Soluble Factor(s) Produced by Adult Bone Marrow Stroma Inhibit In Vitro Proliferation and Differentiation of Fetal Liver BFU-E by Inducing Apoptosis

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Abstract

Hematopoiesis occurs in different organs during fetal development. Several studies suggest that the growth of hematopoietic progenitors at one stage of ontogenic maturation may not be supported by a microenvironment from a different ontogenic stage. To determine if human fetal liver (FL) clonogenic progenitors can develop in an adult bone marrow (ABM) microenvironment, we compared growth of BFU-E and CFU-GM from 7–14-wk-old FL, 11–20-wk-old fetal bone marrow (FBM), umbilical cord blood (UCB), or ABM in clonogenic medium with or without ABM stroma. In contrast to BFU-E from FBM, UCB, or ABM, soluble factor(s) produced by ABM stroma severely suppressed growth of 98% of FL BFU-E by inducing apoptosis of cells beyond early erythroblast stage. The nature of the soluble factor remains unknown, although we have evidence that it is heat labile with molecular mass < 10 kD. Antibody neutralization studies indicate that TGF-β1, IL-1, TNF-α, macrophage inflammatory protein (MIP)-1α, or IFN-γ are not responsible. The observation that FL progenitors may not be capable of differentiating when transferred to an ABM microenvironment may have important implications for FL transplantation into postnatal recipients. Further, this demonstrates that ontogenic stage–specific interactions between hematopoietic progenitors and their microenvironment are important for the normal development of hematopoiesis. (*J. Clin. Invest.* 1997. 100:912–920.) Key words: cell differentiation • BFU-E • bone marrow stroma • apoptosis • hematopoiesis

Introduction

During the initial weeks after conception, hematopoietic stem cells are found in the extraembryonic yolk sac, after which hematopoiesis takes place sequentially in the fetal liver (FL),[1] spleen, and the bone marrow (BM) (1–3). Fetal hematopoiesis, which is almost exclusively erythroid, occurs chiefly in the FL. Although progenitor cells are present in fetal bone marrow (FBM) after 12–14 wk of gestation, they do not provide terminally differentiated cells until late in gestation. At birth, hematopoietic stem cells circulate in the blood of the neonate for the first few days. Afterwards, production of mature blood elements occurs mainly in the bone marrow. Differences in the anatomic and ultrastructural characteristics of the hematopoietic microenvironments in FL, FBM, and adult bone marrow (ABM) have been described (4–9). However, the functional significance of these differences is not clear. Since hematopoiesis at different stages of development occurs in different microenvironments, it is likely that this is due to specific requirements of the hematopoietic progenitors at a certain stage of development to interact with specific components of a given microenvironment.

Several studies suggest that hematopoietic progenitors from one stage of ontogenic maturation may not be able to home and proliferate normally in a microenvironment from a different ontogenic stage. Murine yolk sac hematopoietic stem cells can repopulate newborn mice who have active liver hematopoiesis, but fail to repopulate conditioned adult recipients (10). Conversely, circulating adult stem cells are unable to sustain hematopoiesis in a mouse FL microenvironment (11). Using a human–ovine xenogeneic in utero transplantation model, Zanjani et al. showed that human FL stem cells primarily home to the BM of recipient fetuses. However, although these stem cells appear to expand and proliferate, differentiation and appearance of their progeny in the peripheral blood does not occur until the perinatal period when BM hematopoiesis becomes active (12, 13). In addition, different progenitor subpopulations appear to be uniquely dependent on specific interactions with the microenvironment. For example, although all FL progenitors express very late antigen (VLA)-4, blocking the VLA-4–mediated progenitor–microenvironment interaction in vivo by administering anti–VLA-4 antibodies selectively affects FL erythropoiesis, leaving FL granulopoiesis, and lymphopoiesis intact (14). In contrast, in vivo administration of VLA-4 antibodies to adult animals affects mainly the granulocytic lineage (15). This suggests that interactions between a given microenvironment and a specific progenitor subtype found at a given stage of ontogeny are critically important to support proliferation and differentiation of that progenitor population.

For a number of years, transplantation of hematopoietic stem cells from human FL either in utero or postnatally has been considered for clinical purposes (16–20). However, it is still unclear if hemotopoietic stem cells from FL can establish normal hematopoiesis in adult hematopoietic organs. To address this question, we studied the growth of erythroid burst-

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1. *Abbreviations used in this paper:* 7AAD, 7-amino actinomycin D; ABM, adult bone marrow; BFU-E, erythroid burst-forming units; BM, bone marrow; BMMNC, bone marrow mononuclear cells; CFC, colony-forming cells; CFU-GM, granulocyte colony-forming units; FBM, fetal bone marrow; FL, fetal liver; Hb A, hemoglobin A; Hb F, hemoglobin F; LTC, long-term culture; LTC-IC, long-term culture initiating cells; MIP-1α, macrophage inflammatory protein 1α; PE, phycoerythrin; UCB, umbilical cord blood; VLA-4, very late antigen-4.

forming units (BFU-E) and granulocyte colony-forming units (CFU-GM) obtained from FL, FBM, umbilical cord blood (UCB), and ABM in an ABM microenvironment, mimicked in vitro by a BM stromal layer. FL CFU-GM and more primitive long-term culture initiating cells (LTC-IC) develop normally when placed in an ABM microenvironment. However, ABM stroma inhibits the growth and differentiation of FL BFU-E by inducing their progeny to apoptose, but does not inhibit the same in BFU-E from ontogenically more mature FBM or UCB. This effect is mediated by an as yet unidentified soluble factor(s) produced by ABM stroma.

Methods

Cell source

FL and BM. Human FL tissue (gestational age 7–14 wk) and human FBM (gestational age 12–22 wk) were obtained from the Central Laboratory for Human Embryology (University of Washington, Seattle, WA) or from Advanced Bioscience Resources, Inc. (Alameda, CA) after informed consent according to the guidelines of their institutional Committee on the Use of Human Subjects in Research. FL was disaggregated in a single cell suspension as described (21). FBM was obtained by flushing the long bones with PBS + BSA using a sterile 23–25-gauge needle.

UCB and ABM. UCB was collected after informed consent using guidelines approved by the Committee on the Use of Human Subjects for Research at the University of Minnesota. Heparinized ABM was obtained from the posterior iliac crest of normal, healthy volunteer donors after informed consent using guidelines approved by the Committee on the Use of Human Subjects for Research at the University of Minnesota. UCB mononuclear cells and BM mononuclear cells (BMMNC) were isolated by Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO) density gradient separation (specific gravity 1.077) for 30 min at 37°C and 400 *g*.

