Changes in the Growth Properties of CD34+, CD38− Bone Marrow Progenitors During Human Fetal Development

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We have previously described the isolation of separate populations of CD34+, CD38− stromal and hematopoietic progenitors cells within fetal bone marrow. The CD34+, CD38−, CD50−, HLA-DR+ population contained the majority of primitive hematopoietic progenitor cells, whereas stromal progenitors were contained within the CD34+, CD38−, CD50−, HLA-DR− population. In this study, we compared the frequencies and total numbers of clonogenic CD34+, CD38− stromal and hematopoietic cells as a function of fetal gestational age using single-cell fluorescent-activated cell sorting (FACS). At 14 weeks of gestation, 1/500 fetal bone marrow mononuclear cells were primitive hematopoietic CD34+, CD38+, HLA-DR− progenitor cells, whereas 1/100,000 were stromal progenitors with the CD34+, CD38+, HLA-DR− phenotype. During fetal ontogeny there was a continuous, age-dependent decrease in the frequency of stromal progenitors, such that, at 24 weeks of gestation, only 1/100,000 of bone marrow cells had the CD34+, CD38+, HLA-DR− phenotype and were clonogenic stromal cells when isolated by FACS. In contrast, 1/250 bone marrow cells in a 24-week fetus had the CD34+, CD38+, HLA-DR− phenotype and were clonogenic hematopoietic progenitors. The decrease in the frequency of stromal progenitors was a function of both a decreased frequency of cells with the CD34+, CD38+, HLA-DR− phenotype and a decrease in the growth potential of individual with this phenotype. The total numbers of mononuclear cells and the total numbers of hematopoietic progenitors in two fetal femurs increased in parallel, 100-fold, between 14 and 24 weeks of gestation. In contrast, the total numbers of clonogenic CD34+, CD38+, HLA-DR− stromal progenitor cells remained constant during this period. Although adult bone marrow samples contained stromal progenitor cells at a frequency of approximately 1/7,000 mononuclear cells, clonogenic stromal cells with the CD34+, CD38+, HLA-DR− phenotype could not be isolated by single-cell FACS from these samples. Thus, there are significant differences between the frequencies and biologic characteristics of stromal and hematopoietic stem cells during fetal and postnatal ontogeny.

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MATERIALS AND METHODS

Fluorescent-activated cell sorting (FACS). Human fetal bone marrow from 14- to 24-week gestation fetuses was obtained from Advanced Bioscience Resources, Inc (Alameda, CA). Single-cell suspensions of fetal bone marrow were prepared by flushing the bone marrow cells out of the humerus and femurs using a syringe and a 22-gauge needle into phosphate-buffered saline (PBS) containing 1% wt/vol bovine serum albumin (BSA). The yield of mononuclear cells from each fetal sample were 1 × 10^6 to 100 × 10^6 cells from 2 femurs. Adult bone marrow samples were generously provided by Dr Robert Negrin (Stanford University Hospital, Stanford, CA) and by the Bone Marrow Transplant Program, Emory University (Atlanta, GA) from 16 cancer patients whose bone marrow had been harvested in preparation for autologous bone marrow.
transplantation and from 14 normal individuals who were donating bone marrow for the purpose of allogeneic bone marrow transplantation. The low-density mononuclear cell fraction of bone marrow was obtained by centrifugation of the cell suspension over a Ficoll step gradient (density, 1.077 g/mL) and collecting the buoyant cell fraction. The average number of mononuclear cells from adult bone marrow was 40 × 10⁶/10 mL aspirate. Cell suspensions were stained with saturating concentrations of combinations of CD34 allophycocyanine (APC), CD38 phycoerythrin (PE), and HLA-DR fluorescein (FITC). Single cells with the phenotype CD34+, CD38−, HLA-DR+ and CD34+, CD38−, HLA-DR− were sorted into the individual wells of a 96-well flat-bottom tissue culture plate containing cell culture media using a FACS Vantage cell sorter (Becton Dickinson Immunocytometry Systems [BDIS], San Jose, CA) equipped with an automatic cell deposition unit (ACDU), a Coherent Enterprise laser tuned at 488 nm (100 mW), and a Coherent Spectra laser tuned at 647 nm (100 mW). The analysis of the list mode data files was performed with a custom data presentation software program made by Dr Ben Verwer in the research department of BDIS.

