Functionally Distinct Human Marrow Stromal Cell Lines Immortalized by Transduction With the Human Papilloma Virus E6/E7 Genes

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A replication-defective recombinant retrovirus containing the human papilloma virus E6/E7 genes (LXSN-16 E6E7) was used to immortalize stromal cells from human marrow. The E6/E7 gene products interfere with the function of tumor-suppressor proteins p53 and Rb, respectively, thereby preventing cell cycle arrest without causing significant transformation. Twenty-seven immortalized clones designated HS-1 to HS-27 were isolated, four of which are characterized in this report. Two cell lines, HS-5 and HS-21, appear to be fibroblastoid and secrete significant levels of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (M-CSF), Kit ligand (KL), macrophage-inhibitory protein-1α, interleukin-6 (IL-6), IL-8, and IL-11. However, only HS-5 supports proliferation of hematopoietic progenitor cells when cocultured in serum-deprived media with no exogenous factors. Conditioned media (CM) from HS-5 promotes growth of myeloid colonies to significantly greater extent than a cocktail of recombinant factors containing 10 ng/mL of IL-1, IL-3, IL-6, G-CSF, GM-CSF, and KL and 3 U of erythropoietin (Epo). Two additional clones, HS-23 and HS-27, resemble "blanket" cells, with an epithelioid morphology, and are much larger, broader, and flatter when compared with HS-5 and HS-21. These lines secrete low levels of growth factors and do not support proliferation of isolated progenitor cells in cocultures. CM from HS-23 and HS-27 also fail to support growth of myeloid colonies. Both HS-23 and HS-27 express relatively high levels of VCAM-1, yet HS-27 is the only line that supports the formation of "cobblestone" areas by isolated CD34⁺38⁻ cells. We hypothesize that HS-5, HS-21, HS-23, and HS-27 represent functionally distinct components of the marrow microenvironment.

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DEFINING THE functional components of the marrow microenvironment (ME) is a prerequisite to understanding how the proliferation and differentiation of progenitor cells is coordinately regulated. The cellular complexity of the ME has been shown both in situ and in vitro using a variety of histochemical techniques. It has been established that both hematopoietic and stromal or mesenchymal-derived cells comprise the ME. The stromal cells include endothelial cells that form the sinuses and adventitial reticular cells that have characteristics consistent with adipocytes, fibroblasts, and smooth muscle cells. Numerous advances in recent years have provided considerable information on the ontogeny and development of hematopoietic cells; however, ontogeny of the stromal components and their precise role in controlling hematopoiesis has proven elusive.

Long-term cultures (LTCs) are an in vitro approximation of the in vivo ME and have been informative with respect to the identification of growth factors, adhesion proteins, and extracellular matrix proteins that mediate the interaction between the hematopoietic cells and the stromal elements. One improvement of this system was the use of stromal precursors, positive for the STRO-1 antigen, to establish LTCs that are devoid of myeloid components and extracellular matrix proteins that mediate the interaction between hematopoietic cells and the stromal elements. However, both the STRO-1-initiated cultures and the primary LTCs are too complex to delineate contributions from individual cell types. Moreover, primary cultures can be highly variable and change with time, further complicating the issue.

Immortalized stromal cell lines have been used to circumvent some of these problems. Numerous spontaneous murine marrow line cells have been established; however, unlike mouse lines, human cell lines undergo senescence unless first immortalized with a retrovirus. The few human bone marrow stromal cell lines that are available were established using the SV40 virus large T antigen. Some of these lines are promising with respect to the maintenance of hematopoietic cells; unfortunately, some also display transformed phenotypes, which limits their usefulness for extrapolation to the normal ME.

In this report, a different procedure was used to establish cell lines representative of the heterogeneous ME. Primary LTC were exposed to an amphotropic retrovirus which carries the E6 and E7 open reading frames of the human papilloma virus type 16 (HPV16). The E6 and E7 gene products have been shown in other systems to immortalize differentiated cells without significant transformation. This report provides the initial characterization of HPV16 E6/E7 immortalized human marrow stromal cell clones which support the proliferation of hematopoietic progenitors and maintain colony-forming cells (CFC) for up to 8 weeks in culture.

