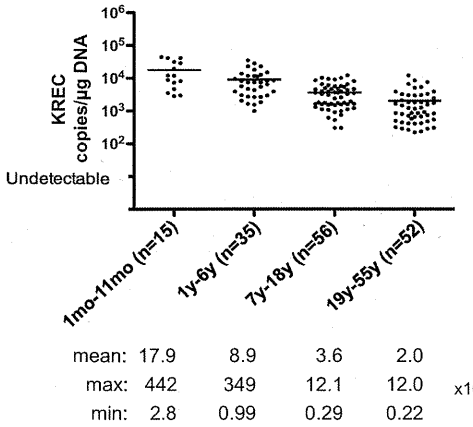

mean:	17.9	8.9	3.6	2.0	
max:	442	349	12.1	12.0	x10^3 copies/µg DNA
min:	2.8	0.99	0.29	0.22	

FIG E2. KREC levels were analyzed in genomic DNA samples extracted from peripheral blood of control subjects at different age groups (n = 158; age range, 1 month to 55 years). KREC levels were significantly higher in infants (17.9 ± 3.9 × 10^3 copies/µg DNA) compared with other children's age groups (8.9 ± 1.3 × 10^3 copies/µg DNA in the 1- to 6-year-old group and 3.6 ± 3.8 × 10^3 copies/µg DNA in the 7- to 18-year-old group) and adults (2.0 ± 3.3 × 10^3 copies/µg DNA; $P < .0001$).