**Tissue culture.** Media consisted of the Terry Fox long-term myeloid media (TF) supplemented with one of two combinations of growth factors: (1) media no. 1, 10 ng/mL insulin-like growth factor-1 (IGF-1) and 2.5 ng/mL basic fibroblast growth factor (bFGF); and (2) media no. 2, a mixture of hematopoietic growth factors consisting of 10 ng/mL interleukin-3 (IL-3), 500 U/mL IL-6, 10 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF), 2.5 U/mL erythropoietin (EPO; Amgen, Thousand Oaks, CA), 50 ng/mL stem cell factor (SCF; Genzyme, Cambridge, MA), and 10 ng/mL IGF-1 and 2.5 ng/mL bFGF. Unless otherwise specified, growth factors were obtained from Becton Dickinson Collaborative Research (Bedford, MA). Ninety-six-well tissue culture plates were kept at 37°C in a 5% vol/vol CO₂ humidified atmosphere. Single cells with the CD34+, CD38−, HLA-DR+ phenotype were deposited into individual wells of a 96-well tissue culture plate containing 200 μL of media no. 1, whereas cells with the CD34+, CD38+, HLA-DR+ phenotype were deposited into individual wells containing 200 μL of media no. 2. The growth of the sorted cell in each well was determined by visual inspection between 14 and 21 days of culture. Wells were scored positive for the growth of either stromal (adherent fibroblast-like cell) or hematopoietic (nonadherent, round cells) colonies when they contained more than 50 cells after 14 to 21 days of culture.

**Poisson analysis of clonogenic cells.** The mononuclear cell fraction of bone marrow cell suspensions were cultured at a range of cell concentrations (10 cells/well to 10⁶ cells/well) in TF media + IGF-1 and bFGF (media no. 1) or, in some of the experiments on adult bone marrow, in TF media alone. For each sample, between 8 and 24 wells were plated at each cell concentration. No difference was noted in the frequency of stromal progenitors between TF media alone and TF media supplemented with IGF-1 and bFGF (data not shown). After 18 to 21 days of culture, the cultures were scored for the presence of more than 50 stromal, fibroblast-like cells. The percentage of negative wells at each cell concentration was plotted against the logarithm of mononuclear cells initially plated in each well, and a straight line was fitted against the points for each type of cultured cells (Poisson analysis). To compare multiple fetal or adult bone marrow samples, the mean frequency (±SEM) of negative wells at each cell concentration was determined, and a straight line was fitted against the mean values. The frequency of clonogenic cells in each population was determined by calculating the intercept on the abscissa at the point at which 37% of the wells failed to show growth.

**RESULTS**

**Fetal bone marrow had a higher frequency of stromal progenitor cells than adult bone marrow.** We and others have previously observed and reported a large difference in the relative frequencies of the CD34+ primitive hematopoietic progenitors in fetal bone marrow compared with adult bone marrow. We investigated the relative frequencies and growth potential of stromal progenitors from fetal and adult bone marrow. On the basis of a limiting dilution of bone marrow cells and a Poisson analysis of the mean frequencies of culture wells that lacked growth of stromal cells, the frequency of stromal progenitors in fetal bone marrow mononuclear cells was 1/7,000 based on 4 samples (17, 20, 21, and 22 weeks of gestation) and 1/7,000 in adult bone marrow mononuclear cells based on the analysis of 30 samples of adult bone marrow (Fig 1). The initial growth pattern of the stromal cells from both fetal and adult sources were similar, ie, swirls of fibroblasts that formed longitudinal arrays. The fibroblast monolayer then developed into strands of fibrous tissue suspended above the bottom of the culture well with loosely adherent clusters of round cells (Fig 2).