MATERIALS AND METHODS

Long-term bone marrow cultures (LTBMCs). Adult BM was obtained from normal donors after informed consent as defined by the Institutional Review Board (IRB) at the Fred Hutchinson Cancer Research Center (FHCRC; Seattle, WA) and LTBMCs were established as described by Gartner and Kaplan. Briefly,uffy coat cells from marrow aspirates were plated in plastic tissue culture dishes at 1 to 2 X 10⁵ cells/mL. Adherent cells were grown in LTC medium containing Iscove’s, 12.5% horse serum, 12.5% fetal calf serum (FCS), L-glutamine (0.4 mmol/L), sodium pyruvate (1 mmol/L), penicillin (100 U/mL), streptomycin sulfate (100 mg/mL), hydrocortisone sodium succinate (10⁻⁴ mol/L), and β-mercaptoethanol (10⁻⁴ mol/L).

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Transduction of LTBM C. The amphotropic retrovirus LXSN-16 E6E7 was packaged in the PA317 murine cell line in the laboratory of Denise Galloway (FHCR C) as previously described. Primary LTCs were exposed to virus in the presence of 4 µM polybrene (Aldrich Chemical Co, Inc, Milwaukee, WI) for 2 hours at 37°C. The virus-containing media was removed and the cells were incubated for an additional 5 hours with media-containing polybrene. Cells were then washed and fed with LTC media and incubated for an additional 48 hours. Cell cultures were then trypsinized and replated at limiting dilution. Transduced clones were selected with 50 µg/mL G418, and resistant colonies were picked and grown in LTC using standard tissue culture techniques. After expansion, most clones were switched to RPMI containing 10% serum and HS-5 was switched to serum-deprived medium containing 1% Nutridoma (Boehringer Mannheim, Indianapolis, IN). 100 mmol/L glutamine, 100 mmol/L sodium pyruvate, and 100 U/mL penicillin-streptomycin in Iscove's media.

Indirect-immunofluorescence staining. The antigenic phenotype of the stromal cell lines was determined by standard immunohistochemistry using 1A4-IgG2a (Sigma, St Louis, MO) for smooth muscle actin; CD14 (leuM3-IgG2b; Becton Dickinson, San Jose, CA) for macrophages; anti-FVIII antigen (human type I; Calbiochem, La Jolla, CA) for endothelial cells; 6.19-IgG2a for fibroblasts, endothelial cells, and adipocytes (C. Frantz, University of Rochester School of Medicine and Dentistry, Rochester, NY); CD34 (12.8, IgM; courtesy of L. Bernstein, FHCR C); PH11 (fibronectin) and PH1-9 (vimentin) from Bill Carter (FHCR C); 60.5 (class I major histocompatibility complex [MHC]; from Dr P. Martin, FHCR C); anti-VLA-4 and anti-VCA-1 (B9 ascites) from PBS Harlan (University of Washington, Seattle, WA); anti-collagen type I (MAB1340, IgG1), type III (MAB1343, IgG1), and type IV (MAB 1910, IgG1) were obtained from Chemicon (Temecula, CA). For immunofluorescence staining, semiconfluent cells were rinsed with warm Hanks' Balanced Salt Solution (HBSS) and fixed for 10 minutes with 1% formaldehyde in phosphate-buffered saline (PBS) at 25°C. The cells were washed with PBS and treated with 0.2 mol/L glycine in PBS for 5 minutes at 25°C. One additional wash was performed with PBS before incubation with a specific antibody or irrelevant nonspecific isotype control antibody for 1 hour at 25°C. After incubation with the primary antibody, the cells were washed three times and incubated with a secondary antibody (goat antimouse IgG antibody fluorescein isothiocyanate [FITC]; Biocytogenics, La Jolla, CA) for 1 hour at 25°C and washed with PBS before viewing with a Nikon Diaphot fluorescent microscope (Chiyoda-Ku, Tokyo, Japan). Primary LTCs and foreskin fibroblasts (FSFs) were used as controls for antibody staining.

Cytotoxicity analysis. Alkaline phosphatase activity was determined using cells fixed with 2% formaldehyde in absolute methanol for 30 seconds at 4°C and then washed with distilled water and air dried. After drying, the cells were incubated at 37°C for 30 minutes in filtered reaction buffer containing 0.2 mol/L Tris HCl (pH 9.1) and 1.0 mg/mL Fastblue BB with or without 0.2 mol/L naphthol AS phosphate in N,N-dimethylethanolamide. After incubation, the cells were washed with distilled water, overlaid with Aqua Mount (Lerner Laboratories, Pittsburgh, PA), and photographed with a Nikon Diaphot microscope.