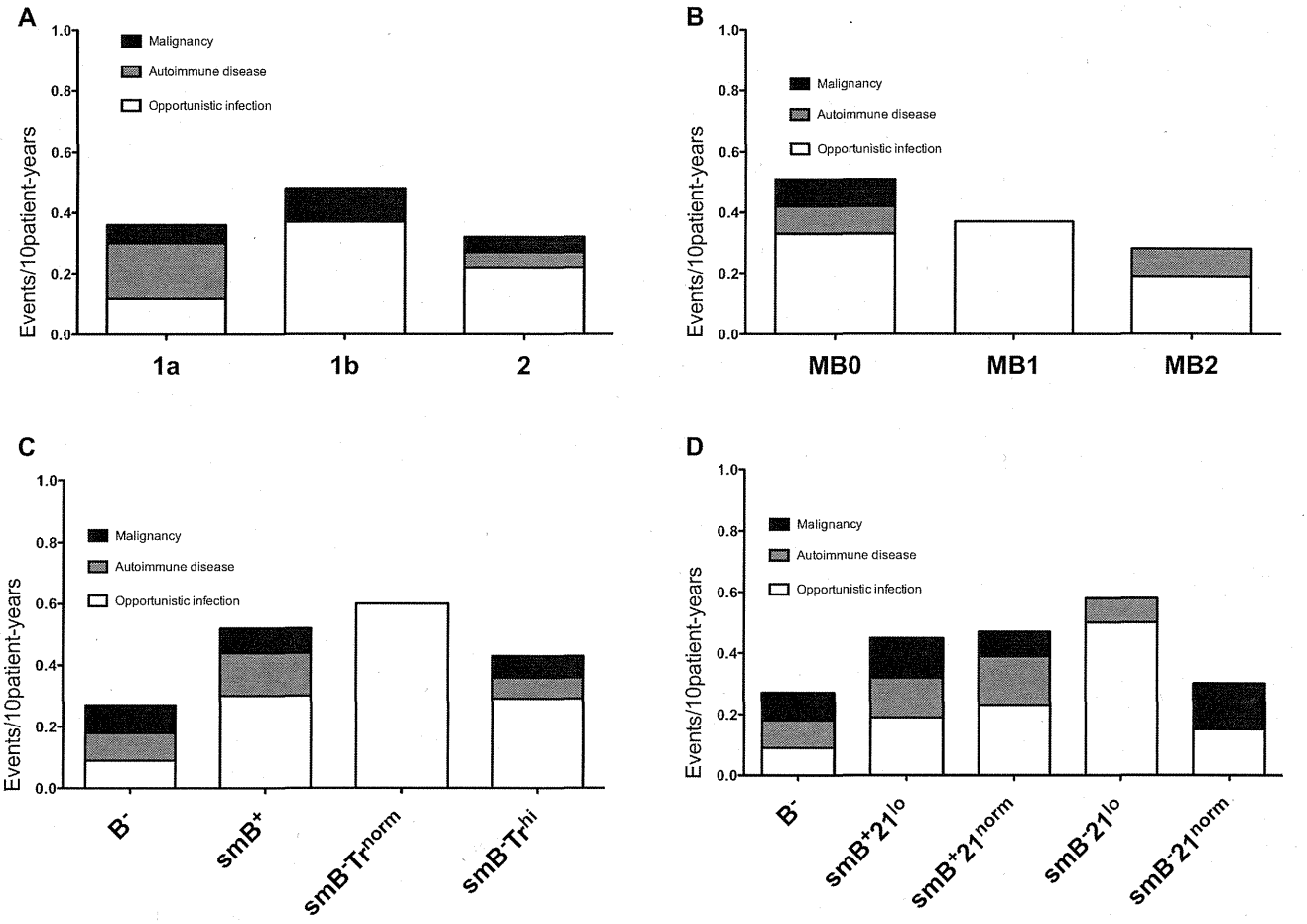

FIG E3. Patients were classified in the following way and analyzed for cumulative incidence of complications: **A,** Freiburg; **B,** Paris; and **C,** EUROclass classifications, according to $CD38^{hi}IgM^{hi}$ transitional B cells (Fig E3, *A-C*) or $CD21^{low}$ B cells **(D).** Five patients were excluded from the Freiburg and Paris classifications because of decreased B-cell numbers (<1%). Additionally, we excluded 4 patients in the Freiburg classification, 1 patient in the Paris classification, and 4 patients in the EUROclass classification for transitional B cells and 8 in the EUROclass classification for $CD21^{low}$ B cells because of lack of data. The following cumulative events/10 patient-years were found. Freiburg classification: 1a, 0.36; 1b, 0.48; 2, 0.32. Paris classification: MB0, 0.50; MB1, 0.37; MB2, 0.28. EUROclass classification according to transitional B cells: B^-, 0.27; smB^+, 0.52; smB^-Tr^{norm}, 0.60; smB^-Tr^{high}, 0.43. EUROclass classification according to $CD21^{lo}$ B cells: B^-, 0.27; smB^+21^{lo}, 0.45; smB^+21^{norm}, 0.47; smB^-21^{lo}, 0.58; smB^-21^{norm}, 0.30. No classification showed any significantly increased events in any particular group according to calculated *P* values, as follows—Freiburg classification: 1a vs 2 = .898, 1b vs 2 = .479, 1a vs 1b = .838; Paris classification: MB0 vs MB2 = .179, MB1 vs MB2 = .654, MB0 vs MB1 = .764; EUROclass classification according to transitional B cells: B^- vs smB^+ = .298, smB^-Tr^{norm} vs smB^+ = .809, smB^-Tr^{hi} vs smB^+ = .702, smB^-Tr^{hi} vs smB^-Tr^{norm} = .641, smB^-Tr^{norm} vs B^- = .329, smB^-Tr^{hi} vs B^- = .508; EUROclass classification according to $CD21^{lo}$ B cells: B^- vs smB^+21^{norm} = .443, smB^+21^{lo} vs smB^+21^{norm} = .930, smB^-21^{lo} vs smB^+21^{norm} = .695, smB^-21^{norm} vs smB^+21^{norm} = .575, B^- vs smB^-21^{norm} = .926, smB^+21^{lo} vs smB^-21^{norm} = .609, smB^-21^{lo} vs smB^-21^{norm} = .399, B^- vs smB^+21^{lo} = 0.474, B^- vs smB^-21^{lo} = 0.270, smB^+21^{lo} vs smB^-21^{lo} = 0.618.