**The phenotypes of CD34+ fetal bone marrow cells varied as a function of gestational age.** To isolate hematopoietic and stromal progenitors within the CD34+ fraction of bone marrow, cell suspensions were stained with CD34 APC, CD38 PE, and HLA-DR FITC and analyzed on a FACS Vantage Cell sorter. The flow cytometric analysis of a bone marrow of a gestational age of 16 and 24 weeks are shown in Fig 3. Figure 3A and D show the forward and orthogonal light scatter of the bone marrow cells of both samples and Fig 3B and E the correlation between CD34 and CD38 expression. The CD34+ cells were depicted black, whereas all other cells were depicted gray. The position of the CD34− B cells and myeloid cells were indicated in Fig 3 with the letters B and M, respectively. In Fig 3C and F the expression of HLA-DR and CD38 is shown only for the CD34+ population. The CD34−, CD38+ cells are shown as small gray dots; the CD34−, CD38−, HLA-DR− cells are shown as large gray pixels in the lower left quadrant; and the CD34−, CD38−, HLA-DR+ cells are shown as large black pixels in the lower right quadrant of each plot. The bone marrow sample with a gestational age of 16 weeks clearly showed a relatively larger proportion of CD34−, CD38−, HLA-DR− cells than the bone marrow sample with a gestational age of 24 weeks. To correlate of the relative proportions of the CD34−, HLA-DR/CD38 subsets with gestational age, the fraction of CD34− cells in each sample was determined, as well as the division of CD34− cells into four subsets: CD38−, HLA-DR−; CD38−, HLA-DR+; CD38−, HLA-DR−; and CD38−, HLA-DR+. The mean frequency of CD34− cells contained within a set of 16 fetal bone marrow samples that ranged in age from 14 to 24 weeks of gestation was 0.12 ± 0.037 (mean ± SD) and did not vary with fetal gestational age. The largest number of fetal CD34− bone marrow cells (80%) of the total number of CD34− cells was contained within the CD38−, HLA-DR− subset, and constituted a mean fraction of 0.1 ± 0.04 (n = 16) of the total number of mononuclear bone marrow cells in fetal bone marrow samples. This cell subset has the lowest growth potential in vitro. The CD34−, CD38−, HLA-DR− and CD34−, CD38−, HLA-DR− cells have limited potential for hematopoietic growth in vitro and significantly less replating potential com-
pared with the CD34+, CD38−, HLA-DR+ cells. In the present study, their mean frequencies within fetal bone marrow mononuclear cells were 0.009 ± 0.005 and 0.005 ± 0.009, respectively (n = 16). The CD34+, CD38+, HLA-DR+ cells, which contained the pluripotent hematopoietic progenitors, were relatively rare and comprised a mean fraction of 0.006 ± 0.004 (n = 16) of the mononuclear cells from fetal bone marrow. There were no significant relationships between the fraction of CD34+, CD38+, HLA-DR+ cells or the fraction of CD34+, CD38−, HLA-DR− cells with fetal gestational age (data not shown). In contrast, the frequency of cells with the CD34+, CD38−, HLA-DR− phenotype declined from approximately 0.02 to 0.002 (1 cell per 50 to 1 cell per 500 mononuclear cells) when multiple fetal bone marrow samples within the range of 14 to 24 weeks gestation were analyzed (middle curve, Fig 4A). The frequency of cells with the CD34+, CD38−, HLA-DR− phenotype remained constant at a fraction of approximately 0.005 of low-density nucleated cells (1 cell per 200) during this period of fetal development (middle curve, Fig 4B).