For analysis of acid phosphatase activity, the cells were fixed with 60% acetone in 0.04 mol/L citrate buffer (pH 5.4) for 30 seconds at 25°C, rinsed with distilled water, air dried, and incubated with the reaction buffer. The reaction buffer was made up in 24 mL of 0.1 mol/L acetate buffer with or without 12.5 mg Naphthal AS-BI phosphate as substrate; 7.5 mg of Fast Garnet GBC dye was added as counterstain. This solution was filtered through a Whatman #4 filter (Whatman Inc, Clifton, NJ) and then incubated with cells for 1 hour at 25°C protected from light. After staining, the cells were washed with distilled water, overlaid with Aqua-mount, photographed (Nikon Diaphot microscope), and scored for the presence of acid phosphatase.

Adipogenesis. Confluent stromal lines were incubated with corticoid steroids for 4 weeks and stained with oil red O to determine if these lines contain adipogenic cells. Cultures were fed weekly with either dexamethasone [10⁻⁷ mol/L], hydrocortisone [10⁻⁴ mol/L], insulin (10 mg/mL), or dexamethasone combined with insulin in RPMI containing 10% FCS. After the incubation period, the cells were washed extensively with PBS and then fixed with 10% formalin in PBS for 30 minutes. The excess formalin was washed off with PBS and the cells were stained for 15 minutes with filtered oil red O (0.3% wt/vol in isopropanol). The stain was then differentiated with 60% isopropanol and washed and the cells were counterstained with Mayers haematoxylin for 30 seconds.

Southern analysis. Genomic DNA was isolated from 1 × 10⁷ stromal cells using a modification of the technique described in Ausbel et al. Before Southern hybridization, 10 µg of genomic DNA was digested with excess EcoRI overnight at 37°C. The DNA was extracted with phenol-chloroform and precipitated. Ten micrograms of the digested genomic DNA was separated on a 0.5% agarose gel in TBE and then transferred to a nylon membrane according to manufacturer's specifications (Hybond; Amersham, Arlington Heights, IL). The membrane was hybridized with random primed probes generated against the E6E7 insert. A total of 50,000 cpm was hybridized overnight at 42°C and washed twice with 0.2× SSPE at 25°C and then washed two more times with 0.2× SSPE containing 0.1% sodium dodecyl sulfate (SDS) at 60°C before autoradiography.

Isolation of CD34+38+ cells. Adult marrow from cadaveric donors was obtained from the Northwest Tissue Center (Seattle, WA). The mononuclear cells were isolated by Ficoll density centrifugation and red blood cells (RBCs) were removed by hemolysis with 150 mmol/L NaCl at 37°C. Marrow mononuclear cells were stored frozen in RPMI, 36% FCS, 10% dimethylsulfoxide (DMSO), 90 U penicillin, 90 mg/mL streptomycin sulfate, and 0.36 mg/mL glutamine. The stored cells were thawed at 37°C and slowly diluted on ice to a final DMSO concentration less than 1%. After washing, the CD34+ cells were labeled with anti-CD34 conjugated to fluorescein isothiocyanate (FITC; HPCA-2 [IgG]; Becton Dickinson, San Jose, CA) for 20 minutes on ice, washed with PBS containing 1% bovine serum albumin (BSA), and then labeled with rat antimouse IgG1 conjugated to superparamagnetic microspheres (Milenyi Biotec GmbH, Bergisch Gladbach, Germany). The CD34+ cells were positively selected using High-Gradient Magnetic Cell Sorting (Milenyi Biotec GmbH). The CD34+ enriched population was then incubated with anti-CD38 conjugated to phycocerythrin (PE; leu-17; Becton Dickinson) for 20 minutes on ice, washed, and sorted using a FACStar plus (Becton Dickinson). Cells with medium to high forward light scatter and low side scatter were selected and both the 38° and the 38° populations of CD34+ cells were collected.