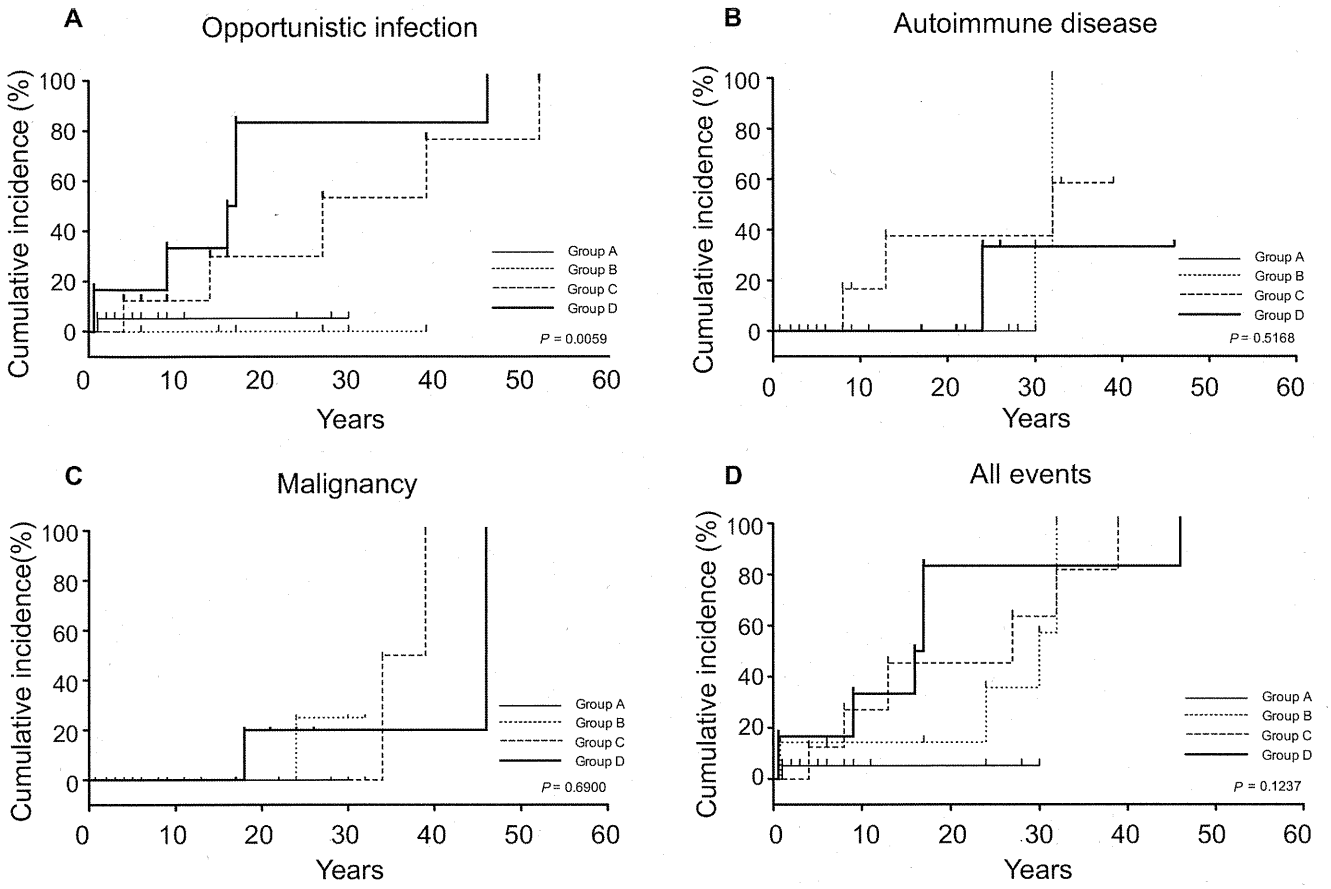

FIG E4. Comparing longitudinal cumulative incidence of complication events among groups. Cumulative incidence was estimated separately and longitudinally by using the Kaplan-Meier method and statistically compared between groups by using the log-rank test. The cumulative incidence of opportunistic infections **(A)**, autoimmune diseases **(B)**, malignancies **(C)**, and all events **(D)** is shown.

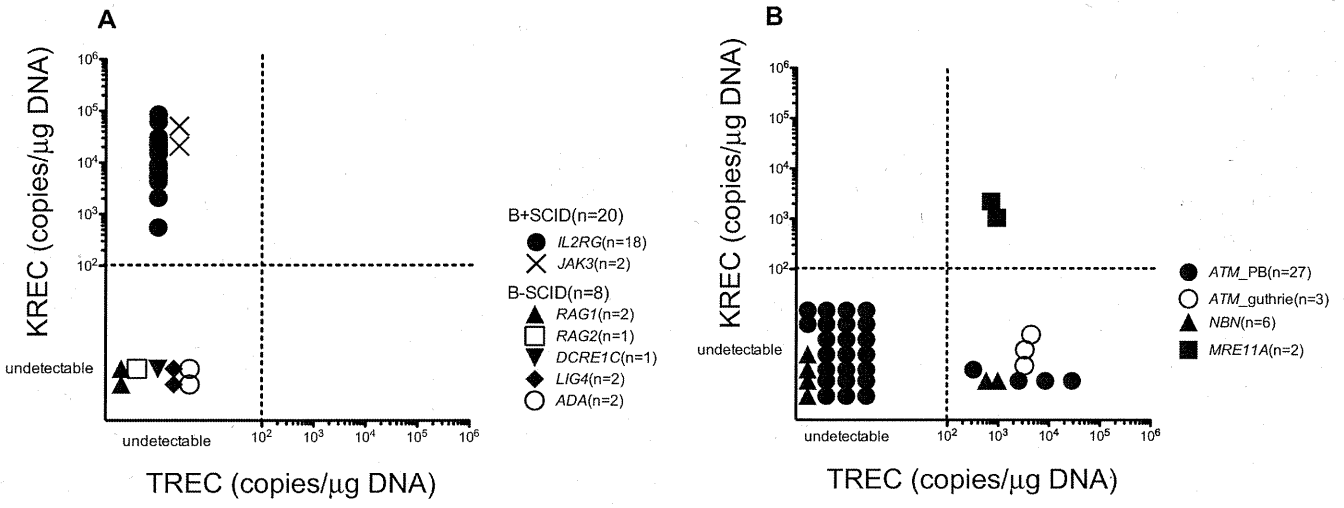

FIG E5. TREC and KREC quantification classifies patients with SCID, AT, NBS, or ataxia-telangiectasia–like disease *(ATLD)* into 4 groups. **A,** Patients with B^+SCID (n = 20) were classified as group C, and patients with B^-SCID (n = 8) were classified as group D; these patients were included in the previous studies.[5,6] **B,** Although most patients with AT (n = 23) and patients with NBS (n = 4) were classified as group D, TRECs were detected in peripheral blood samples (n = 4 in patients with AT and n = 2 in patients with NBS) and neonatal Guthrie cards (n = 3) of some patients with AT, who were classified as group B. Patients with ATLD with *MRE11A* mutations were classified as group A.

