completely inhibited colony-stimulating activity of the corresponding cytokine (Supplementary Fig. 1A-C), and the antibody against GM-CSF at concentrations higher than 1 μg/mL completely neutralized the activity of 10 ng/mL GM-CSF (Supplementary Fig. 1D). Then, we tested the effects of these antibodies on the culture supernatants of the FL stromal cells (2 samples, FL1 and FL2), using suspension culture and colony assay of TAM blasts obtained from 3 patients. The antibody against GM-CSF alone almost completely inhibited both cell growth in suspension culture (Fig. 4A, C and Supplementary Fig. 2A) and colony formation in methylcellulose culture (Fig. 4B, D and Supplementary Fig. 2B) of TAM blasts with all FL culture supernatants and TAM patient samples. In the colony assay of patient TAM-4, the antibody against GM-CSF inhibited colony formation to levels even below that of the negative control (no added culture supernatants and antibodies) (Fig. 4D), suggesting that TAM blasts of this patient may produce a small amount of GM-CSF in an autocrine fashion and that such activity was also neutralized by the antibody. On the other hand, the antibodies against G-CSF and SCF and the control non-immune IgG did not significantly affect the growth of TAM blasts (Fig. 4 and Supplementary Fig. 2).

Effects of IGF2 on the growth of TAM blasts

IGF2 is produced in fetal organs, including the liver [Pollak, 2008; Zhang and Lodish, 2004], and it has been shown that abnormalities in the IGF2 signaling pathway may be involved in the pathogenesis of TAM and AMKL-DS [Klusmann et al., 2010]. However, our previous study showed that growth medium supplemented with FCS, which contains a high concentration of IGF2 as shown in Table 2, alone does not significantly stimulate the growth of TAM blasts [Miyauchi et al., 2010]. To ascertain if IGF2 has any growth-stimulating activity for TAM blasts, we tested the

in vitro effects of IGF2 on TAM blasts in 2 patients. Both colony formation in methylcellulose culture (data not shown) and recovery of blast progenitors from suspension culture only slightly increased when IGF2 was added at high concentration (100 ng/mL), but the maximum activity was still much lower than that of other hematopoietic growth factors such as IL-3 and TPO in both patients (Fig. 5). Furthermore, no synergistic effects were detected with any combinations of IGF2 and another hematopoietic growth factor (data not shown). Although the experiments were performed in the presence of 10% FCS, these findings indicate that IGF2 on its own has only modest growth-stimulating activity on TAM blasts.

DISCUSSION

The present coculture experiments demonstrated that in vitro growth of blast progenitors in TAM is dependent on stromal cells of the FL, but not FBM, indicating that the microenvironment of the FL could play an important role in the pathogenesis of TAM. This result is consistent with the data described by Tunstall-Pedoe et al. [2008] that FLs, but not FBMs, in DS patients exhibit perturbed myeloid hematopoiesis with a higher frequency of megakaryocyte-erythroid progenitor cells compared with normal individuals, indicating that trisomy 21 provides a background for leukemogenesis in the FL preceding the acquisition of *GATA1* mutations. These data support the hypothesis previously proposed by us that TAM is a special form of leukemia arising in the FL [Miyauchi et al., 1992]. Although the number of TAM samples used in this study is small, we have previously shown that growth characteristics of TAM blast progenitors in response to hematopoietic growth factors are quite uniform with very little patient heterogeneity [Miyauchi et

al., 2010]. This could be a reflection of the relatively simple cytogenetic background of TAM, in which the rate of mutations other than *GATA1* is very low [Nikolaev et al., 2013; Yoshida et al., 2013] and a variety of *GATA1* mutations all result in an uniform abnormality of GATA1 protein, namely, generation of GATA1s and abrogation of full-length GATA1 [Gurbuxani et al., 2004]. Therefore, our present findings may represent the general biological nature of TAM blasts. Furthermore, our present in vitro findings are consistent with the in vivo observations of human patient samples that blasts and/or atypical megakaryocytes proliferated predominantly in the liver in autopsy cases of stillborns or liveborn infants with DS and TAM, which corroborate our present data [Becroft and Zwi, 1990; Ishigaki et al., 2011; Miyauchi et al., 1992; Ruchelli et al., 1991].