Stromal cell functional analysis. Screening was initiated by plating stromal cells at a density of 600 to 1,000 per well in terasaki 96-well plates (Nunc, Naperville, IL) 2 days before the addition of approximately 200 hematopoietic cells. 38° and 38° cells were added to the cultures in serum-deprived medium (Nutridoma-HU; Boehringer Mannheim, Indianapolis, IN) and incubated at 37°C in a 5% CO₂ humidified incubator for 5 days. The viability and proliferation of progenitors was scored after the addition of 5 µL of a staining mixture that contained 2.5% India ink, 250 µg/mL ethidium bromide, and 75 µg/mL acridine orange in HBSS. The number of viable cells was determined for each well by inverted fluorescence microscopy (Nikon Diaphot microscope).

Long-term support of CFCs. Two- to four-week-old primary LTCs, established and maintained according to previously published guidelines, and stromal cell lines were irradiated at 2,000 cGy.
and plated into 24-well plates at least 24 hours before the addition of hematopoietic cells. Irradiated stromal cells were plated at sufficient cell densities to insure formation of monolayers. The stromal cultures were seeded with 1,000 to 3,000 38<sup>th</sup> cells and were demidepleted weekly for 5 or 8 weeks. The nonadherent and adherent cells were harvested and analyzed for CFCs using colony assays (described below).

Conditioned media (CM). Media were conditioned by exposure to semiconfluent cultures for 1 week. Both RPMI containing 10% FCS and serum-deprived (1% Nutridoma-HU) media were used. The culture debris was pelleted by centrifugation at 2,000 rpm for 10 minutes and the supernatant was then aliquoted and frozen at -20°C. CM were thawed only once before use. Concentrated CM was obtained using Amicon centriprep 10 concentrator (Amicon, Beverly, MA) according to the manufacturer’s specifications, and protein content was determined using the BioRad protein assay (BioRad Laboratories, Richmond, CA). The same batch of CM was assayed for colony-stimulating activity in standard colony assays and for cytokine content with enzyme-linked immunosorbent assays (ELISAs; see below). In addition to these assays, 38<sup>th</sup> cells were incubated with CM or GF mix for 5 days and then stained for viability as described above.

Colony assays. Colony assays were performed using a stock of 1.2% methylcellulose, 2.5% BSA, 25% FCS (Hyclone 796), 100 U penicillin, 100 μg/mL streptomycin sulfate, and 0.1 mol/L β-mercaptoethanol. Colony-stimulating activity was provided either by a growth factor mix containing 10 ng/mL interleukin-1 (IL-1), IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage-CSF (GM-CSF), Kit ligand (KL), and 3 μM/L erythropoietin (Epo) or by 10% CM. Hematopoietic cells (38<sup>th</sup> or 38<sup>th</sup>) plus 100 μL growth factor mix or CM was added to 0.9 mL of the methylcellulose stock. Colony formation was scored at day 14 and designations were periodically confirmed by Wright-Giemsa staining of colony cytospins.

ELISAs. Cytokine content of the CM was determined with Quantikine kits (R & D Systems, Minneapolis, MN) according to the manufacturer’s specifications. Supernatants were analyzed neat at 1:2 and at 1:5 dilutions.

RESULTS

Immortalization of BM cell lines. LTBMCs were infected with the LXSN-16 E6/E7 retrovirus that was packaged in the PA317 cell line, and infected cells were plated at limiting dilutions. Twenty-seven foci were identified and isolated using cloning rings to establish stromal cell lines (HS-1 to HS-27), of which 24 were retained and proved to be resistant to G418 at 50 μg/mL. All lines were initially characterized morphologically and histochemically, screened for maintenance and/or proliferation of hematopoietic progenitors (HPS; see below), and then frozen. Several clones, designated HS-5, HS-21, HS-23, and HS-27, were selected for more detailed analysis and have been maintained in continuous culture for up to 20 months with periodic analysis of their phenotypes.

Based on morphology, two distinct cell types were observed, small fibroblastic (HS-5 and HS-21) and large flattened epithelioid-like (HS-23 and HS-27). The two fibroblastic lines, although similar in morphology, differed in regards to growth patterns. HS-5 forms a reticulum of over-lapping cells, reminiscent of astrocytes, whereas HS-21 cells are well separated and line up in parallel arrays. At higher densities, HS-5 forms a dense “net” of cells, whereas HS-21 forms a contiguous monolayer with discernible cell boundaries. HS-23 and HS-27 form large flattened polygonal shaped cells that exemplify “blanket” cells and maintain numerous intercellular contacts with neighboring cells. HS-23 and HS-27 will also form monolayers; however, because of their flattened morphology, it is difficult to identify distinct cell boundaries.