CD34 enrichment

FL, FBM, and UCB. FL cells, FBM cells, and UCB mononuclear cells were enriched for $CD34^+$ cells using the MACS® CD34 isolation kit (Miltenyi Biotec Inc., Sunny Vale, CA) as described (21). In some experiments, selected $CD34^+$ cells were passed over the MACS® column a second time to obtain highly enriched (90–97% pure) CD34 cells.

ABM. A CD34-enriched population was obtained from BMMNC using a biotinylated anti-CD34 mAb and a Ceprate® avidin–biotin immunoadsorption column (CellPro, Inc., Bothell, WA) per manufacturer's instructions as described (21).

FACS® sorting

In some experiments, CD34-enriched populations from FL or ABM were washed in PBS with 0.3% BSA and labeled either with FITC-conjugated mouse anti-CD34 antibody ($CD34^{FITC}$) and phycoerythrin (PE)-conjugated mouse anti-CD38 antibody ($CD38^{PE}$), or with PE-conjugated mouse anti-CD34 antibody ($CD34^{PE}$) and FITC-conjugated anti–HLA-DR antibody ($HLA\text{-}DR^{FITC}$) (all antibodies from Becton Dickinson Advanced Cellular Biology, San Jose, CA). Labeled cells were subjected to FACS® sorting on a FACStar$^{PLUS®}$ flow cytometry system (Becton Dickinson) equipped with a CONSORT 32 computer. PE- and FITC-conjugated isotype-matched mouse IgGs (Becton Dickinson) were used as negative controls. Sorting windows were set for four parameters: forward and orthogonal light scatter, and FITC and PE fluorescence as described previously (21), to detect $CD34^+/CD38^-$ and $CD34^+/CD38^+$ or $CD34^+/HLA\text{-}DR^+$ and $CD34^+/HLA\text{-}DR^-$ cells.

Stromal feeders

Stromal layers. Stromal layers were generated by plating FL, FBM, or adult BMMNC in long-term culture (LTC) medium (IMDM with 12.5% FCS; Hyclone, Logan, UT), 12.5% horse serum (Terry Fox Laboratories, Vancouver, BC), 2 nM L-glutamine, 1,000 U/ml penicillin, and 100 U/ml streptomycin (GIBCO BRL, Gaithersburg, MD), and 10^{-6} M hydrocortisone (A-hydrocort; Abbott Laboratories Diagnostic Division, North Chicago, IL), as described (22). Once confluent, stroma was irradiated with 2,000 rads, and stromal cells were recovered by treatment of the adherent layer with trypsin and subcultured at 0.35×10^6/ml in 24- or 6-well plates with LTC medium.

Glutaraldehyde fixation of stromal feeders. Irradiated stromal layers were treated with 2% glutaraldehyde (Sigma Chemical Co.) in 0.1 M Sorenson's buffer for 5 min and washed four times with IMDM as described (23). We have shown that adhesive ligands are still recognized by specific mAbs but that the fixed stromal cells are no longer metabolically active (23).

Cultures

Methylcellulose cultures. 1,000–5,000 column selected or FACS®-sorted $CD34^+$ cells or subpopulations were plated in clonogenic methylcellulose culture supplemented with 3 IU/ml erythropoietin (Amgen Biologicals, Thousand Oaks, CA) and 2.5 ng/ml IL-3 (R&D Systems, Inc., Minneapolis, MN) as described (22). Cells were first plated in LTC medium in stroma-free wells or in contact with ABM stroma for 2 h. $CD34^+$ cells or $CD34^+$ cells with ABM stromal cells were then harvested by short-term trypsinization and replated in methylcellulose assay. Alternatively, cells were plated in methylcellulose clonogenic culture medium in direct contact with viable or fixed ABM stromal feeders in 6-well plates or in a transwell placed above viable or fixed ABM stromal feeders. For the latter culture, 3 ml methylcellulose medium was placed on the stromal feeder, a transwell with a 0.4-μm filter membrane (Costar Corp., Cambridge, MA) was placed above the methylcellulose medium, and $CD34^+$ cells were plated in 2 ml methylcellulose medium in the transwell. This allows soluble factors produced by the stroma to diffuse to the progenitor cells in the transwell but prevents direct contact between the progenitors and stroma.

Liquid clonogenic culture. For liquid clonogenic cultures, culture medium consisted of FCS and growth factors in concentrations similar to the culture medium used in semisolid cultures but without methylcellulose. Cells were placed in a transwell suspended in 1 ml of culture medium over viable or fixed ABM stromal layers in 24-well plates.

Conditioned medium culture. Liquid clonogenic medium was cultured for 14 d over viable or fixed ABM stroma. The medium was collected (conditioned medium) and frozen at −20°C. Subsequent clonogenic cultures were initiated by plating 1,000–5,000 cells in 0.5 ml of conditioned medium in wells of a 24-well plate.

Serum free medium culture. For these cultures, the medium consisted of IMDM-based defined media containing BSA (20 mg/ml) (GIBCO BRL), insulin (10 mg/ml), transferrin (200 mg/ml) (Sigma Chemical Co.), 10–4 M 2-mercaptoethanol, penicillin 100 U/ml, and streptomycin 100 U/ml (GIBCO BRL). The following cytokines were added: 3 IU/ml erythropoietin (Amgen Biologicals) and 2.5 ng/ml IL-3 (R&D Systems). Cells were placed in a transwell suspended in 1 ml of culture medium above viable or fixed ABM stromal layers in 24-well plates.

Long-term BM culture. 10,000 FL $CD34^+/CD38^-$ cells were cultured in a transwell placed over irradiated ABM stroma in 24-well plates in LTC medium. Cultures were fed at weekly intervals by removing half the cell-free supernatant and replacing it with fresh LTC medium. After 1 or 2 wk, the cells were collected and plated either in semisolid methylcellulose medium in contact with viable or fixed ABM stromal layers, or in liquid clonogenic medium in transwells above ABM stroma, to determine the effect of ABM stroma on secondary colony-forming cells (CFC).