Although we used TAM samples enriched for >95% blasts morphologically, a minor proportion of normal hematopoietic progenitors may have coexisted in the samples and formed colonies in culture. However, all the patient samples enriched for blasts harbored *GATA1* mutations and one of them (TAM-4) exhibited only mutated *GATA1* sequences both before and after culture with hematopoietic growth factors [Miyauchi et al., 2010], indicating that all colonies of this patient sample should be derived from TAM blasts. Since coculture results of the other patients were very similar to that of this particular patient, the influence of coexisting non-leukemic hematopoietic progenitors, if any, seems to be small and negligible.

MSCs are mesodermal progenitors with the potential to differentiate to multilineage mesenchymal cells [Pittenger et al., 1999]. Perivascular cells, or pericytes, which reside ubiquitously around endothelial cells of capillaries and microvessels in many organs, have been shown to represent ancestors of, or basically the same population as, MSCs [Covas et al., 2008;

Crisan et al., 2008] and to be capable of supporting hematopoietic stem cells (HSCs) [Corselli et al., 2013]. In addition to the originally detected endosteal niche, the existence of a perivascular niche for HSCs in the bone marrow, such as CXCL2-expressing reticular cells [Sugiyama and Nagasawa, 2012] or nestin-expressing MSCs [Mendez-Ferrer et al., 2010], has been demonstrated. Concerning the liver, perisinusoidal hepatic stellate cells, residing in the space of Disse, express antigens associated with MSCs, produce cytokines, including GM-CSF, and support HSCs [Kordes et al., 2013], suggesting that these cells may be liver-resident perivascular MSCs that form an HSC niche in the liver. The FL stromal cells that we used expressed CD146, nestin and α-SMA, which is consistent with hepatic pericytes/MSCs [Gerlach et al., 2012]. Although these cells might correspond to the immature form of hepatic stellate cells since only one of the stellate cell-associated antigens (α-SMA) was positive, this point needs further confirmation. In addition to the mesenchymal antigens, these cells also expressed CK8, an epithelial cell marker, indicating that they could belong to the unique category of cells called EMT cells. It has been shown that such cells are present in the fetal liver and associated with florid hematopoiesis [Chagraoui et al., 2003]. However, only a minor fraction of our FL stromal cells expressed CK8 and did not express other epithelial markers, even after treatment with OSM, which is different from the results of murine fetal liver stromal cell lines with EMT cell natures described by Chagraoui et al. The exact reason for this discordance is not clear, but it could be due to the difference of species.

Although it has been shown that perivascular MSCs maintain the stemness of HSCs by cell-to-cell contact, cell contact between TAM blasts and FL stromal cells in our study did not enhance, or even reduced, the recovery of TAM blast progenitors, indicating that humoral factors

produced by the stromal cells stimulated the growth of TAM blasts. This is in agreement with previous findings that fetal HSCs pass through the cell cycle at a higher frequency during fetal development than adult HSCs, which are largely quiescent [Martin and Bhatia, 2005]. However, it is also possible that FL stromal cells provide a hematopoietic niche for a minor population of quiescent leukemic stem cells in TAM and support their long-term survival through cell-to-cell contact in vivo.