Changes in the growth properties of hematopoietic and stromal progenitors during fetal ontogeny. To determine whether there were functional differences between cells isolated from fetal samples of different gestational ages, we measured the cloning efficiency of FACS-sorted single cells with either the stromal cell progenitor phenotype (CD34+, CD38−, HLA-DR+) or the hematopoietic cell phenotype (CD34+, CD38−, HLA-DR+). The two populations with the phenotypes shown as large gray or black pixels in Fig 3C and F were sorted using orthogonal sort gates, as we have previously described. The probability that any singly sorted CD34+, CD38−, HLA-DR− cells yielded stromal cultures in media no. 1 (which supports stromal cell growth) decreased over a 10-fold range, from 0.2 (1 in 5 sorted cells) to approximately 0.02 (1 in 50 sorted cells) during this period of fetal development (upper curve, Fig 4A), whereas the fraction of CD34+, CD38−, HLA-DR− cells that formed hematopoietic colonies in media no. 2 (containing hematopoietic growth factors) ranged from 0.3 to 0.78 during the same period (a mean at 6 cells of every 10 cells sorted), with a trend toward an increasing cloning efficiency during the latter stages of fetal development (upper curve, Fig 4B). The difference between the mean frequency of clonogenic stromal cells among the singly sorted CD34+, CD38−, HLA-DR− cells in fetal samples less than 18 weeks of gestation (0.14, n = 4) compared with fetal samples greater than or equal to 18 weeks of gestation (0.04, n = 12) was highly significant by the Student’s t-test (P < .002). Based on the absolute frequencies of cells with the CD34+, CD38−, HLA-DR− (stromal) and CD34+, CD38−, HLA-DR+ (hematopoietic) phenotypes in fetal bone marrow and the relative cloning efficiency of cells with these phenotypes when isolated by FACS, we calculated the frequencies of clonogenic stromal and hematopoietic progenitor cells. We defined this as the product of the frequency of bone marrow cells with a given phenotype.
multiplied by the fraction of singly sorted cells with this phenotype that grew in culture after single-cell FACS. The frequency of clonogenic CD34+, CD38+, HLA-DR+ stromal progenitors decreased by 99% during this period of fetal development, from 0.001 (1/1000) to 0.00001 (1/100,000) (lower curve, Fig 4A). There was a statistically significant difference between the mean fractions of bone marrow cells that were both CD34+, CD38+, HLA-DR+ and clonogenic stromal progenitors in the younger fetal samples (0.002) compared with the older fetal samples (0.0001; P < .05). In contrast, the frequency of clonogenic CD34+, CD38+, HLA-DR+ cells increased slightly, from 0.002 (1/500) to 0.004 (1/250) of nucleated cells between 14 and 24 weeks of gestation (lower curve, Fig 4B).

The total number of CD34+, CD38+, HLA-DR+ hematopoietic progenitors increased during fetal development, whereas the number of CD34+, CD38+, HLA-DR+ stromal progenitors remained constant. The total number of clonogenic CD34+, CD38+, HLA-DR+ stromal progenitors and the total number of clonogenic CD34+, CD38+, HLA-DR+ hematopoietic progenitors contained within two fetal femurs were calculated by determining the product of the frequency of clonogenic stromal or hematopoietic progenitor cells (lower curves, Fig 4A and B), with the total number of nucleated cells obtained from two femurs. The calculated number of total stromal progenitors with the CD34+, CD38+, HLA-DR+ phenotype remained constant at ca. 2,500 cells per two fetal femur bones (lower curve, Fig 4C) during the period of fetal development between 14 and 24 weeks of gestation. Because the CD34+, CD38+, HLA-DR+ population was heterogeneous and contained separate populations of stromal (CD50+) and hematopoietic (CD50−) progenitors, we calculated the number of clonogenic CD34+, CD38+, HLA-DR− (CD50−) hematopoietic cells for each of the fetal samples studied and found that it remained constant at 3,000 cells per two femurs during this period of fetal development (data not shown). In contrast, the calculated number of clonogenic CD34+, CD38+, HLA-DR− (CD50−) hematopoietic cells increased exponentially 100-fold, from approximately 5,000 cells to 500,000 cells per two fetal femurs (middle curve, Fig 4C) and in parallel with the increase in the total number of nucleated cells recovered from two fetal femurs (upper curve, Fig 4C).