Clonal analysis of stromal cell lines. Southern hybridization of genomic DNA from stromal cell lines using a radiolabeled probe directed against the E6E7 genes. Bands represent products from EcoRI digestion at a site within the retroviral insert and a site provided by the flanking genomic DNA. The lane labeled E6E7 is an EcoRI digest of the pLXSN16E6E7 construct that generates a 6.8-kb band and FSF is genomic DNA from a nontransformed foreskin fibroblast line. The molecular weight markers (M) are ethidium bromide-stained λ HindIII DNA fragments.

![Cell Lines](Fig 1. Southern hybridization of genomic DNA from stromal cell lines using a radiolabeled probe directed against the E6E7 genes. Bands represent products from EcoRI digestion at a site within the retroviral insert and a site provided by the flanking genomic DNA. The lane labeled E6E7 is an EcoRI digest of the pLXSN16E6E7 construct that generates a 6.8-kb band and FSF is genomic DNA from a nontransformed foreskin fibroblast line. The molecular weight markers (M) are ethidium bromide-stained λ HindIII DNA fragments.)

All cell lines were negative for major histocompatibility complex (MHC) class II (DR) and CD14, a macrophage-specific marker (Table 1), and positive for antigens normally associated with nonhematopoietic stromal cells. All lines expressed collagen III and IV, with low levels of collagen I detected on HS-5 and HS-27. Analysis of VCAM-1 showed that HS-5 and HS-21 expressed low levels, HS-23 was heterogeneous positive, and HS-27 was homogeneously strongly positive. All lines were positive for acid phosphatase, with some differences in the degree of staining. In contrast, HS-5 was negative for alkaline phosphatase, whereas all others were heterogeneously positive.

These cell lines were also tested for their ability to undergo
lipogenesis in response to corticosteroids. Dexamethasone, hydrocortisone, insulin, and a combination of dexamethasone and insulin were added to confluent cell layers for 4 weeks, at which point the cultures were stained for lipid vacuoles with oil red O. HS-5 and HS-21 did not accumulate lipids, whereas a few cells (approximately 1% to 2%) from HS-23 formed lipid vacuoles in the presence of dexamethasone only. However, none of the lines acquired the extent of proliferation beyond that observed with HS-5.

A rapid screening assay was developed to assess the viability and expansion of hematopoietic cells when cocultured with the cell lines. Both 38' and 38+ cells were cocultured on stromal cell lines in serum-deprived medium (Natriodma-HU) for 5 days with and without IL-3 (10 ng/mL) and then stained to differentiate stromal from hematopoietic cells and viable cells from dead cells.

All 24 cell lines maintained the viability of both 38' and 38+ subpopulations of CD34+ cells for 5 days. When IL-3 was added to the cocultures, the 38+ cells increased in number in all cases. However, one cell line, HS-5, was able to induce the 38+ cells to proliferate without exogenous IL-3, and the addition of IL-3 to HS-5 coculture did not increase the extent of proliferation beyond that observed with HS-5 alone. Figure 2 shows the difference between the maintenance of 38' cells on HS-21 and the proliferation of these cells on HS-5. No proliferation of 38+ cells was observed within the 5-day time span of this experiment.

Long-term hematopoiesis. To determine whether these lines could support less mature hematopoietic cells, we monitored their ability to maintain or produce CFCs from the 38' population after 5 and 8 weeks (Fig 3). The two representative experiments are indicative of the range of CFC production and show that the stromal lines can maintain CFCs at levels comparable to primary LTCs for up to 8 weeks. Interestingly, HS-27, which expresses the highest levels of VCAM-1, was the only cell line to establish cobblestone regions when incubated with 38+ cells (Fig 4).