Like the case of TAM, the growth of AML blasts in adult patients was supported more efficiently by stromal cells of the FL than those of the FBM, and adult bone marrow stromal cell line KM101 also supported the growth of TAM blasts. Hence, FL stromal cells do not exclusively support the growth of fetal hematopoietic cells and the growth of TAM blasts is not exclusively dependent on FL stromal cells but also supported by adult bone marrow stromal cells. These results can be explained by the present findings that FL stromal cells produce hematopoietic growth factors, including GM-CSF, G-CSF and SCF, that stimulate the growth of adult AML blasts and by the data of other investigators showing that KM101 cells produce GM-CSF [Nakajima et al., 1994] that stimulates the growth of TAM blasts. These data do not support the hypothesis that we previously proposed [Miyauchi et al., 1992] that the cessation of fetal liver hematopoiesis and a shift of major hematopoietic organ from the liver to the bone marrow after birth cause the inhibition of TAM blast growth and spontaneous resolution of TAM because of the loss of a suitable microenvironment. Spontaneous resolution of TAM therefore appears to be more likely associated with a change of the intrinsic genetic program controlling fetal hematopoiesis rather than a shift of the site of hematopoiesis after birth. The FBM stromal cells that we used were not very active in

supporting the growth of leukemic blasts in either TAM or adult AML, indicating that FBM stromal cells at the 13th and 16th weeks of gestational age may still be immature and incapable of constituting a functional hematopoietic microenvironment. The low concentrations of secreted hematopoietic growth factors, particularly GM-CSF and G-CSF, in the culture supernatants of the FBM stromal cells suggest this possibility.

We showed in the present study that the FL stromal cells produce several hematopoietic growth factors and secrete them into the culture medium. We have previously shown that IL-3, GM-CSF and SCF are the major growth factors for TAM blasts in vitro, all of which directly and potently stimulate the growth of TAM blasts [Miyauchi et al., 2010]. However, IL-3 has not been described to be produced in the liver and was not detected in the culture supernatants of the FL stromal cells. Although low concentrations of SCF were detected in the culture supernatants of FL stromal cells, the anti-SCF antibody did not significantly affect the growth of TAM blasts in the presence of FL stromal cell culture supernatants, indicating that SCF secreted into the culture supernatants at these concentrations did not potently stimulate the growth of TAM blasts. Since the antibody against GM-CSF alone significantly inhibited the growth-stimulating activity of the FL culture supernatants on TAM blasts, it seems highly likely that, among the cytokines produced by FL stromal cells, GM-CSF is the most important hematopoietic growth factor for TAM blasts and might play an important role in the pathogenesis of TAM. However, megakaryoblastic features of TAM blasts are hardly ascribable to the function of GM-CSF alone since GM-CSF is not a potent inducer of megakaryocytic differentiation. We have previously shown that TPO has such an activity for TAM blasts, although its growth-stimulatory effect is weaker than that of GM-CSF

[Miyauchi et al., 2010]. Since hepatocytes are known to be the major source of TPO and to release it into the blood [Sungaran et al., 1997] and TPO was not detected in the culture supernatants of our FL stromal cells, it seems plausible that megakaryocytic differentiation of TAM blasts in vivo is induced by TPO produced by fetal hepatocytes or hepatoblasts, while their growth is stimulated by GM-CSF produced by FL stromal cells. Although it has been shown that IGF2 is produced by fetal hepatocytes and involved in the pathogenesis of TAM and AMKL-DS [Klusmann et al., 2010], it seems unlikely that IGF2 is a major growth regulator of TAM blasts since our data indicate that IGF2 on its own is only a modest growth stimulator of TAM blasts in vitro.

In conclusion, our present study demonstrated that FL stromal cells with unique immunophenotypic features of EMT cells constitute a functional hematopoietic microenvironment that supports the expansion of TAM clones originating from the fetal liver and that GM-CSF produced by FL stromal cells may play an important role in the pathogenesis of TAM.

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AUTHORSHIP

J.M. designed and performed the research, analyzed the data and wrote the manuscript. H.K. contributed designing of the research and data analysis.

