C. L., Poursine-Laurent, J., Liu, F., and Link, D. C. (1999) *Immunity* **11**(2), 153-161
2. Arcasoy, M. O., Maun, N. A., Perez, L., Forget, B. G., and Berliner, N. (2001) *Eur J Haematol* **67**(2), 77-87
3. Hsu, C. L., Kikuchi, K., and Kondo, M. (2007) *Blood* **110**(5), 1420-1428
4. Buitenhuis, M., Verhagen, L. P., van Deutekom, H. W., Castor, A., Verploegen, S., Koenderman, L., Jacobsen, S. E., and Coffer, P. J. (2008) *Blood* **111**(1), 112-121
5. Radomska, H. S., Basseres, D. S., Zheng, R., Zhang, P., Dayaram, T., Yamamoto, Y., Sternberg, D. W., Lokker, N., Giese, N. A., Bohlander, S. K., Schnittger, S., Delmotte, M. H., Davis, R. J., Small, D., Hiddemann, W., Gilliland, D. G., and Tenen, D. G. (2006) *J Exp Med* **203**(2), 371-381
6. Yamamoto Y, K. H., Nakano Y, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Saito K, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Saito H, Ueda R, Ohno R, Naoe T. (2001) *Blood* **97(8)**, 6
7. Levis, M., Tse, K. F., Smith, B. D., Garrett, E., and Small, D. (2001) *Blood* **98**(3), 885-887
8. Griffith, J., Black, J., Faerman, C., Swenson, L., Wynn, M., Lu, F., Lippke, J., and Saxena, K. (2004) *Mol Cell* **13**(2), 169-178
9. Bene, M. C., Bernier, M., Casasnovas, R. O., Castoldi, G., Knapp, W., Lanza, F., Ludwig, W. D., Matutes, E., Orfao, A., Sperling, C., and van't Veer, M. B. (1998) *Blood* **92**(2), 596-599
10. Nagata, H., Worobec, A. S., Oh, C. K., Chowdhury, B. A., Tannenbaum, S., Suzuki, Y., and Metcalfe, D. D. (1995) *Proc Natl Acad Sci U S A* **92**(23), 10560-10564
11. Longley, B. J., Tyrrell, L., Lu, S. Z., Ma, Y. S., Langley, K., Ding, T. G., Duffy, T., Jacobs, P., Tang, L. H., and Modlin, I. (1996) *Nat Genet* **12**(3), 312-314
12. Ikeda, H., Kanakura, Y., Tamaki, T., Kuriu, A., Kitayama, H., Ishikawa, J., Kanayama, Y., Yonezawa, T., Tarui, S., and Griffin, J. D. (1991) *Blood* **78**(11), 2962-2968
13. Longley, B. J., Jr., Metcalfe, D. D., Tharp, M., Wang, X., Tyrrell, L., Lu, S. Z., Heitjan, D., and

Ma, Y. (1999) *Proc Natl Acad Sci U S A* **96**(4), 1609-1614

14. Fritsche-Polanz, R., Jordan, J. H., Feix, A., Sperr, W. R., Sunder-Plassmann, G., Valent, P., and Fodinger, M. (2001) *Br J Haematol* **113**(2), 357-364

15. Furitsu, T., Tsujimura, T., Tono, T., Ikeda, H., Kitayama, H., Koshimizu, U., Sugahara, H., Butterfield, J. H., Ashman, L. K., Kanayama, Y., and et al. (1993) *J Clin Invest* **92**(4), 1736-1744

16. Cools, J., Stover, E. H., Boulton, C. L., Gotlib, J., Legare, R. D., Amaral, S. M., Curley, D. P., Duclos, N., Rowan, R., Kutok, J. L., Lee, B. H., Williams, I. R., Coutre, S. E., Stone, R. M., DeAngelo, D. J., Marynen, P., Manley, P. W., Meyer, T., Fabbro, D., Neuberg, D., Weisberg, E., Griffin, J. D., and Gilliland, D. G. (2003) *Cancer Cell* **3**(5), 459-469