Culture maintenance. All cultures were incubated in a humidified atmosphere at 37°C and 5% CO_2 and assayed after 12–14 d. For methylcellulose-containing cultures, CFU-Mix, CFU-GM, and BFU-E were enumerated as described (22). For liquid cultures, cells were

harvested at various time points and analyzed for cell number, morphology, surface antigen phenotype, and signs of apoptosis.

Morphology

Cytospin preparations of cells harvested from liquid clonogenic cultures were stained with Wright Giemsa and examined for morphology. Differential counts were determined by counting at least 100 cells per slide.

Analysis of cell phenotype

Expression of surface antigens on cultured cells was determined by labeling the harvested cells with $CD71^{FITC}$ and $CD33^{PE}$ (Becton Dickinson), or glycophorin-A^{PE} (Immunotech, Marseille, France) and $CD33^{FITC}$ (Coulter Immunology, Hialeah, FL). FITC- or PE-coupled isotype-matched antibodies were used as controls. Phenotypic analysis was performed with a FACStar$^{PLUS®}$ flow cytometer equipped with a CONSORT 32 computer.

Analysis for apoptotic cells

Staining with 7-amino actinomycin D (7AAD) (Cal-Biochem Corp., La Jolla, CA) followed by FACS® analyses to analyze cellular DNA content was used to detect apoptosis (24). 5–10 $\times$ 10^4 cells collected from liquid clonogenic cultures were suspended in 200 μL PBS and incubated on ice for 20 min with 20 μg/ml 7AAD in PBS. Cells incubated without 7AAD served as control. After incubation, cells were washed once in cold PBS, resuspended in 200 μL 2% paraformaldehyde (Sigma Chemical Co.), and analyzed within 1 h on a FACStar$^{PLUS®}$ flow cytometer equipped with a CONSORT 32 computer. All nucleated cells were analyzed. Scattergrams were generated for forward light scatter and 7AAD fluorescence. Distinct cell populations with negative, dim, or bright 7AAD fluorescence representing viable, early apoptotic, and late apoptotic cells, respectively, were identified and their frequency noted. Cell populations were collected and examined after Giemsa staining for morphological features of apoptosis.

Hemoglobin identification

To determine the type of hemoglobin synthesized by the progenitors in culture, 50–200 $\times$ 10^3 $CD34^+$ cells from FL, FBM, UCB, or ABM were cultured in liquid clonogenic medium without ABM stroma. Cultures were maintained for 2 wk, after which all cells were collected and washed with PBS at 4°C. The cell pellet was suspended in 50 μl PBS and lysed using hypotonic solution. Hemoglobin identification was then performed by subjecting the sample, along with markers of known hemoglobin types, to isoelectric focusing.

Cytokine assay and neutralization

Levels of IL-1b (sensitivity 0.3 pg/ml), TNF-α (sensitivity < 10 pg/ml), TGF-β1(sensitivity 5.0 pg/ml), macrophage inflammatory protein (MIP)-1α (sensitivity 3.0 pg/ml), and IFN-γ (sensitivity 3.0 pg/ml) in stroma-conditioned media were assayed by ELISA using cytokine analysis kits (R&D Systems) as per manufacturer's instructions. Neutralizing antibodies, or the corresponding chicken IgY or goat IgG control antibodies, were used singly or together in liquid clonogenic cultures or conditioned media cultures: anti–TGF-β1 (50 μg/ml, which neutralizes 10–30 ng/mL TGF-β1), anti–MIP-1α (20 μg/ml, which neutralizes 20–40 ng/ml MIP-1α), anti–TNF-α (10 μg/ml, which neutralizes 30–62.5 ng/ml TNF-α), anti–IL-1 (10 μg/ml, which neutralizes 12.5–30 ng/ml IL-1), and anti–IFN-γ (10 μg/ml, which neutralizes 2.5–3.5 ng/ml IFN-γ) (all neutralizing antibodies from R&D Systems). Antibodies were added before the addition of cells and replenished at 3-d intervals. Cultures were maintained as described earlier and analyzed after 14 d.

Heat inactivation and size fractionation of conditioned media

Aliquots of viable and fixed ABM stroma–conditioned media were heated at 60°C for 40 min to assess the effect of heat treatment on inhibitory activity. To characterize the size of the inhibitor present in medium conditioned by viable ABM stroma, we size-fractionated viable and fixed stroma–conditioned media by ultrafiltration (Amicon Corp., Beverly, MA). Media were ultrafiltered through a 100-kD (YM100) membrane (concentrate = fraction > 100 kD). The eluate was then ultrafiltered through a 10-kD (YM10) membrane (concentrate = fraction > 10 kD). The final eluate was equal to fraction < 10 kD. We have shown previously by gel electrophoresis that most molecules > 20 kD were excluded from the < 10-kD fraction, and that most molecules > 140 kD were absent from the 10–100-kD fraction. The concentrates were then resuspended in fresh IMDM + IL-3 (2.5 ng/ml) and erythropoietin (3 IU/ml) at a final dilution equal to the total volume of the conditioned media used to prepare the concentrates. As control, media conditioned by fixed stromal feeders were size-fractionated using similar filter membranes, and the fractions were resuspended in fresh IMDM + IL-3 and erythropoietin.

Statistical analysis

Results of experimental points obtained from multiple experiments were reported as the mean±SEM. Significance levels were determined by two-sided Student's *t* test analysis.

Results

In initial studies, we compared the number of BFU-E, CFU-Mix, and CFU-GM that could be identified when FL or ABM $CD34^+$ cells were cocultured with or without irradiated viable ABM stromal cells. Cells were plated in LTC medium in stroma-free wells or in contact with irradiated ABM stromal feeder for 2 h. $CD34^+$ cells or $CD34^+$ cells combined with stromal cells were harvested and plated in methylcellulose assay. As we have previously shown (23, 25), coculture of ABM $CD34^+$ cells with ABM stroma inhibited CFC outgrowth by 30–50%. This inhibition was similar for BFU-E, CFU-Mix, and CFU-GM. Likewise, coculture of FL CD34 cells with ABM stroma significantly decreased the number of detectable CFC (47.1±6.1% of CFC in nonstroma culture) ($P = 0.039$). In con-