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FIGURE LEGENDS

Figure 1. Immunocytochemistry of FL and FBM stromal cells

Stromal cells of the FL and FBM cultured on culture slides were immunostained for α-SMA (A, B), nestin (C, D), CK8 (E) and CD10 (F). (A), (C) and (E) are FL stromal cells, and (B), (D) and (F) are FBM stromal cells. Scale bars indicate 500 μm (A-D & F) and 200 μm (E).

Figure 2. Effects of FL and FBM stromal cells on the growth of TAM blast progenitors

(A)-(D) TAM blasts of 4 patients (TAM-1 through TAM-4) were cocultured with the FL or FBM stromal cells or KM101 cells in the presence or absence of the transwell system for 7 days. The number of TAM blast progenitors (clonogenic cells) recovered from the culture is shown. The stromal cells of FL1 and FBM1 were used in (A) and (B) and those of FL2 and FBM2 in (C) and (D). *Abbreviations*: Memb, microporous membrane separating TAM blasts from the stromal cells; NA, non-adherent layer; AD, adherent layer; NG, no growth factor; BM, bone marrow; ND, not determined. Statistically significant difference is indicated by asterisks (*P<0.05) while NS indicates no significant difference.

Figure 3. Effects of FL and FBM stromal cells on the growth of AML blast progenitors

AML blasts of 2 adult patients (AML-1 and AML-2) were cocultured with the FL or FBM stromal cells in the presence or absence of the transwell system for 7 days. The number of AML blast progenitors (clonogenic cells) in patients AML-1 (A) and AML-2 (B) recovered from the culture is shown. The stromal cells of FL2 and FBM2 were used in both (A) and (B). Abbreviations are the

same as for Figure 1. Statistically significant difference is indicated by asterisks (*P<0.05) while NS indicates no significant difference.

Figure 4. Effects of neutralizing antibodies on culture supernatants of FL stromal cells

TAM blasts were cultured in suspension or in methylcellulose with 20% culture supernatants of FL1 or FL2 stromal cells in the presence of a neutralizing antibody against G-CSF (αG-CSF, 2.5 μg/mL), GM-CSF (αGM-CSF, 2.5 μg/mL) or SCF (αSCF, 5.0 μg/mL), or non-immune IgG (IgG), or in the absence of culture supernatants and any antibody (None). The total number of cells in patient samples TAM-2 and TAM-4 after suspension culture is shown in (A) and (C), and the number of colonies after methylcellulose culture in (B) and (D), respectively. Statistically significant difference is indicated by asterisks (*P<0.05). Double asterisks in (D) indicate significant difference compared with the culture without the supernatants and antibody (None).

Figure 5. Effects of IGF2 on the growth of TAM blast progenitors in suspension culture

TAM blasts were cultured in suspension with IGF2, IL-3 or TPO, or in the absence of growth factors (NG) for 7 days. The number of TAM blast progenitors (clonogenic cells) in patient samples TAM-2 (A) and TAM-4 (B) recovered from the culture is shown. Statistically significant difference is indicated by asterisks (*P<0.05).

Table 1. Immunocytochemistry of the stromal cells isolated from FL and FBM

Markers	FL	FBM
Mesenchymal cell / Perivascular cell	+++	+++
vimentin	+++	++
α-smooth muscle actin (αSMA)	++	++
CD146	++	++

nestin		
Epithelial cell	–/+	–
cytokeratin 8 (CK8)	–	–
cytokeratin 18 (CK18)	–	–
E-cadherin	–	–
α-fetoprotein (AFP)	–	–
hepatocyte antigen (Hep/Par1)		
Endothelial cell	–	–
CD34	–	–
CD31		
Hepatic sinusoidal endothelial cell	–	–
CD54 (ICAM-1)	–	–
CD4		
Hepatic stellate cell (Ito cell)	+++	++
α-smooth muscle actin (αSMA)	–	–
desmin	–	–
glial fibrillary acidic protein (GFAP)	–	–
synaptophysin	–	–
CD56 (NCAM)		
Kupffer cell / Macrophage	–	–
Lysozyme	–	–
CD68		
Hematopoietic cell	–	–
CD34	–	–
CD45		
Bone marrow reticular cell	–	+
CD10		