Article

Fetal liver stromal cells support blast growth in transient abnormal myelopoiesis in Down syndrome through GM-CSF†

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Abstract

Transient abnormal myelopoiesis (TAM) in neonates with Down syndrome, which spontaneously resolves within several weeks or months after birth, may represent a very special form of leukemia arising in the fetal liver (FL). To explore the role of the fetal hematopoietic microenvironment in the pathogenesis of TAM, we examined the in vitro influences of stromal cells of human FL and fetal bone marrow (FBM) on the growth of TAM blasts. Both FL and FBM stromal cells expressed mesenchymal cell antigens (vimentin, α-smooth muscle actin, CD146 and nestin), being consistent with perivascular cells/mesenchymal stem cells that support hematopoietic stem cells. In addition, a small fraction of the FL stromal cells expressed an epithelial marker, cytokeratin 8, indicating that they could be cells in epithelial-mesenchymal transition (EMT). In the coculture system, stromal cells of the FL, but not FBM, potently supported the growth of TAM blast progenitors, mainly through humoral factors. High concentrations of hematopoietic growth factors were detected in culture supernatants of the FL stromal cells and a neutralizing antibody against granulocyte-macrophage colony-stimulating factor (GM-CSF) almost completely inhibited the growth-supportive activity of the culture supernatants. These results indicate that FL stromal cells with unique characteristics of EMT cells provide a pivotal hematopoietic microenvironment for TAM blasts and that GM-CSF produced by FL stromal cells may play an important role in the pathogenesis of TAM.

INTRODUCTION

In mammals as well as non-mammalian vertebrates, the first wave of hematopoiesis ("primitive erythropoiesis") begins in the extra-embryonic yolk sac, originating from a common mesodermal precursor for hematopoietic and endothelial cell lineages [Dzierzak and Speck, 2008]. Various types of hematopoietic precursors produced later in the yolk sac, allantois and aorta-gonad-mesonephros region migrate to the liver, where the second wave of hematopoiesis (adult-type or "definitive hematopoiesis") begins and continues until birth. The major site of adult-type hematopoiesis finally shifts to the bone marrow afterwards, where it continues throughout life. Leukemia generally arises in the bone marrow, but certain types of leukemia have been shown to arise in utero. These include infantile and childhood acute lymphoblastic leukemia (ALL) with *MLL* gene rearrangements [Ford et al., 1993] or *TEL-AML1* rearrangements [Ford et al., 1998; Wiemels et al., 1999] and transient abnormal myelopoiesis (TAM) in neonates with Down syndrome (DS) [Ahmed et al., 2004]. In such cases, the microenvironment of prenatal hematopoietic organs, not bone marrow, might play an important role in the leukemogenesis.

Children with DS have a higher risk of developing leukemia [Hitzler and Zipursky, 2005; Roy et al., 2009]. ALL is the major form of leukemia in patients with DS at the age of 3 years or older, as in the case of non-DS patients, while acute myeloid leukemia (AML) is as commonly seen as ALL under the age of 3 years, and acute megakaryoblastic leukemia (AMKL), a very rare subtype of AML in non-DS children, comprises the majority of AML cases. In 4-10% of neonates with DS, abnormal blasts indistinguishable from those of AMKL in DS (AMKL-DS) appear in the blood, but usually spontaneously disappear within several weeks or months. A variety of terms have been given to this special disorder, including TAM, transient myeloproliferative disorder

(TMD) and transient leukemia (TL) [Hitzler and Zipursky, 2005; Roy et al., 2009; Roy et al., 2012; Zipursky, 2003]. AMKL arises in 20-30% of patients with TAM after spontaneous remission by the age of 4 years. Somatic mutations affecting the *GATA1* gene, which encodes one of the GATA family of zinc-finger transcription factors, have been detected exclusively in AMKL-DS and TAM in nearly all cases [Hitzler et al., 2003; Mundschau et al., 2003; Wechsler et al., 2002]. A variety of *GATA1* mutations have been reported, but almost all of them result in the lack of the 50-kD full-length GATA1 protein and the generation of a 40-kD short isoform of GATA1, called GATA1s, lacking the N-terminal activation domain [Gurbuxani et al., 2004]. The precise role of GATA1s in the pathogenesis of TAM and AMKL-DS remains largely unknown.