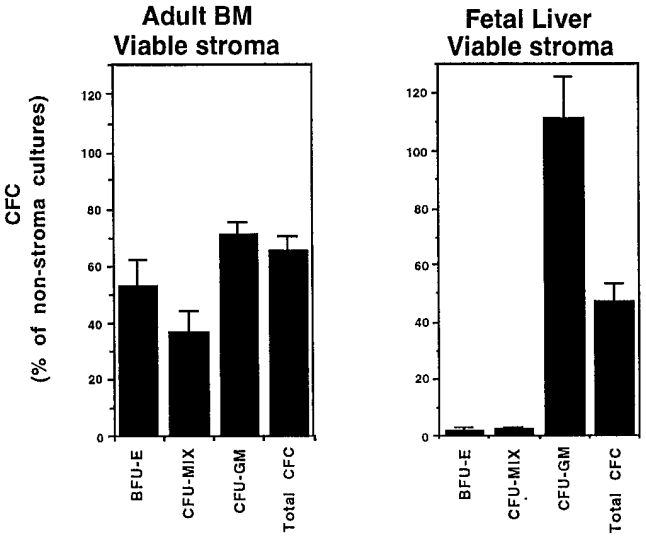

Figure 1. ABM stroma inhibits FL BFU-E significantly more than ABM BFU-E. FL $CD34^+/CD38^+$ cells were plated in LTC medium in stroma-free wells or in contact with viable ABM stroma for 2 h. Hematopoietic cells and stromal cells were recovered by short-term trypsinization and plated in methylcellulose assay. Colonies were enumerated after 12–14 d of culture. CFC obtained in stromal cultures are expressed as the fraction (%) of CFC in nonstromal cultures ($n = 5$).

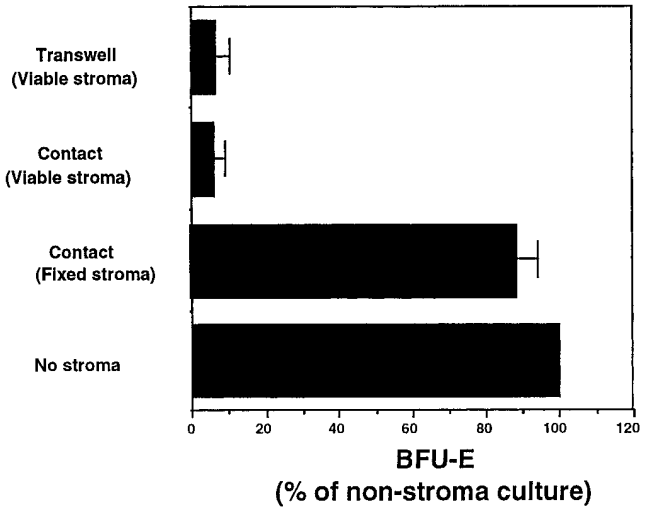

Figure 2. Direct contact with BM stroma is not required for the inhibition of FL BFU-E. FL CD34$^+$/CD38$^+$ cells were plated in methylcellulose cultures either in a stroma-free well, in direct contact with viable or fixed ABM stroma, or in transwells above viable or fixed ABM stroma. BFU-E obtained in viable or fixed stromal cultures are expressed as the fraction (%) of BFU-E in nonstromal cultures ($n = 4$).

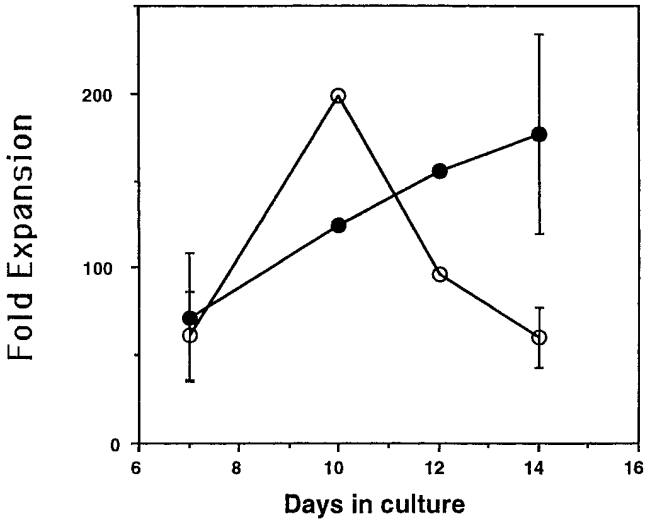

Figure 3. FL CD34$^+$/CD38$^+$ cells cultured in transwells above fixed BM stroma expand significantly more than when cultured in transwells above viable stroma. FL CD34$^+$/CD38$^+$ cells were cultured in transwells above viable or fixed ABM stroma in liquid clonogenic culture medium. Cells were harvested on days 7, 10, 12, and 14 and enumerated. Cells proliferated to a similar extent under both culture conditions for 7–10 d, after which the cells in cultures containing fixed stroma expanded significantly more compared to cultures containing viable stroma ($n = 4$–6). Viable stroma, ○. Fixed stroma, ●.

trast to ABM CD34$^+$ cells, similar numbers of FL CFU-GM were present in cultures containing stroma or stroma-free cultures, but few or no BFU-E (2.0±1.1%) or CFU-Mix (2.7±0.7%) grew in stroma-containing cultures (Fig. 1). This suggests that ABM stroma preferentially inhibits the growth of FL BFU-E and CFU-Mix.

To determine the role of direct contact with stroma versus the effect of soluble factors produced by the ABM stroma in mediating this inhibition, FL CD34$^+$ cells were plated in methylcellulose containing clonogenic medium either in a stroma-free well, in direct contact with viable stroma or with metabolically inactivated glutaraldehyde-fixed ABM stroma, or in transwells placed over viable or fixed ABM stroma. The transwells allow soluble factors produced by stroma to diffuse to the progenitor cells in the transwell, but prevent direct contact between the progenitors and stroma. A similar decrease in BFU-E colonies was observed after 14 d, when FL CD34$^+$ cells were cultured in transwells above viable stroma, as in cultures in which FL CD34$^+$ cells were cultured in direct contact with viable ABM stroma (Fig. 2). Growth of FL BFU-E was not inhibited when cultured either in contact with or separated by a transwell from fixed ABM stroma. Serial observation of the colonies growing in viable stroma–containing wells over the 14-d culture period demonstrated that nonhemoglobinized erythroid colonies were identifiable by days 5–7. However, their number decreased over the next 2–3 d so that by days 10–12, no erythroid colonies were identifiable. These experiments demonstrate that soluble factor(s) produced by ABM stroma, rather than contact-mediated events, are responsible for the inhibitory effect of ABM stroma on FL BFU-E growth.