+++, 100% positive; ++, 50-99% positive; +, 10-50% positive; –/+, 1-10% positive;

–, negative

Table 2. ELISA assay of culture supernatants of FL and FBM stromal cells

Hematopoietic growth factor	FL1	S/N	FBM1	S/N	FL2	S/N	FBM2	S/N	αMEM + 10% FCS

GM-CSF	6,100	19	1,210	12	UD
G-CSF	131,000	UD	12,000	UD	UD
SCF	240	43	220	190	UD
IGF2	14,345	6,500	14,285	19,060	6,770
IL-3	UD	UD	UD	UD	UD
TPO	UD	UD	UD	UD	UD

The values in the table are given in pg/mL. *Abbreviations*: S/N, supernatant; FL1 and FBM1 represent FL and FBM stromal cells of fetus 1, respectively. Likewise, FL2 and FBM2 represent those of fetus 2. UD, undetectable (below the sensitivity level).

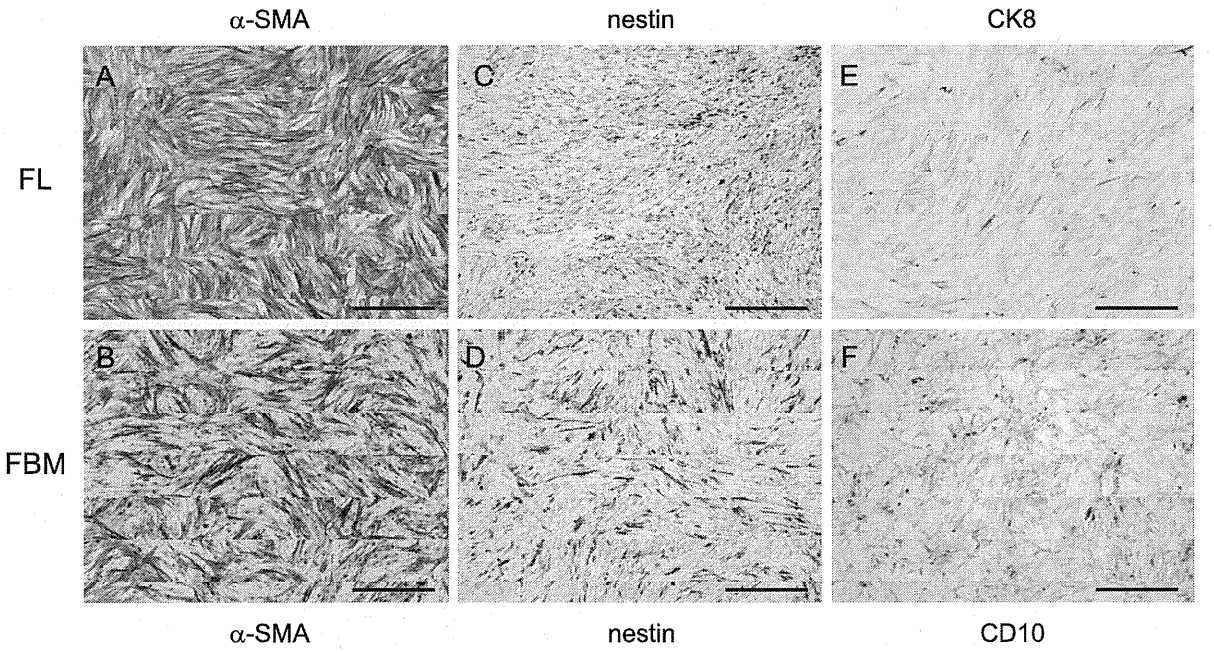

Figure 1

Figure 1.