Myelofibrosis is a common complication of AMKL and is thought to be caused by cytokines, including transforming growth factor β (TGF-β), that are produced by leukemic megakaryoblasts and stimulate fibroblasts to induce fibrosis in the bone marrow [Terui et al., 1990]. Although TAM blasts have the features of megakaryoblasts similar to those of AMKL-DS blasts, myelofibrosis is absent in most cases but, instead, hepatic fibrosis is often found in fatal cases of TAM [Miyauchi et al., 1992; Schwab et al., 1998]. On the basis of these findings and considering that TAM is a disorder of neonates, we hypothesized that TAM may be a very special form of leukemia arising in the fetal liver (FL) but not in the bone marrow and that cytokines produced by TAM blasts in the FL cause hepatic fibrosis in a similar manner to myelofibrosis in AMKL [Miyauchi et al., 1992]. Consistent with this hypothesis, the expression of TGF-β has been immunohistochemically demonstrated in megakaryocytic cells in hepatic sinusoids of patients with TAM [Arai et al., 1999]. Furthermore, using a *GATA1* knock-in allele, the dominant action of

N-terminus-truncated GATA1 protein, leading to the hyperproliferation of hematopoietic progenitor cells in the yolk sac and FL, but not the bone marrow, has been shown [Li et al., 2005], indicating that the source of TAM is hematopoietic progenitor cells of embryonic/fetal origin. If the above hypothesis is the case and the growth of blast progenitors in TAM is dependent on the microenvironment of FL, cessation of fetal hematopoiesis in the liver after birth might cause spontaneous resolution of TAM [Miyauchi et al., 1992]. To explore the role of the fetal hematopoietic microenvironment in the pathogenesis of TAM directly, we investigated the in vitro influence of stromal cells derived from human FL and fetal bone marrow (FBM), the major hematopoietic organs in fetal stage, on the growth of blasts in TAM. The obtained findings indicate that blast progenitors in TAM are dependent on the microenvironment of the FL and that granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by FL stromal cells is particularly important for the growth of TAM blasts.

MATERIALS AND METHODS

Cells

TAM blasts were obtained from the peripheral blood of 4 patients with TAM (TAM-1 through TAM-4) after obtaining the informed consent of their parents. The clinical profile of these patients and *GATA1* mutations of the blasts have been published (TAM-1 through TAM-4 correspond to patient 1 through 4 in the reference, respectively) [Miyauchi et al., 2010]. AML blasts from 2 adult patients (AML-1 and AML-2; FAB subtypes, M5a and M1, respectively) were also obtained from the peripheral blood after obtaining informed consent. Blast cells were enriched by depleting

monocytes and lymphocytes by plastic adherence and immuno-magnetic bead methods, respectively, as previously described [Miyauchi et al., 2010], and these patient samples were confirmed to contain more than 95% blasts morphologically and to possess *GATA1* mutations.

Stromal cells of human FL and FBM were obtained from two fetuses (FL1/FBM1 from fetus 1; FL2/FBM2 from fetus 2) that had been artificially aborted during the 16th and 13th weeks of gestation, respectively, because of maternal health problems, after informed consent for the use of fetal tissues for research purposes had been obtained. Single-cell suspensions of FL and FBM stromal cells were prepared as previously described [Campagnoli et al., 2001]. Briefly, FBM cells were flushed out of the femur using a syringe with a 23-gauge needle into the growth medium [α-minimum essential medium (αMEM) supplemented with 10% fetal calf serum (FCS)], whereas FL was minced with scalpels, incubated in 0.25% trypsin solution containing 1 mM EDTA, filtered through a 70-μm nylon mesh and suspended in the growth medium. These cells were incubated at 37°C in 5% CO_2. Nonadherent cells were removed after 48 h and adherent cells were expanded in fresh growth medium for 2-3 weeks with 3-4 passages. The confluent adherent cell layers in primary cultures of the FL and FBM, consisting of morphologically homogeneous populations of fibroblast-like spindle cells, were trypsinized and cryopreserved.

A stromal cell line, KM101, which is derived from human adult bone marrow and known to support the growth of hematopoietic progenitors [Harigaya and Handa, 1985], was provided by Prof. K. Harigaya at Chiba University, Japan, and stem-cell-factor (SCF)-dependent leukemic cell line KPAM1 [Toki et al., 2009], derived from AMKL-DS, was provided by Dr. T. Toki and Prof. E. Ito at Hirosaki University, Japan. The experimental procedures using human cells described

above were approved by the Ethics Committee of Tokyo Dental College Ichikawa General Hospital.