17. Klion, A. D., Noel, P., Akin, C., Law, M. A., Gilliland, D. G., Cools, J., Metcalfe, D. D., and Nutman, T. B. (2003) *Blood* **101**(12), 4660-4666

18. Stover, E. H., Chen, J., Lee, B. H., Cools, J., McDowell, E., Adelsperger, J., Cullen, D., Coburn, A., Moore, S. A., Okabe, R., Fabbro, D., Manley, P. W., Griffin, J. D., and Gilliland, D. G. (2005) *Blood* **106**(9), 3206-3213

19. Cools, J., DeAngelo, D. J., Gotlib, J., Stover, E. H., Legare, R. D., Cortes, J., Kutok, J., Clark, J., Galinsky, I., Griffin, J. D., Cross, N. C., Tefferi, A., Malone, J., Alam, R., Schrier, S. L., Schmid, J., Rose, M., Vandenberghe, P., Verhoef, G., Boogaerts, M., Wlodarska, I., Kantarjian, H., Marynen, P., Coutre, S. E., Stone, R., and Gilliland, D. G. (2003) *N Engl J Med* **348**(13), 1201-1214

20. Griffin, J. H., Leung, J., Bruner, R. J., Caligiuri, M. A., and Briesewitz, R. (2003) *Proc Natl Acad Sci U S A* **100**(13), 7830-7835

21. La Starza, R., Specchia, G., Cuneo, A., Beacci, D., Nozzoli, C., Luciano, L., Aventin, A., Sambani, C., Testoni, N., Foppoli, M., Invernizzi, R., Marynen, P., Martelli, M. F., and Mecucci, C. (2005) *Haematologica* **90**(5), 596-601

22. Buitenhuis, M., Verhagen, L. P., Cools, J., and Coffer, P. J. (2007) *Cancer Res* **67**(8), 3759-3766

23. Bonnet, D., and Dick, J. E. (1997) *Nat Med* **3**(7), 730-737

24. Hope, K. J., Jin, L., and Dick, J. E. (2004) *Nat Immunol* **5**(7), 738-743

25. Sutherland, H. J., Blair, A., and Zapf, R. W. (1996) *Blood* **87**(11), 4754-4761

26. Blair, A., Hogge, D. E., and Sutherland, H. J. (1998) *Blood* **92**(11), 4325-4335

27. Cozzio, A., Passegue, E., Ayton, P. M., Karsunky, H., Cleary, M. L., and Weissman, I. L. (2003) *Genes Dev* **17**(24), 3029-3035

28. Huntly, B. J., Shigematsu, H., Deguchi, K., Lee, B. H., Mizuno, S., Duclos, N., Rowan, R., Amaral, S., Curley, D., Williams, I. R., Akashi, K., and Gilliland, D. G. (2004) *Cancer Cell* **6**(6), 587-596

29. Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000) *Nature* **404**(6774), 193-197

30. Segawa, K., Matsuda, M., Fukuhara, A., Morita, K., Okuno, Y., Komuro, R., and Shimomura, I. (2008) *The Journal of endocrinology*

31. Okitsu, Y., Takahashi, S., Minegishi, N., Kameoka, J., Kaku, M., Yamamoto, M., Sasaki, T., and Harigae, H. (2007) *Biochemical and biophysical research communications* **364**(2), 383-387

32. Smith, V. M., Lee, P. P., Szychowski, S., and Winoto, A. (1995) *The Journal of biological chemistry* **270**(4), 1515-1520

33. Ezoe, S., Matsumura, I., Nakata, S., Gale, K., Ishihara, K., Minegishi, N., Machii, T., Kitamura, T., Yamamoto, M., Enver, T., and Kanakura, Y. (2002) *Blood* **100**(10), 3512-3520