In subsequent experiments, FL CD34$^+$ cells were cultured in transwells above viable or fixed ABM stroma in clonogenic culture medium that did not contain methylcellulose. Cultures were sampled on days 7, 10, and 14 and analyzed for cell number, morphology, and surface antigen phenotype. FL cell expansion in viable (61.5±17.5-fold) and fixed (71.5±36.5-fold, NS) stromal cultures was similar until day 7. From days 7–14 of culture, the cells in transwells above fixed stroma continued to expand, whereas cells in transwells above viable stroma did not. This resulted in an almost 3-fold higher cell number in fixed compared to viable stroma cultures on day 14 (176.6±56.9-fold vs. 60.2±17.5-fold, $P = 0.05$) (Fig. 3). Morphologic examination on day 7 showed that under both conditions the vast majority of cells were early erythroid lineage cells (nonhemoglobinized early erythroblasts). After 14 d, 60–80% of cells recovered from transwells above fixed ABM stroma expressed CD71 and glycophorin-A and were morphologically recognizable as normoblasts or RBC. In contrast, < 10% of cells recovered after 14 d from transwells above via-

Table I. FL CD34$^+$/ CD38$^+$ Cells Cultured in Transwells Above Viable ABM Stroma Expand Significantly Less and Give Rise to Only Rare Erythroid or Glycophorin-A$^+$ Cells

	Viable ABM stroma	Fixed ABM stroma	$P =$
Fold expansion	60.2±17.5	176.6±5.9	0.05
% Erythroid	12.3±3.7	67±9.7	0.006
% Glycophorin-A$^+$	7.0±2.3	77.6±1.0	0.01

FL CD34$^+$/CD38$^+$ cells were plated in transwells above viable or fixed ABM stroma in liquid clonogenic culture medium. After 14 d, cells were harvested and examined for expansion, glycophorin-A expression, and erythroid morphology ($n = 5$).

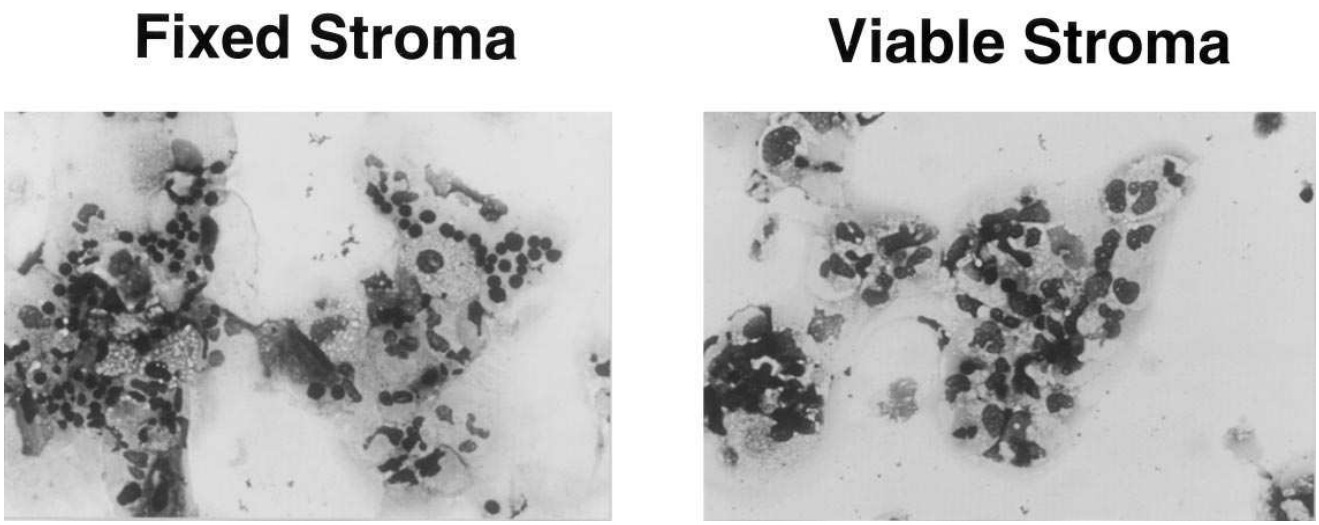

Figure 4. FL $CD34^+/CD38^+$ cells cultured in transwells above viable BM stroma give rise to significantly fewer erythroid cells than those cultured above fixed stroma. FL $CD34^+/CD38^+$ cells were cultured in transwells above viable or fixed ABM stroma in liquid clonogenic culture medium. Cells were harvested after 14 d and examined by light microscopy after Giemsa staining. Cells obtained from viable stromal cultures contained significantly fewer erythroid cells (12.3±3.7%) than those from fixed stromal cultures (67±9.7%; $P = 0.006$) ($n = 5$).

ble ABM stroma were glycophorin-A positive or morphologically identifiable as erythroid cells (Fig. 4 and Table I). Thus, soluble factor(s) from viable stroma inhibit the growth and differentiation of FL-derived erythroid cells after ~ 7 d in culture, approximately the time when hemoglobinization begins to occur.

To investigate the mechanism responsible for this inhibition, we examined the cells obtained on days 7, 10, and 14 from transwell cultures for evidence of apoptosis. Cells were labeled with 7AAD, and the proportion of cells with 7AAD fluorescence above control, representing apoptotic cells, was assessed by FACS® and confirmed by morphology after sorting. On day 14, a significantly higher proportion of cells in transwells above viable ABM stroma were apoptotic compared to cells cultured in transwells above fixed ABM stroma (38.8±4.4 vs. 13.8±2.3%, $n = 5$; $P = 0.002$) (Fig. 5).

To further characterize the nature of the inhibitor, liquid clonogenic culture medium was conditioned for 14 d with viable and fixed ABM stroma. When FL $CD34^+$ cells were cultured for 14 d with viable stroma–conditioned medium, but not fixed stroma–conditioned medium, we again saw a decrease in cell expansion after 14 d of culture (fold expansion 29±9.6 vs. 244.8±39.5; $P = 0.002$) ($n = 5$). Only rare erythroid cells were present in viable stroma–conditioned medium cultures. When conditioned medium was diluted with an equal volume of IMDM, significantly less inhibition of BFU-E growth was seen (fold expansion 202±10.5 vs. 220±9.5; $P = 0.035$). These data confirm that a soluble inhibitory factor(s) secreted by ABM stroma is responsible for the inhibition of FL BFU-E growth. Inhibition was also observed when FL $CD34^+$ cells were cocultured with viable ABM stroma in serum-free medium for 14 d (fold expansion 38±6 vs. 125±19, $n = 2$), suggesting that serum is not required for the production of inhibitor by ABM stroma.