DISCUSSION

This study correlates the frequency of clonogenic hematopoietic and stromal cells within the CD34+, CD38− phenotype with fetal gestational age using data collected from single-cell sorting involving 16 different fetal bone marrow samples. These data support previous findings showing that the phenotypes and the developmental potentials of hematopoietic progenitors change as a function of fetal gestational age2,9,10,15 and extend the application of this principle to human bone marrow stromal progenitors. Blast-forming unit-fibroblast (BFU-F), as used in this study, was defined operationally as a stromal progenitor cell with the ability to produce more than 50 fibroblast-like progeny in culture media containing 25% vol/vol serum and supplemented with bFGF and IGF-1.12 We measured the frequencies and the total numbers of CD34+, CD38− hematopoietic and stromal progenitors contained within the bone marrow of two fetal femurs. We found, as expected, that the total number of nucleated cells in the bone marrow from two femurs increased exponentially with gestational age. The number of clonogenic hematopoietic progenitor cells with the phenotype CD34+, CD38−, HLA-DR− increased in parallel with the total number of mononuclear bone marrow cells. In contrast, the frequency of clonogenic stromal progenitors with the phenotype CD34+, CD38−, HLA-DR− decreased by 99% during the period of fetal development between 14 weeks and 24 weeks of gestational age. Because the total number of mononuclear bone marrow cells increased 100-fold during this period, the net effect was that the total number of CD34+, CD38−, HLA-DR− stromal progenitors remained constant. Although the adult bone marrow samples we studied contained BFU-F at the same mean frequencies as the fetal bone marrow samples by limiting dilution analyses as well as rare cells with the CD34+, CD38−, HLA-DR− phenotype, we could not isolate clonogenic stromal cell progenitors with this phenotype by single-cell FACS (data not shown).

There are two types of explanations that are consistent
Fig 3. FACS analysis of the relative expression of HLA-DR and CD38 in the CD34⁻ fraction of fetal bone marrow. Fetal bone marrow samples of 16 and 24 weeks of gestational age were stained with monoclonal antibodies to CD34, CD38, and HLA-DR as described in Materials and Methods. In (A), (B), (D), and (E) all bone marrow cells are shown and the CD34⁺ cells are represented by small black crosses. In (C) and (F) only the CD34⁺ cells are displayed; the CD34⁺, CD38⁺, HLA-DR⁺ cells are represented by heavy black dots in the lower right quadrant; and the CD34⁺, CD38⁻, HLA-DR⁻ cells are represented by large gray dots in the lower left quadrant. The remainder of the CD34⁺ cells from these two samples are represented by small gray dots. The absolute fractions of total CD34⁺ cells, the CD34⁺, CD38⁻, HLA-DR⁻ cells and the CD34⁺, CD38⁺, HLA-DR⁻ cells from these two samples were 0.114, 0.006, and 0.006 (16 weeks) and 0.114, 0.01, and 0.001 (24 weeks), respectively.

with the discordant changes in the frequencies of hematopoietic and stromal progenitors during development that we observed. First, the findings are a result of technical limitations of the methodology that we used, such that the recovery of clonogenic CD34⁺, CD38⁻, HLA-DR⁻ stromal progenitors from bone marrow suspensions by FACS becomes increasingly inefficient during fetal and postnatal development. Second, there are significant changes in the phenotypes and/or biologic properties of stromal progenitors during development.