34. Ono, R., Ihara, M., Nakajima, H., Ozaki, K., Kataoka-Fujiwara, Y., Taki, T., Nagata, K., Inagaki, M., Yoshida, N., Kitamura, T., Hayashi, Y., Kinoshita, M., and Nosaka, T. (2005) *Mol Cell Biol* **25**(24), 10965-10978

35. Ezoe, S., Matsumura, I., Gale, K., Satoh, Y., Ishikawa, J., Mizuki, M., Takahashi, S., Minegishi, N., Nakajima, K., Yamamoto, M., Enver, T., and Kanakura, Y. (2005) *The Journal of biological chemistry* **280**(13), 13163-13170

36. Matsumura, I., Kawasaki, A., Tanaka, H., Sonoyama, J., Ezoe, S., Minegishi, N., Nakajima, K., Yamamoto, M., and Kanakura, Y. (2000) *Blood* **96**(7), 2440-2450

37. Matsumura, I., Kitamura, T., Wakao, H., Tanaka, H., Hashimoto, K., Albanese, C., Downward, J., Pestell, R. G., and Kanakura, Y. (1999) *The EMBO journal* **18**(5), 1367-1377

38. Doornbos, R. P., Theelen, M., van der Hoeven, P. C., van Blitterswijk, W. J., Verkleij, A. J., and van Bergen en Henegouwen, P. M. (1999) *The Journal of biological chemistry* **274**(13), 8589-8596

39. Tanaka, H., Matsumura, I., Itoh, K., Hatsuyama, A., Shikamura, M., Satoh, Y., Heike, T., Nakahata, T., and Kanakura, Y. (2006) *Stem Cells* **24**(11), 2592-2602

40. Abkowitz, J. L., Golinelli, D., Harrison, D. E., and Guttorp, P. (2000) *Blood* **96**(10), 3399-3405

41. Huang, S., Law, P., Francis, K., Palsson, B. O., and Ho, A. D. (1999) *Blood* **94**(8), 2595-2604

42. Stover, E. H., Chen, J., Folens, C., Lee, B. H., Mentens, N., Marynen, P., Williams, I. R., Gilliland, D. G., and Cools, J. (2006) *Proc Natl Acad Sci U S A* **103**(21), 8078-8083

43. Heinrich, M. C., Corless, C. L., Duensing, A., McGreevey, L., Chen, C. J., Joseph, N., Singer, S., Griffith, D. J., Haley, A., Town, A., Demetri, G. D., Fletcher, C. D., and Fletcher, J. A. (2003) *Science* **299**(5607), 708-710

44. Heldin, C. H., and Westermark, B. (1999) *Physiol Rev* **79**(4), 1283-1316

45. McNagny, K., and Graf, T. (2002) *J Exp Med* **195**(11), F43-47

46. Iwasaki, H., Mizuno, S., Arinobu, Y., Ozawa, H., Mori, Y., Shigematsu, H., Takatsu, K., Tenen, D. G., and Akashi, K. (2006) *Genes Dev* **20**(21), 3010-3021

47. Du, J., Stankiewicz, M. J., Liu, Y., Xi, Q., Schmitz, J. E., Lekstrom-Himes, J. A., and Ackerman, S. J. (2002) *The Journal of biological chemistry* **277**(45), 43481-43494

48. Duan, W., Chan, J. H., Wong, C. H., Leung, B. P., and Wong, W. S. (2004) *J Immunol* **172**(11), 7053-7059

49. Kampen, G. T., Stafford, S., Adachi, T., Jinquan, T., Quan, S., Grant, J. A., Skov, P. S., Poulsen, L. K., and Alam, R. (2000) *Blood* **95**(6), 1911-1917

50. Wong, C. K., Zhang, J. P., Ip, W. K., and Lam, C. W. (2002) *Clin Exp Immunol* **128**(3), 483-489

51. Daley, G. Q., Van Etten, R. A., and Baltimore, D. (1990) *Science (New York, N.Y* **247**(4944), 824-830

52. Yamada, Y., Rothenberg, M. E., Lee, A. W., Akei, H. S., Brandt, E. B., Williams, D. A., and Cancelas, J. A. (2006) *Blood* **107**(10), 4071-4079