To characterize the nature of the inhibitor, we examined its heat stability and its approximate size. When FL $CD34^+$ cells

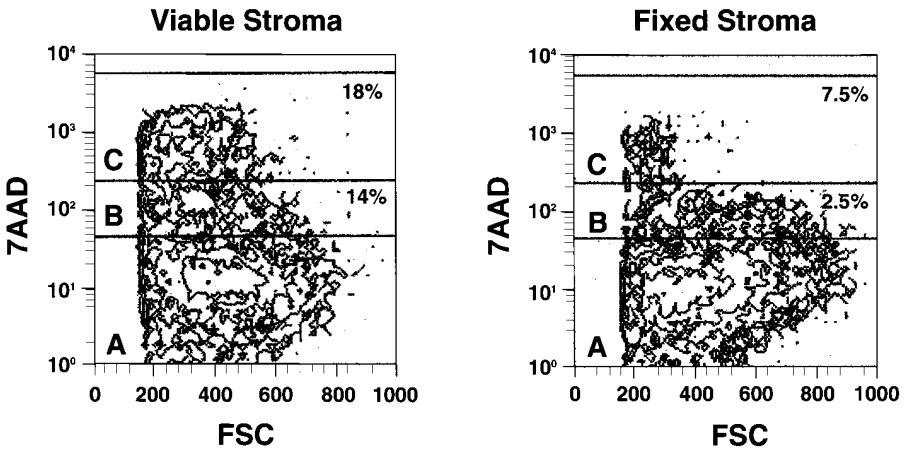

Figure 5. Soluble factor(s) produced by ABM stroma induce apoptosis of proliferating FL $CD34^+$ cells. Staining with 7AAD followed by FACS® analyses to analyze cellular DNA content was used to detect apoptosis (24). Scattergrams were generated for forward light scatter and 7AAD fluorescence. Distinct cell populations with negative (box *A*), dim (box *B*), or bright (box *C*) 7AAD fluorescence representing viable, early apoptotic, and late apoptotic cells, respectively, were identified. After 14 d of culture, significantly higher proportions of cells in transwells above viable ABM stroma were apoptotic (38.8±4.4%) compared to cells cultured in transwells above fixed ABM stroma (13.8±2.3%) ($n = 6$).

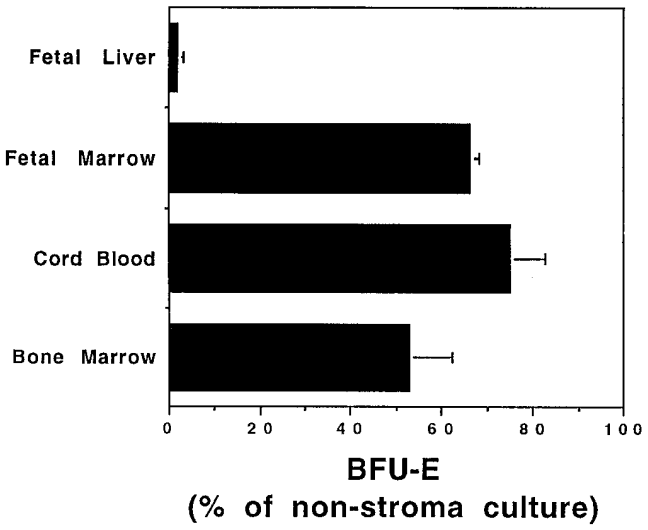

Figure 6. FL BFU-E but not FBM, cord blood, or ABM BFU-E are inhibited by ABM stroma. FL, FBM, cord blood, or ABM $CD34^+$ cells were plated in LTC medium in stroma free wells or in contact with viable ABM stroma for 2 h. Hematopoietic cells and stromal cells were then recovered by short-term trypsinization and plated in methylcellulose assay. Colonies were enumerated after 12–14 d of culture. BFU-E obtained in stromal cultures are expressed as the fraction (%) of BFU-E in nonstromal cultures (n = 3–7).

were cultured with heat-treated media conditioned by viable ABM feeders (60°C for 40 min), no effect on expansion in cell numbers was observed. In fact, cell expansion in cultures supported by heat-treated viable stroma–conditioned media was similar to that observed in cultures supported by media conditioned by fixed ABM stroma, both before and after heat inactivation. This indicated that the inhibitor is heat labile. To assess its approximate size, FL $CD34^+$ cells were cultured in media conditioned by ABM stromal feeders after size separation by Amicon ultrafilteration. These studies demonstrate that expansion of FL $CD34^+$ cells in the < 10-kD fraction of media conditioned by viable stroma, but not the < 10-kD fraction of media conditioned by fixed ABM stroma, was significantly inhibited. However, the 10–100-kD fraction of media conditioned by viable ABM stroma or media conditioned by fixed ABM stroma did not inhibit FL $CD34^+$ cell expansion. These studies indicate that the size of the inhibitor is likely to be < 10–20-kD (Table II). We then examined the concentration of cytokines with known inhibitory properties present in medium conditioned for 14 d with viable or fixed ABM stroma. Significantly higher levels of TGF-β1 were present in

Table II. The Inhibitory Activity Is Contained in the Small Molecular Mass (< 10 kD) Fraction of Viable Stroma–conditioned Medium

Molecular mass fraction	Fold expansion of viable ABM stroma	Fold expansion of fixed ABM stroma	P =
< 10 kD	44±18	755±138	0.05
10–100 kD	567±183	635±127	NS
> 100 kD	725±155	737±26	NS

To characterize the nature of the inhibitor produced by ABM stroma, we fractionated viable and fixed ABM stroma–conditioned media based on molecular size. FL $CD34^+$ cells cultured in < 10-kD fraction of viable ABM stroma–conditioned medium expanded significantly less than those cultured in same–size fraction of fixed ABM stroma–conditioned medium. Similar degrees of expansion occurred in 10–100-kD and > 100-kD fractions of both viable and fixed ABM stroma–conditioned media.

the medium conditioned by viable than in that by fixed ABM stroma (P = 0.008). No significant differences in the levels of IL-1, TNF-α, MIP-1α, or IFN-γ were seen (Table III). To determine if these cytokines are responsible for the inhibition of BFU-E growth, neutralizing antibodies against TGF-β1 singly or in combination with neutralizing antibodies against IL-1, TNF-α, MIP-1α, and IFN-γ were added to cultures with viable ABM stroma–conditioned medium. No significant effect of the neutralizing antibodies, singly or in combination, was observed on the inhibitory activity of viable ABM stroma–conditioned medium on FL BFU-E.