A number of theoretical limitations of using FACS technology to analyze bone marrow suspensions could result in lower recovery of BFU-F cells from older fetuses and adult bone marrow samples. In contrast to the hematopoietic stem cells and cells of the various hematopoietic lineages, all of which can be found both in the bone marrow aspirate and the peripheral blood, stromal cells are adherent to the bone matrix. The extent to which they can be recovered in a bone marrow aspirate likely reflects their stromal lineage and their degree of differentiation. Certain stromal progenitors from
growth potentials of the stromal progenitors. Comparing the frequencies of stromal progenitors in the 14 normal bone marrow samples to the 16 samples from cancer patients, we found that both groups were heterogeneous, with a range of stromal progenitors from 1/1500 mononuclear cells (0.0007) to 1/150,000 mononuclear cells (7 \times 10^{-5})). The median frequencies of stromal progenitors for bone marrow samples from the 14 normal donors (1/6,000) and the 16 samples from cancer patients (1/5,000) were similar to what has been previously reported for adult bone marrow. The reproducibility of the limiting dilution assay was assessed by setting up duplicate 96-well culture plates with samples. In these cases the calculated frequency of stromal progenitors did not vary more than twofold between duplicate plates in four experiments (data not shown). Thus, the heterogeneity of stromal progenitors among different individuals may represent, in some cases, the effects of chemotherapy on the numbers and growth potentials of stromal progenitors.

Comparing the frequencies of stromal progenitors in the 14 normal bone marrow samples to the 16 samples from cancer patients, we found that both groups were heterogeneous, with a range of stromal progenitors from 1/1500 mononuclear cells (0.0007) to 1/150,000 mononuclear cells (7 \times 10^{-5})). The median frequencies of stromal progenitors for bone marrow samples from the 14 normal donors (1/6,000) and the 16 samples from cancer patients (1/5,000) were similar to what has been previously reported for adult bone marrow. The reproducibility of the limiting dilution assay was assessed by setting up duplicate 96-well culture plates with samples. In these cases the calculated frequency of stromal progenitors did not vary more than twofold between duplicate plates in four experiments (data not shown). Thus, the heterogeneity of stromal progenitors among different individuals may represent, in some cases, the effects of chemotherapy on the numbers and growth potentials of stromal progenitors and other, as yet unknown, physiologic effects.
The frequencies of clonogenic CD34', CD38', HLA-DR' stromal cell progenitors varied by 100-fold between 14 and 24 weeks of gestation and were undetectable in samples of adult bone marrow. Although the single-cell cloning assay by FACS may have underestimated the numbers of stromal progenitors from older fetal and adult bone marrow samples, because of suboptimal culture conditions or damage to clonogenic stromal progenitors caused by cell sorting, the magnitude of the difference between young fetal samples and older fetal samples suggests that there are fundamental changes in the biologic properties or phenotypes of BFU-F during fetal and postnatal development. Significant differences between the growth potentials of stromal versus hematopoietic stem cells could account for the relatively constant numbers of CD34', CD38', HLA-DR' stromal progenitors compared with the increasing numbers of hematopoietic stem cells during fetal development. A fixed number of stromal progenitor cells, with a high potential for self-renewal, may be sufficient to maintain the stem cell pool for the bone and connective tissue compartments, whereas increasingly numbers of hematopoietic progenitor cells are needed to maintain the stem cell pool for blood cells during fetal development. The result would be that the numerical expansion of BFU-F during fetal and postnatal bone marrow development is significantly less than the expansion of hematopoietic progenitors. In addition, we have assumed that a "single hit" of a clonogenic cell per culture well is sufficient to initiate a stromal culture. The observed decrease in the clonogenicity of CD34', CD38', HLA-DR' cells isolated by FACS from older fetal samples (and their absence in adult bone marrow) could represent the requirement for the presence of other supportive cell types in the culture. Thus, a single CD34', CD38', HLA-DR' cell from a young fetal sample might be capable of producing a stromal culture, but a similar cell, isolated from adult bone marrow, might not grow without the presence of additional supportive cells.