Biologic activity of CM. Only CM from HS-5 induced proliferation of 38' cells in the absence of stromal cells. Figure 5 shows the extent of proliferation induced by CM from HS-5 compared with that induced by CM from HS-21 and a recombinant growth factor mix. Additionally, we determined whether CM from HS-5, HS-21, HS-23, and HS-27 could support colony formation using colony assays. Consistent with the results of the shorter assay, only CM from HS-5 supported the growth of colonies from the 38' population and the 38+ population. Figure 6 is a comparative analysis between the activity of HS-5 CM, HS-21 CM (with and without serum), and growth factor mix (GF mix). HS-5 CM, independent of serum content, generated an equivalent number of G/GM colonies from 38' cells as the GF mix; however, HS-5 CM generated significantly more colonies from 38+ cells (Fig 6A). In contrast, CM from HS-21 supported significantly fewer G/GM from both CD3+ subpopulations compared with HS-5 or GF mix. The relative numbers of CD3+ cells were also significantly different and paralleled the observations with the G/GM colonies (Fig 6B). However, the GF mix generated significantly more BFU-E from 38' cells than any CM.

Cytokine analysis in CM. The CMs from four cell lines were assayed for G-CSF, GM-CSF, KL, leukemia-inhibitory factor (LIF), IL-6, IL-1α, IL-3, and IL-11. Only HS-5 and HS-21 CM contained significant amounts of these cytokines and were additionally tested for the presence of IL-1β, IL-1RA, IL-2, IL-8, epidermal growth factor (EGF), tumor necrosis factor α (TNFα), transforming growth factor α (TGFα), and macrophage-inhibitory protein-1α (MIP-1α). Figure 7 shows that the majority of the cytokines are present in HS-5 and HS-21 supernatants at similar levels. However, HS-5 additionally secretes IL-1α, IL-1β, IL-1RA, and LIF. IL-3 was not found in any supernatant and was not detectable using reverse-transcriptase polymerase chain reaction for IL-3 message in mRNA from the HS-5 or HS-21 cell lines (data not shown). Although it has been reported that IL-3 mRNA can be detected in primary LTCs, it is not clear what the cellular source is. Given that the low amounts detected may be produced by lymphocytes present in LTCs, it is not surprising that HS-5 does not produce IL-3.

DISCUSSION

The hematopoietic ME is essential for controlling differentiation and proliferation of progenitor cells. The ME is approximated in vitro by the LTC system, which generates complex stromal cell layers that are composed of multiple cell types reflecting the in vivo heterogeneity. Unfortunately this complexity complicates efforts to define specific cellular components that are required for regulation of hematopoiesis. However, investigators have used this system to define cytokines, adhesion molecules, and matrix compo-
IMMORTALIZED HUMAN STROMAL CELL LINES

Fig 2. Coculture of 38+ cells with the HS-5 and HS-21 stromal cell lines for 5 days in the absence of serum or additional cytokines. Small round cells are viable hematopoietic cells that have accumulated acridine orange and fluoresce green, whereas the large flat cells are stromal cells. Nonviable cells have incorporated ethidium bromide and fluoresce orange. The same number of 38+ cells were plated into each culture at the initiation of the experiment. The micrograph shows a portion of a terasaki well by inverted fluorescence microscopy (original magnification ×40).

nents that contribute to hematopoietic regulation. A further dissection of this system into functional components, such as the cell lines described here, is needed to determine what each component contributes to the overall ME and how these activities are regulated.

Human BM stromal cell lines have been generated with the SV40 large T antigen, whereas attempts for transformation with c-myc, N-ras, and v-ras have not proven successful. Several labs using different SV40 constructs have generated cell lines that differ in their morphology and functional activity in ways that appear to be influenced by the SV40 construct. Although these lines display proteins that are indicative of nontransformed cell lines, they also exhibit properties of transformed cells and some are not stable. To eliminate or reduce these effects, investigators have used the metallothionein-regulated promoter system that allows cells to return to their “normal” phenotype; however, the residual effects of transformed growth cannot be assessed.

Previous reports indicated that epithelial cells immortalized by the HPV16 E6 and E7 genes can retain their differentiated phenotype, although aneuploidy is observed in late passage cells. At the molecular level, E6 binds to and degrades p53, which functions to prevent cell entry into S phase in the event of DNA damage. E7 alone has the ability to immortalize cells; however, when combined with E6 in LXSN-16 E6E7, they immortalize epithelial cells at a higher frequency. E7 functions by inhibiting the hypophosphorylated form of Rb, which binds E2F, thus preventing cell cycle progression. Rb, by virtue of binding E2F, is central to cell cycle regulation and, more specifically, entry into S
they may alter cellular phenotypes sufficiently to prevent characterization of the specific cell types and an understanding of their normal function.