53. McPherson, T., Cowen, E. W., McBurney, E., and Klion, A. D. (2006) *Br J Dermatol* **155**(4), 824-826

54. Robyn, J., Lemery, S., McCoy, J. P., Kubofcik, J., Kim, Y. J., Pack, S., Nutman, T. B., Dunbar, C., and Klion, A. D. (2006) *Br J Haematol* **132**(3), 286-292

55. Capovilla, M., Cayuela, J. M., Bilhou-Nabera, C., Gardin, C., Letestu, R., Baran-Marzak, F., Fenaux, P., and Martin, A. (2008) *Eur J Haematol* **80**(1), 81-86

56. Jovanovic, J. V., Score, J., Waghorn, K., Cilloni, D., Gottardi, E., Metzgeroth, G., Erben, P., Popp, H., Walz, C., Hochhaus, A., Roche-Lestienne, C., Preudhomme, C., Solomon, E., Apperley, J., Rondoni, M., Ottaviani, E., Martinelli, G., Brito-Babapulle, F., Saglio, G., Hehlmann, R., Cross, N. C., Reiter, A., and Grimwade, D. (2007) *Blood* **109**(11), 4635-4640

57. Simon, H. U., Plotz, S. G., Dummer, R., and Blaser, K. (1999) *N Engl J Med* **341**(15), 1112-1120

58. Roufosse, F., Cogan, E., and Goldman, M. (2003) *Annu Rev Med* **54**, 169-184

59. Roufosse, F. E., Goldman, M., and Cogan, E. (2003) *N Engl J Med* **348**(26), 2687; author reply 2687

60. Iwasaki, H., Mizuno, S., Mayfield, R., Shigematsu, H., Arinobu, Y., Seed, B., Gurish, M. F., Takatsu, K., and Akashi, K. (2005) *J Exp Med* **201**(12), 1891-1897

61. Rekhtman, N., Radparvar, F., Evans, T., and Skoultchi, A. I. (1999) *Genes Dev* **13**(11), 1398-1411

62. Zhang, P., Behre, G., Pan, J., Iwama, A., Wara-Aswapati, N., Radomska, H. S., Auron, P. E., Tenen, D. G., and Sun, Z. (1999) *Proc Natl Acad Sci U S A* **96**(15), 8705-8710

63. Nerlov, C., Querfurth, E., Kulessa, H., and Graf, T. (2000) *Blood* **95**(8), 2543-2551

64. Smith, L. T., Hohaus, S., Gonzalez, D. A., Dziennis, S. E., and Tenen, D. G. (1996) *Blood* **88**(4), 1234-1247

65. Hohaus, S., Petrovick, M. S., Voso, M. T., Sun, Z., Zhang, D. E., and Tenen, D. G. (1995) *Mol Cell Biol* **15**(10), 5830-5845

66. Wang, N. D., Finegold, M. J., Bradley, A., Ou, C. N., Abdelsayed, S. V., Wilde, M. D., Taylor, L. R., Wilson, D. R., and Darlington, G. J. (1995) *Science* **269**(5227), 1108-1112

67. Querfurth, E., Schuster, M., Kulessa, H., Crispino, J. D., Doderlein, G., Orkin, S. H., Graf, T., and Nerlov, C. (2000) *Genes Dev* **14**(19), 2515-2525

68. McNagny, K. M., and Graf, T. (2003) *Blood* **101**(3), 1103-1110

69. Kulessa, H., Frampton, J., and Graf, T. (1995) *Genes Dev* **9**(10), 1250-1262

## FOOTNOTES

This work was supported by grants from the Ministry of Education, Science, Sports, and Culture and Technology of Japan. Abbreviations; LTK, leukemogenic tyrosin kinase; PDGFRα , platelet-derived factor receptor alpha unit; PDGFRβ , platelet-derived factor receptor beta unit; KSL, $Lin^{-}Sca^{-}1^{hi}c\text{-}Kit^{hi}$ cell, CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, common granulocyte/monocyte progenitor; HSC, hematopoietic stem cell; HPC, hematopoietic progenitor cell; C/EBP, CCAAT/enhancer-binding protein.