Coculture of blasts and stromal cells

Stromal cells of the FL and FBM were irradiated with 15 Gy, while KM101 cells were with 10 Gy, and seeded onto 12-well plates (Corning, Lowell, MA) in 1.6 ml of growth medium. The following day, half of the medium was removed and the same volume of fresh growth medium containing 8 x 10^5 TAM or AML blasts was placed on the confluent monolayer of the adherent stromal cells. In another set of cultures, chambers with a microporous membrane (Transwell, pore size 0.4 μm; Corning) were placed in culture wells and blasts were placed into the chambers. For controls, blasts were cultured without the stromal cell layer in the presence or absence of hematopoietic growth factors. After 7 days of culture, cells were harvested and counted. Blasts cultured without a microporous membrane were treated as follows: 1) blasts that were not attached to the stromal cell layer were harvested as nonadherent cells, and 2) the remaining cells, including the adherent stromal cells and blasts that were attached to them, were trypsinized, harvested together, and the number of blasts was counted, with co-existing stromal cells being easily recognized by their larger size and excluded. Co-existing stromal cells were removed by culturing the cells for 3 h, allowing them to adhere again. The remaining cells were washed and plated in methylcellulose for subsequent colony assay. The number of blast progenitors (clonogenic cells) that had been recovered from these cultures was calculated by multiplying the plating efficiency in the colony assay by the total number of cells after coculture.

Suspension culture

TAM blasts were cultured at 2.5-5 x 10^5 cells/mL in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) or Linbro/Titertek 96-well plates (MP Biomedicals, Solon, OH) in the growth medium with or without hematopoietic growth factors or culture supernatants of FL stromal cells. The recovery of blast progenitors from these cultures was evaluated as described above using subsequent colony assay.

Colony assay

Colony formation of blast progenitors was assessed using methylcellulose culture as previously described [Miyauchi et al., 1987]. Blasts were plated at a concentration of 5 x 10^3 cells per well in 96-well plates in 0.1 mL of growth medium. As a growth stimulant, interleukin-3 (IL-3) was used for TAM blasts since it has been shown to be the most powerful growth stimulator for TAM blasts [Miyauchi et al., 2010], whereas granulocyte-CSF (G-CSF) or IL-3 was used for G-CSF- or IL-3-dependent AML blasts, respectively.

Immunocytochemistry

The stromal cells of the FL and FBM, seeded onto Culture Slides (BD Biosciences, Bedford, MA) and cultured, were air-dried, fixed with buffered formalin-acetone for 30 sec and subjected to immunocytochemistry for analysis of their phenotypic antigen expression. The cell samples were pretreated for antigen retrieval with 0.1 M citrate buffer (pH 6.0 or 9.0) in a microwave oven or a pressure cooker according to the manufacturers' instructions and immunocytochemistry was performed using an automated slide preparation system (Ventana Japan, Yokohama, Japan). The

antibodies (mouse monoclonal, unless otherwise stated) used are as follows: anti-vimentin (clone V9; Nichirei, Tokyo, Japan), anti-CD146 (clone EPR3208; Abcam, Tokyo, Japan), anti-nestin (clone 2C1.3A11; Abcam), anti-α-smooth muscle actin (α-SMA) (clone 1A4; DAKO, Tokyo, Japan), anti-cytokeratin 8 (CK8) (clone C-43; Abcam), anti-CK18 (clone DC10; DAKO), anti-α-fetoprotein (AFP) (clone ZSA06; Nichirei), anti-E-cadherin (clone NCH-38; DAKO), anti-HepPar1 (clone OCH1E5; DAKO), anti-CD31 (clone JC70A; DAKO), anti-CD34 (clone QBEnd-10; DAKO), anti-CD54 (rabbit polyclonal; Cell Signaling, Boston, MA), anti-CD4 (clone SP35; Roche, Tokyo, Japan), anti-desmin (clone D3; Nichirei), anti-glial fibrillary acidic protein (GFAP) (rabbit polyclonal; DAKO), anti-synaptophysin (rabbit polyclonal; Invitrogen, Tokyo, Japan), anti-CD56 (NCAM) (clone CD564; Leica, Newcastle, UK), anti-lysozyme (rabbit polyclonal; DAKO), anti-CD68 (clone PGM1; DAKO), anti-CD45 (clone PD7/26, 2B11; Nichirei) and anti-CD10 (clone 56C6; Nichirei).

Hematopoietic growth factors

G-CSF was provided by Chugai Pharmaceutical (Tokyo, Japan) and the other hematopoietic growth factors were purchased from various manufacturers: IL-3 (Strathmann Biotech AG, Hamburg, Germany), GM-CSF (Prospec-Tany, Rehovot, Israel), SCF (BioVision, Mountain View, CA), thrombopoietin (TPO) (PeproTech EC, London, UK) and insulin-like growth factor 2 (IGF2) (Abcam). IL-3, GM-CSF, SCF, TPO and G-CSF were used at a final concentration of 50 ng/ml unless otherwise stated, whereas IGF2 was used at 100 ng/mL based on the result of titration experiments (data not shown).