Consistent with the E6/E7 immortalization of epithelial cells, the stromal cell lines reported here have increased growth rates, do not undergo senescence (some have been in continuous culture for 2 years), and retain characteristics of normal differentiated BM stromal cells. Positive staining with antibody 6.19 (specific for fibroblasts, endothelial, and adipocytes), P4.1 (CD10, endopeptidase), and P1H11 (vimentin) and the absence of a macrophage marker (CD14) indicate that the cells are mesenchymal in origin. The lack of FVIII antigen indicates that they are not endothelial; however, all lines express collagen type IV, which is consistent with the endothelial nature of BM stroma. Only the HS-23 cell line responds to dexamethasone, suggesting that it may be preadipocytic. The cell lines display a normal staining pattern for smooth muscle actin, vimentin, and cell-associated fibronectin and growth is inhibited at confluency. CD34 and STRO-1 are absent, which is consistent with the loss of these markers in normal BM cultures after several weeks of growth. All 24 lines, except HS-5, were heterogeneously positive for both alkaline phosphatase and acid phosphatase. Similar to HS-5, alkaline phosphatase-negative cell lines were identified by Lanotte et al in their attempt to generate spontaneous human lines.

Overall, the morphologic and phenotypic characteristics of these cell lines are similar to those of murine BM stromal

phase by controlling the expression of genes such as c-myc. In the LXSN-16 E6E7 retroviral construct, E7 is downstream of E6, resulting in lower expression of E7. This level of expression is still sufficient to facilitate cell cycle progression without significant transformation of the cell. The SV40 large T antigen also binds Rb, thereby facilitating entry into S phase; however, numerous additional activities have been attributed to the large T antigen. Although these activities are not intrinsically involved in transformation,
Fig 5. Small-scale expansion of 38- cells with GF mix (A), HS-5 CM (B), and HS-21 CM (C). The same number of cells were added per well at time 0, expanded with different media for 5 days, and stained with ethidium bromide and acridine orange.

Fig 6. Number of hematopoietic colonies grown from 38- or 38+ cells in the presence of GF mix, HS-5 CM, and HS-21 CM with serum (s) or serum deprived (sd). (A) Granulocytic/monocytic colony numbers (G/GM); (B) erythroid bursts (BFU-E). RPMIs represents RPMI media supplemented with 10% FCS. Results significantly different from HS-5 are designated 1. Results significantly different from GF mix are designated 2. Error bars represent SEM. *P < .01; **P < .05.
increase in clonogenic cells (data not shown). Additional experiments are necessary to evaluate the retention or expansion of LTCIC with this CM. In contrast to HS-5 and HS-21, the “blanket” cell lines HS-23 and HS-27 only secrete small quantities of cytokines and, as expected, CM from these lines do not support colony growth (data not shown).

Both the fibroblastic and “blanket” cell lines supported CFC for 5 to 8 weeks at levels comparable to those of primary LTC. These results indicate that, independent of phenotypic differences and detectable cytokine secretion, these diverse stromal cell lines produce hematopoietic main-

tenance factors at levels that are sufficient to support immature pluripotent progenitors. Previous investigations have also indicated a lack of correlation between cytokine secretion and support of hematopoiesis by stromal cells.27,42 Also consistent with these observations is the ability of a murine cell line (MS-5) to maintain immature human hematopoietic cells,43 which accounts for the ability of fixed 3T3 cells to support murine hematopoietic cells.49 The support of cobblestone regions by the HS-27 cell line indicates that it provides additional signals for the burrowing of precursors and their subsequent proliferation beneath the cell layer. This line should be useful for the identification and isolation of the in vivo equivalent of the blanket cell29 and the signals required for cobblestone formation.

Together, these observations suggest that the factors responsible for differentiation and proliferation of committed progenitors are distinct from those required for maintenance of the immature pre-CFC pool. Moreover, it suggests that these maintenance factors are ECM or membrane associated. Continued analysis of the differences between these diverse cell lines will be useful in determining the role of different stromal elements in the regulation of hematopoiesis.

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