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## FIGURE LEGENDS

Fig. 1. **The effects of leukemogenic tyrosine kinases on proliferation and survival of hematopoietic stem/progenitor cells.** ***A,*** KSLs were isolated from murine bone marrow mononuclear cells. After the retrovirus (Mock, FIP1L1-PDGFRα, or TEL-PDGFRβ) infection, retrovirus-infected KSLs were sorted as $GFP^{+}$ cells and cultured with (*left panel*) or without (*right panel*) SCF, TPO, FLT3L and IL-6 for 96 h. During these cultures, total viable cell numbers were counted at the time indicated. ***B,*** KSLs infected with each retrovirus were sorted and seeded into the methylcellulose medium containing EPO, TPO, SCF, G-CSF, and IL-3. Colony numbers were counted on day 12. ***C,*** Immortalization assays for retrovirus-infected KSLs and CMPs. Retrovirus-infected KSLs and CMPs ($10^3$ cells) were plated into methylcellulose medium and colony

numbers were counted after 1 week. Then, single-cell suspensions of colonies ($10^3$ cells) were serially replated every week in the same way. Bars indicate the number of colonies obtained after each round of replating in methylcellulose as means ± SD ($n$=3). **, $p<0.01$ compared with the value of Mock-transduced cells.

Fig. 2. **Eosinophil development from KSLs.** ***A,*** After retrovirus transduction, KSLs were cultured with SCF, TPO, IL-6, and FLT3L, and FACS analysis was performed after 4 days (upper panel) and 6 days (lower panel). GFP$^+$ cells were gated and the expression of Gr-1 and CD125 was analyzed. **, $p<0.01$ compared with the value of Mock-transduced cells ($n$=3). ***B,*** After 6-day cultures with SCF, TPO, IL-6, and FLT3L, retrovirus-infected KSLs were further cultured with a cytokine cocktail containing IL-5 for 5 days. Transduced cells were subjected to the May-Giemsa staining (*upper panel*) and eosinostain (*lower panel*). ***C,*** After 10-day cultures with TPO, IL-6, FLT3L, and SCF, GFP-positive cells were sorted and the expression of eosinophil-related genes was analyzed by RT-PCR analysis.

Fig. 3. **FIP1L1-PDGFRα-induced eosinophil development from CMPs, MEPs, and CLPs.** ***A,*** Isolation of GMPs/CMPs/MEPs and CLPs from murine mononuclear cells by a FACS cell sorter using several markers. ***B,*** Mock-, FIP1L1-PDGFRα- or TEL-PDGFRβ-transduced CMPs were cultured with SCF, IL-6, FLT3L and TPO for 6 days. Then, the expression of CD125 and Gr-1 was analyzed by flowcytometry. ***C,*** Mock-, FIP1L1-PDGFRα- or TEL-PDGFRβ-transduced MEPs were cocultured with OP-9 cells in the presence of EPO and SCF for 8 days, and then subjected to FACS analysis. ***D, E,*** Mock- or FIP1L1-PDGFRα-transduced MEPs were cultured in serum-free medium supplemented with TPO and IL-11 for 9 days and subjected to FACS analysis. Transduced cells were observed with differential interferance contrast (DIC) and fluorescence microscopy. Mock- and FIP1L1-PDGFRα-transduced GFP-positive cells (arrows) were sorted and subjected to the May-Giemsa staining. ***F,*** Retrovirus-transduced CLPs were cocultured with OP-9 cells in the presence of SCF, IL-7, and FLT3L for 2 days. Then, GM-CSF was added into the medium. and cells were cultured for 8 more days. **, $p<0.01$ compared with the value of Mock-transduced cells ($n$=3).