ELISA assay

Culture supernatants of the FL and FBM stromal cells were harvested 3 days after the cells had become confluent and floating cell debris was removed by centrifugation and microfiltration with a 0.2 μm Acrodisc Syringe Filter (PALL, Ann Arbor, MI). Concentrations of IL-3, GM-CSF, G-CSF, TPO, SCF and IGF2 in the culture supernatants of the FL and FBM stromal cells were measured with ELISA kits (IL-3, GM-CSF, G-CSF, TPO and SCF: R&D Systems, Minneapolis, MN; IGF2: Mediagnost, Reutlingen, Germany) according to the manufacturers' instructions.

Neutralizing antibodies

The antibodies against GM-CSF and G-CSF were purchased from PeproTech (Rocky Hill, NJ) and the antibody against SCF was purchased from Abcam. These antibodies were used at concentrations above the one-half maximal inhibition doses described in the manufacturers' instructions. Before being added to the cultures, these antibodies were incubated with the culture supernatants or hematopoietic growth factors at 4°C for 4 h.

RESULTS

Characterization of FL and FBM stromal cells

Prior to the experiments using the FL and FBM stromal cells, phenotypic antigen expression of these cells on culture slides was determined by immunocytochemistry (Table 1). Both FL and FBM stromal cells were intensely positive for vimentin (a general mesenchymal cell marker), α-SMA (an antigen expressed in hepatic stellate cells and mesenchymal stem cells (MSCs)) (Fig. 1A, B),

CD146 and nestin (antigens expressed in perivascular cells/pericytes and MSCs) [Corselli et al., 2013; Covas et al., 2008; Crisan et al., 2008; Gerlach et al., 2012; Mendez-Ferrer et al., 2010] (Fig. 1C, D). In addition, a small fraction of the FL stromal cells weakly expressed CK8 (an epithelial antigen expressed in hepatoblasts and hepatocytes) (Fig. 1E), although they were negative for other epithelial markers such as CK18, E-cadherin, AFP and HepPar1 (antigens expressed in hepatoblasts and/or hepatocytes). The FBM stromal cells were positive for CD10 (an antigen expressed in bone marrow reticular cells) (Fig. 1F), but negative for epithelial markers. Both types of stromal cell were negative for other antigens examined, namely, general endothelial markers (CD34, CD31), markers of hepatic sinusoidal endothelial cells (CD54/ICAM-1, CD4), those of Kupffer cells (lysozyme, CD68) and those of hepatic stellate cells (also called Ito cells) (desmin, GFAP, synaptophysin, CD56/NCAM), except for α-SMA (Table 1). These findings indicate that both the FL and the FBM stromal cells that we used represent the perivascular MSCs, with the former corresponding to hepatic pericytes and the latter being consistent with bone marrow reticular cells. The FL stromal cells also appeared to have some features of cells in epithelial-mesenchymal transition (EMT cells), known to be seen under certain conditions, including developing organs [Choi and Diehl, 2009; Kalluri and Weinberg, 2009] and to support fetal hematopoiesis in the liver [Chagraoui et al., 2003].

Since it has been shown that murine fetal liver EMT cells are induced to differentiate into hepatocytes and lose their hematopoiesis-supporting ability in the presence of oncostatin M (OSM), we tested whether our FL stromal cells also exhibit such changes in response to OSM treatment according to a method previously described [Chagraoui et al., 2003]. Although the in

vitro growth rate of the FL stromal cells was suppressed by the addition of 1 ng/mL OSM, spindle cell morphology and immunophenotypic features were largely unchanged, with no obvious enhancement or induction of epithelial cell marker expression and only a slight decrease in α-SMA expression.

Coculture of TAM blasts and stromal cells

To examine the influence of cells constituting the fetal hematopoietic microenvironment on the in vitro growth of TAM blasts, we analyzed the growth-supporting ability of stromal cells of the FL and FBM obtained from two human fetuses by coculturing them with TAM blasts obtained from 4 patients. To eliminate the influence of cell-to-cell contact, a transwell coculture system with a microporous membrane, separating blasts from stromal cells and allowing only the passage of humoral factors between the two chambers, was used. To compare the functions of stromal cells of the fetal and postnatal hematopoietic microenvironment, we also examined the effects of an adult human bone marrow-derived stromal cell line, KM101, which is known to be capable of supporting hematopoiesis. Since we found in the preliminary experiments that KM101 cells form colonies in methylcellulose, which makes it difficult to analyze the data of TAM blast colony assay if these cells coexist in the same samples, KM 101 cells were used only in the presence of transwells. When TAM blasts were cocultured with the FL stromal cells in the presence of a transwell, the growth of TAM blast progenitors was potently supported in all patients and the numbers of blast progenitors recovered to levels comparable to those of the cultures with IL-3 in 3 patients (Fig. 2A-C), although it was slightly below the level of IL-3 in one patient (Fig. 2D). After the coculture of TAM blasts and the FL stromal cells in the absence of transwells, numerous viable TAM cells were present in