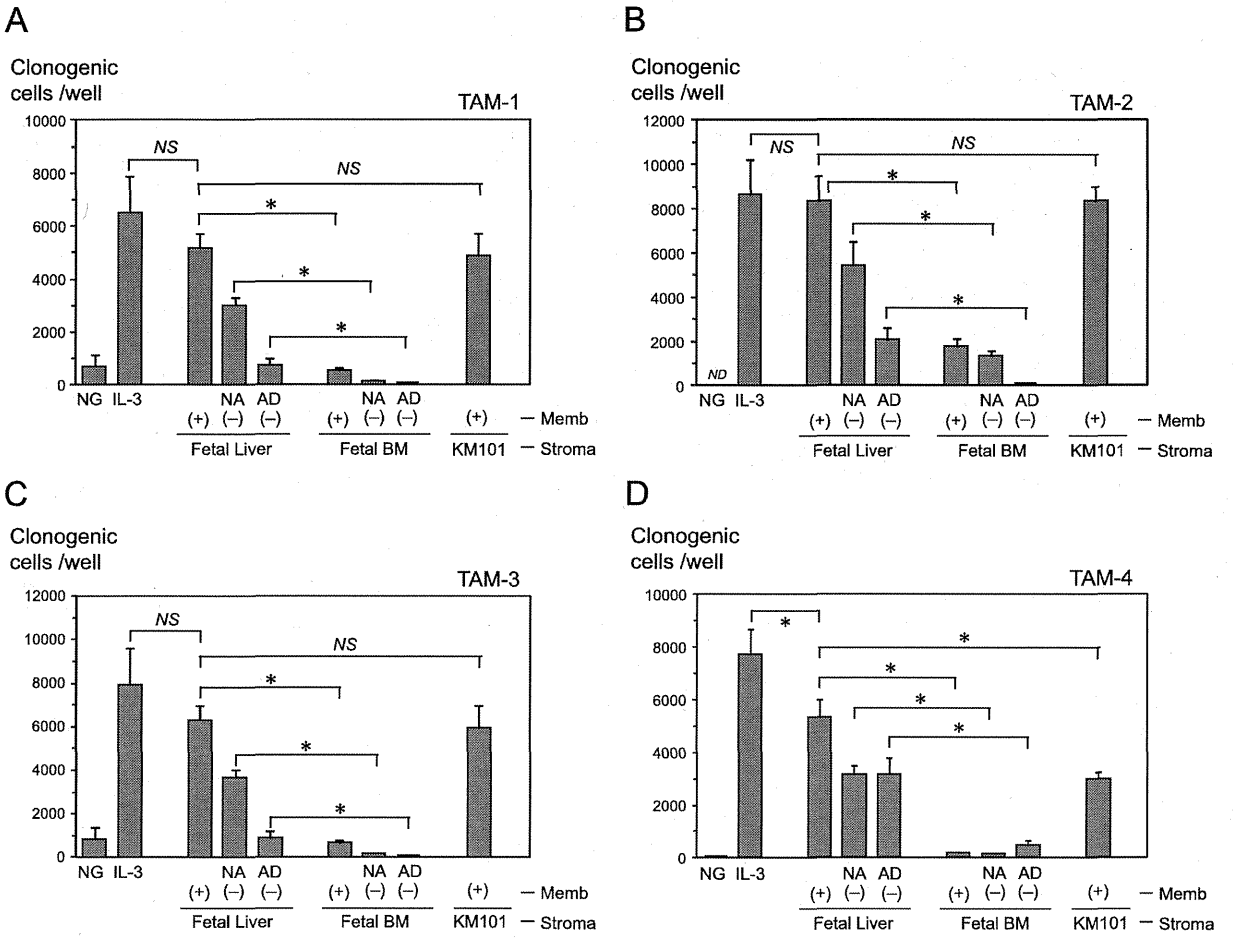

Figure 2

Figure 2.