We also examined if the growth of FBM or UCB BFU-E is inhibited to the same extent by ABM stroma as that of FL BFU-E. We cocultured FBM and UCB $CD34^+$ cells with ABM stroma. Similar to what we had seen for ABM BFU-E, ABM stroma inhibited the growth of FBM BFU-E (33.7±2.0%) or UCB BFU-E (25.1±7.8%) significantly less than that of FL BFU-E (98±1.1%) (Fig. 6). Since the inhibition of BFU-E coincides with the time of hemoglobinization, we evaluated the type of hemoglobin generated in vitro by FL, FBM, UCB, or ABM erythroid cells cultured in the absence of ABM stroma. Cultures initiated with $CD34^+$ cells from FL, FBM, and UCB contained predominantly or almost exclusively hemoglobin F (Hb F), while ABM cultures contained both Hb F and hemoglobin A (Hb A) (Fig. 7). This indicates that the observed inhibition of erythroid differentiation occurs irrespective of the type of hemoglobin in the progenitors. To determine if the factor is produced by ABM stroma only, we also examined the effect of FBM stroma on FL $CD34^+$ cells in liquid cultures. As

Table III. Significantly Higher Levels of TGF-β Are Present in Viable Stroma–conditioned Medium than in Fixed Stroma–conditioned Medium

	TGF-β1	MIP-1α	IL-1α	TNF-1α	IFN-γ
	pg/ml	*pg/ml*	*pg/ml*	*pg/ml*	*pg/ml*
Live stroma	2456.9±226	35.03±28.6	0.169±0.074	0.43±0.33	1.02±0.38
Fixed stroma	1794.1±259.1	12.64±4.06	0.158±0.047	3.3±3.16	1.04±0.45
P =	0.008	0.42	0.83	0.41	0.96

Liquid clonogenic medium was conditioned for 14 d over viable or fixed ABM stroma. Levels of IL-1, TNF-α, TGF-β1, MIP-1α, and IFN-γ in stroma–conditioned medium were assayed by ELISA (n = 7).

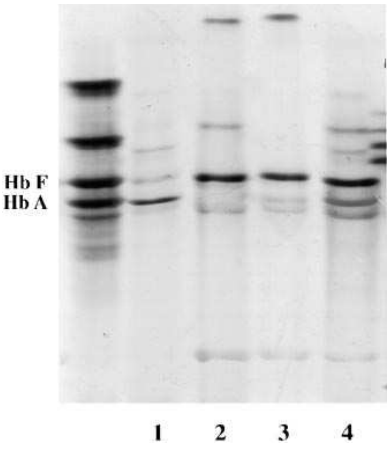

Figure 7. FL, FBM, and cord blood $CD34^+$ cells predominantly or exclusively synthesize Hb F while ABM $CD34^+$ cells synthesize mainly Hb A in liquid clonogenic culture. $50–200 \times 10^3$ $CD34^+$ cells from ABM (*1*), FL (*2*), FBM (*3*), or UCB (*4*) were cultured in liquid clonogenic medium without ABM stroma. After 2 wk, cells were collected, pelleted, resuspended in 50 μl PBS, and lysed using a hypotonic solution. Hemoglobin identification was performed by subjecting the sample, along with markers of known hemoglobin types, to isoelectric focusing ($n = 2–3$).

seen for coculture with ABM stroma, FL cells cultured with viable FBM stroma expanded significantly less than those cultured with fixed FBM stroma (16.8±5.9-fold vs. 83.7±29.5-fold, $P = 0.04$) ($n = 3$). This indicates that inhibition of FL erythroid progenitors occurs both in an ABM and FBM microenvironment.

We have previously reported that FL $CD34^+$ cells contain LTC-IC (25). In these studies, FL $CD34^+/CD38^-$ or $CD34^+/HLA\text{-}DR^+$ cells were cultured for 5 wk with viable M2-10B4 stroma, and progeny assessed for BFU-E and CFU-GM. M2-10B4, a mouse BM–derived fibroblast cell line supports growth and differentiation of ABM LTC-IC to the same extent as human marrow–derived stromal feeders (26). Since some BFU-E were detected after 5 wk of culture, this suggests that BFU-E generated from primitive LTC-IC may not be inhibited. To assess the effect of ABM stroma on BFU-E generated from FL $CD34^+/CD38^-$ cells, we cultured FL $CD34^+/CD38^-$ cells in LTC medium in a transwell above ABM stromal layers. After 2 wk, the progeny of these cultures were harvested and subcultured in clonogenic methylcellulose medium or in transwells in liquid clonogenic culture medium above viable or fixed ABM stromal feeders. The erythroid cells obtained after 2 wk of subculture contained almost exclusively Hb F ($n = 2$). Significantly fewer BFU-E were detected in secondary methylcellulose cultures with viable ABM stroma than in those without stroma when week 2 progeny were assessed (4.2±1.1% vs. 29.5±4.5%, $P = 0.02$). Expansion and generation of morphologically recognizable mature erythroid cells were also significantly lower when week 2 progeny were cultured in transwells above viable than above fixed ABM stroma (51.2±15-fold vs. 104±26-fold, $P = 0.04$). However, the difference in the number of BFU-E present in viable and fixed stromal cultures initiated with week 2 progeny of FL $CD34^+/CD38^-$ cells (9–15-fold), was significantly less pronounced than when freshly sorted FL $CD34^+$ cells were cultured under the same conditions (28–140-fold, $P = 0.03$).