culture and the growth of blast progenitors, particularly in non-adherent layers, was efficiently supported although to lower levels than that of the cultures with transwells in all patients (Fig. 2A-D). The immunophenotypic features of the FL stromal cells were unchanged before and after the coculture (data not shown). In contrast, after the culture with FBM stromal cells, TAM blasts dramatically decreased in number and the recovery of TAM blast progenitors was very poor and significantly lower than in the cultures with the FL stromal cells under all culture conditions irrespective of the presence or absence of transwells (Fig. 2A-D). After the coculture of TAM blasts with KM101 cells, the growth of TAM blast progenitors was supported to levels similar to, or slightly lower than, those of the FL stromal cells in the presence of transwells (Fig. 2A-D). These findings indicate that the growth of TAM blast progenitors is dependent on the stromal cells of FL, but not FBM, and supported mainly by humoral factors produced by the FL stromal cells, and that adult bone marrow stromal cells are also capable of supporting the growth of TAM blast progenitors.

Coculture of AML blasts and stromal cells

To determine if the growth-supporting ability of the FL stromal cells is limited to hematopoietic cells of FL origin such as TAM blasts and to test whether FBM stromal cells are capable of supporting the growth of leukemic cells derived from the bone marrow, we next examined the influence of the FL and FBM stromal cells on the growth of AML blasts in adult patients. Since the growth patterns of adult AML blasts in response to hematopoietic growth factors are heterogeneous among patients, two patient samples were selected for this experiment; blast progenitors in patient AML-1 are dependent on IL-3, GM-CSF and SCF as in the case of TAM, whereas those of patient

AML-2 are dependent almost exclusively on G-CSF and SCF. These AML blasts were cultured with the FL or FBM stromal cells in the same way as for TAM blasts. The growth of blast progenitors in AML-1 and AML-2 was stimulated by IL-3 and G-CSF, respectively, as expected (Fig. 3A, B). In both cases, the FL stromal cells in the presence, but not the absence, of a transwell stimulated the growth of AML blast progenitors more effectively than G-CSF or IL-3 alone. The FBM stromal cells also exhibited some degree of growth-supporting activity on AML blasts in the presence of transwells, particularly for those of AML-1, but the activity was still significantly weaker than that of the FL stromal cells. Hence, it was shown that the growth-supporting ability of the FL stromal cells is not limited to fetal hematopoietic cells but is also valid for adult leukemic blasts originating in the bone marrow, whereas the stromal cells of the FBM that we used, at the gestational ages of 13 and 16 weeks, do not support the growth of either fetal or adult myeloid leukemia cells as efficiently as the FL stromal cells do, possibly due to their functional immaturity.

Hematopoietic growth factors produced by stromal cells

Since the growth of TAM and AML blasts was potently supported by the FL stromal cells in transwell cultures with a microporous membrane hindering cell-to-cell contact between blasts and stromal cells, humoral factors must be secreted from the stromal cells into the culture medium. In order to identify the humoral factors produced by the stromal cells, we measured the concentrations of six major human hematopoietic growth factors: IL-3, GM-CSF, G-CSF, SCF, TPO and IGF2, in culture supernatants of the FL and FBM stromal cells by ELISA. High concentrations of GM-CSF, G-CSF, SCF and IGF2 were detected in the culture supernatants of the FL stromal cells obtained from both of the two fetuses (FL1 and FL2) (Table 2). Compared with the culture supernatants of

the FL stromal cells, similar or even higher amounts of SCF and IGF2 were detected in those of the FBM stromal cells, but GM-CSF was barely detectable and G-CSF was undetectable. Notably, IGF2 was detected even in the control growth medium containing 10% FCS, but its concentration was lower than in most of the FL and FBM stromal cell culture supernatants. IL-3 and TPO were not detected in any culture supernatants of the stromal cells. Among these hematopoietic growth factors, the key factors that caused the big difference in growth-supporting ability for TAM blast progenitors between the FL and FBM stromal cells should be those that are present at higher concentrations in the stromal cell culture supernatants of FL than those of FBM. Since the concentrations of SCF and IGF2 were not uniformly higher in the FL stromal cell culture supernatants and our previous study showed that G-CSF is not a very active stimulator of the growth of TAM blasts [Miyauchi et al., 2010], we speculated that GM-CSF might be the most likely candidate for such a factor.

Effects of neutralizing antibodies

To ascertain the important hematopoietic growth factor(s) for the growth of TAM blasts that are produced by the FL stromal cells, we tested the effects of neutralizing antibodies against GM-CSF, G-CSF and SCF on the culture supernatants of the FL stromal cells. Antibodies against IL-3, TPO and IGF2 were not included because neither IL-3 nor TPO was detected in any culture supernatants and IGF2 was not shown to be a potent growth stimulator for TAM blasts in our experiments as described below. Prior to the experiments, the neutralizing ability of these antibodies was confirmed using colony assay of three AML cell lines, OCI-AML1, MO7e and KPAM1, the growth of which is dependent on G-CSF, GM-CSF and SCF, respectively. Each antibody