Fig. 4. **Function of FIP1L1 and PDGFRα in FIP1L1-PDGFRα-induced eosinophil development.** ***A,*** Schematic representation of FIP1L1-PDGFRβ and TEL-PDGFRα, PDGFRαD561V and PDGFRαD842V. In FIP1L1-PDGFRβ and TEL-PDGFRα, FIP1L1 in FIP1L1-PDGFRα and TEL in TEL-PDGFRβ were completely replaced each other. Splicing sites are indicated with black arrows and point mutation sites are indicated with vacant arrows. TM, transmembrane domain; JM, juxtamembrane domain. ***B,*** Murine KSLs were infected with the retrovirus as indicated, and cultured with SCF, TPO, IL-6, and FLT3L for 6 days. Then, expression of CD125 and Gr-1 was analyzed by flow cytometry. **, $p<0.01$ compared with the value of Mock-transduced cells ($n$=3).

Fig. 5. **Roles of signal cascades in FIP1L1-PDGFRα-induced eosinophil development.** ***A,*** Murine KSLs were infected with the retrovirus indicated, and cultured with SCF, TPO, IL-6, and FLT3L with or without kinase inhibitors as indicated, and then subjected to FACS analysis. * indicates $p<0.05$ and ** $p<0.01$ compared with the value of DMSO-treated cells ($n$=4). ***B,*** FIP1L1-PDGFRα- or TEL-PDGFRβ- or MOCK- transduced KSLs were cultured for 2 days and the phosphorylation status of ERK1/2, p38MAPK, and STAT5 was analyzed using phosflow technology.

Fig. 6. **Effects of FIP1L1-PDGFRα and its downstream molecules on the expressions of eosinophil-related transcription factors, and effects of inhibition of these molecules.** ***A,*** The expressions of eosinophil-related transcription factors in KSLs were analyzed by RT-PCR analysis 48 h after retrovirus transfection. PCR products were electrophoresed and visualized by ethidium bromide staining (*left panel*) and their intensities were quantified using Fluor Imager595 and ImageQuant software. Relative intensities to the products from Mock-transduced cells were indicated (*right panel*). * indicates ** $p<0.01$ as compared with the value in Mock-transduced cells. Data represent means ±SD ($n$=3). ***B,*** Murine KSLs were infected with lentivirus expressing noncoding or encoding shRNA against C/EBPα or GATA-2 to evaluate the suppression efficacy of each shRNA. After 48-h culture,

cells were subjected to RT-PCR analyses (*upper panel*). Next, FIP1L1-PDGFRα–transduced murine KSLs were further infected with these shRNAs, and cultured with SCF, TPO, IL-6 and FLT3L, which were subjected to FACS analyses on the expression of CD125 and Gr-1 (*lower panel*). * indicates $p<0.05$ and ** $p<0.01$ as compared with the value in the cells coexpressing FIP1L1-PDGFRα and noncoding shRNA ($n=3$). ***C***, FIP1L1-PDGFRα-transduced murine KSLs were further infected with retrovirus encoding Mock or a dominant negative form of GATAs (GATA-3KRR). ** indicates $p<0.01$ as compared with the value in FIP1L1-PDGFRα- and Mock-cotransduced cells ($n=3$).

Fig. 7. **Effects of FIP1L1-PDGFRα and its downstream molecules on the activities of eosinophil-related transcription factors.** The activities of GATA-1, GATA-2, PU.1, and C/EBPα were analyzed by luciferase assays. After transfection of several effector genes and the appropriate reporter genes as indicated, NIH3T3 cells were cultured for 48 h, and subjected to luciferase assays. 3×MαP-luciferase, 3×MHC-luciferase, and 1×MPO-luciferase contain biding sites for GATA, PU.1, and C/EBPα, respectively. * indicates $p<0.05$ and ** $p<0.01$. Data represent means ±SD ($n=3$).