Discussion

Our studies demonstrate that ABM stroma inhibits the proliferation and differentiation of human FL BFU-E in vitro but does not affect FL CFU-GM growth. Unlike the contact-mediated inhibitory effect of ABM stroma on ABM CFC (23, 25), the inhibition of FL BFU-E is mediated by soluble factor(s) produced by ABM stroma but not contact factors. These factor(s) mediate inhibition by inducing apoptosis of proliferating FL BFU-E. Growth of FL BFU-E is equally inhibited by coculture with FBM stroma. A similar soluble factor–mediated inhibition is not seen for FBM, UCB, or ABM BFU-E. Although the identity of these factor(s) is not known at present, we have evidence that several inhibitory cytokines (IL-1, TNF-α, TGF-β1, MIP-1α, and IFN-γ) do not appear to be responsible, as their neutralization in culture medium does not block the inhibitory activity of ABM stroma. Further, we show that the inhibitory factor is heat labile, has a molecular mass < 10 kD, and serum-containing medium is not required for its elaboration by ABM stromal cells.

A similar FL $CD34^+$ cell expansion is seen in the presence of fixed or viable ABM stroma until day 7–10 of culture, at which time immature nonhemoglobinized erythroid progenitors are recognizable. However, ABM stroma inhibits subsequent proliferation and differentiation of FL progenitors into mature hemoglobinized erythroid elements by inducing their apoptosis. This suggests that erythroid progenitors at a specific stage of differentiation only are affected. In vitro, hemoglobinization of erythroid progenitors occurs after 7–10 d in culture. Since this coincides with the time when the inhibition becomes manifest, this raises the possibility that the hemoglobinization process itself, or some other cellular event that temporally correlates with hemoglobinization, is affected by the ABM stroma–derived soluble factor(s) leading to cell death. In vivo, FL erythroid cells produce almost exclusively Hb F, while ABM erythroid cells produce predominantly Hb A. In culture, progenitors from FL, FBM, and UCB contained almost exclusively Hb F, whereas ABM progenitors contained mainly Hb A. Since FL BFU-E and not BFU-E from FBM or UCB are inhibited, differences in the type of hemoglobin produced cannot explain why FL BFU-E only are inhibited. Transcription factor GATA-1 plays a central role in erythroid differentiation by regulating the expression of several erythroid-specific genes (26, 27). Absence of GATA-1 results in maturation arrest of erythroid precursors at the proerythroblast stage followed by their apoptosis (28). We hypothesize that the apoptosis of FL erythroid cells induced by ABM stromal factor(s) may be the result of alterations in GATA-regulated erythroid genes induced by these soluble factors, a hypothesis which is currently being examined.

Hematopoiesis in the fetus and adult differ, partly in response to differing needs of the organism during development. During fetal development, when the primary hematopoietic need of the fetus is for oxygen-delivering RBCs, fetal hematopoiesis is almost exclusively erythroid. Granulocytic precursors are present in the fetus, but granulopoiesis is minimal under steady state conditions (29). In the adult, challenges posed by exposure to several allergens and infectious agents create a higher demand for hematopoietic elements other than RBCs. This is reflected in the relatively smaller erythroid component in the adult marrow. While different demands on hematopoiesis may provide a teliologic explanation for the differences observed between fetal and adult hematopoiesis, mechanism(s) underlying the preferential lineage differentiation or reasons why different microenvironments are used are unknown. The observation that both FBM and ABM stroma inhibit FL

BFU-E may explain why during early fetal life, when hematopoiesis is almost exclusively erythroid, hematopoiesis is restricted to the FL.

Several differences between FL and ABM hematopoiesis have been described. These include in vivo differentiation almost exclusively along the erythroid pathway (see p. 51 in ref. 30), rapid cycling rate and accelerated in-vitro maturation time (31), greater sensitivity to erythropoietin (31–33), and decreased sensitivity to GM-CSF (34). In vivo, FL hematopoiesis is almost entirely restricted to the erythroid lineage. However, we and others have shown that granulocytic progenitors are present in FL (21, 35), but these progenitors do not seem to proliferate in the in vivo FL microenvironment. In contrast, significantly more granulocytic than erythroid progenitors are recovered from FBM. This suggests that the BM microenvironment may favor granulocytic differentiation and possibly inhibit erythroid differentiation, whereas the FL favors erythroid but not granulocytic differentiation. This is consistent with the observation that when FL progenitors are cultured in long-term cultures with ABM stroma, the relative proportion of BFU-E generated declines rapidly with time. After 2 wk, only rare BFU-E are present while CFU-GM continue to be generated for many months (6, 36). Although a number of cytokines inhibitory to hematopoiesis, such as MIP-1α, TGF-β, IFN-γ and TNF-α, may differentially affect subpopulations of FL or UCB $CD34^+$ cells (37), our studies suggest that these factors may not be responsible for the inhibition of BFU-E development in an ABM microenvironment.

In contrast to FL BFU-E, FL CFU-GM are inhibited to a significantly lesser extent by ABM microenvironment, which is consistent with the predominantly myeloid nature of ABM hematopoiesis. We have shown previously that ABM stroma supports the proliferation and differentiation of FL LTC-IC and primitive natural killer cell precursors (21). Progeny of FL LTC-IC obtained after 5 wk of culture do contain BFU-E, albeit in low frequency, suggesting that these may not be inhibited by ABM stroma. However, some inhibition of BFU-E was seen for progenitors derived from FL $CD34^+/CD38^-$ cells cultured for only 2 wk in an ABM stroma microenvironment, even though the degree of inhibition was lower than that seen for $CD34^+$ BFU-E cultured immediately in an ABM stroma–containing methylcellulose assay. This is not related to the type of hemoglobin produced, since both the BFU-E directly obtained from FL and those derived from FL $CD34^+/CD38^-$ cells cultured for 2 wk in an ABM stroma microenvironment contained almost exclusively Hb F. This may indicate either that more primitive progenitors are less inhibited by ABM stroma or that, as has been suggested for yolk sac progenitors (10), adaptation to an ontogenically more mature microenvironment occurs, which ultimately allows normal development of ontogenically immature progenitors in an adult microenvironment.

Thus, we have shown that soluble factor(s) produced by ABM stroma inhibit proliferation and differentiation of BFU-E present in FL but not BFU-E from FBM, UCB, or ABM. Other classes of progenitors in FL, including CFU-GM, LTC-IC, and natural killer cell progenitors, are not inhibited by ABM stroma. This demonstrates that ontogenic stage–specific progenitor–microenvironment interactions are important for normal hematopoietic development. In the last several years, FL hematopoietic progenitors have been used for in utero or postnatal transplantation (19, 38). Failure of FL BFU-E to differentiate in an ABM microenvironment may have important implications for such transplantations and should be kept in mind when FL transplants into adult recipients are contemplated.

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