A change in the phenotype of the BFU-F during fetal and postnatal development could account for the decreases in the frequency of stromal progenitors in the CD34', CD38', HLA-DR' population that we observed. The delineation of the phenotype of BFU-F is confounded by the fact that the phenotypes of hematopoietic and stromal progenitors partially overlap. Hematopoietic progenitor cells in fetal bone marrow with the highest initial cloning efficiency and with high long-term proliferative capacity are CD34', CD38', HLA-DR'. However, the CD34', CD38', HLA-DR' subset contains separate populations of both hematopoietic and stromal progenitors. Hematopoietic progenitors constitute only 5% of the CD34', CD38', HLA-DR' subset, but these cells have a long-term proliferative potential approximately equivalent to that of the CD34', CD38', HLA-DR' cells. These data indicate that the lack of HLA-DR expression does not discriminate stromal from hematopoietic progenitors in fetal bone marrow. Indeed, some investigators consider pluripotent hematopoietic stem cells from adult bone marrow to be HLA-DR' some claim that they are HLA-DR'.20-24 Some present evidence that they are HLA-DR'25-28 and some present evidence that they are HLA-DR'29,30 CD50 (ICAM-3) can positively identify hematopoietic progenitors within both the HLA-DR' and HLA-DR' fractions of fetal and adult bone marrow, such that hematopoietic stem cells with the CD34', CD38', HLA-DR' phenotype uniformly express CD50, whereas the stromal progenitors are CD50'. A description of developmental changes in the patterns of expression STRO-1 and CD49b markers that may positively identify stromal progenitors may help elucidate stromal cell differentiation. Changes in the relative numbers of CD50'/CD50' cells within the CD34', CD38', HLA-DR' population during development could account for some of the decrease in the frequency of stromal progenitors that we observed. Direct measurements of the frequency of CD50' hematopoietic progenitors contained within the CD34', CD38', HLA-DR' population of fetal bone marrow did not show any correlation with increasing gestational age (data not shown). Therefore, the declining frequency of stromal progenitors within this population is not simply a result of increasing numbers of hematopoietic progenitors.

We conclude that a large fraction of the stromal progenitors that exist within adult bone marrow (and some of the stromal progenitors in bone marrow samples from older fetuses) are likely to have a different phenotype than CD34', CD38', HLA-DR'. Stromal progenitors could be contained within another CD34' subset of bone marrow or within a CD34' cell population. The mean frequency of BFU-F (of any phenotype) from four fetal bone marrow samples studied was approximately 1 cell per 7,000 (Fig 1). Bone marrow from an "average" fetal bone marrow sample of 20 weeks of gestation contains 40,000,000 mononuclear cells, 0.0025 of which are CD34', CD38', HLA-DR' (100,000 cells); approximately 0.027 of these CD34', CD38', HLA-DR' cells are clonogenic stromal progenitors (2,700 cells) (Fig 4). Using the Poisson analysis shown in Fig 1 to estimate
the total number of stromal progenitors, such a sample should contain a total number of approximately 5,700 stromal progenitors. This finding suggests that about 50% of the total number of clonogenic fetal bone marrow stromal cells are contained within the CD34+, CD38+, HLA-DR+ population, and 50% have a different phenotype. Indeed, sorting CD34+ and CD34− populations from fetal bone marrow samples has shown that approximately 50% of stromal progenitors are CD34+ and that the great majority of these are Thy-1+, CD34+, CD38−, HLA-DR− (Sheehan, Guo, and Waller, manuscript in preparation). In adult bone marrow, uncertainty as to the fraction of the total marrow volume contained in a particular aspirate prevents a reliable estimate of the total number of stromal progenitors. However, an average 10 ml samples contained approximately 5,700 total stromal progenitors per 40,000,000 mononuclear cells (Fig 1), none of which were CD34+, CD38−, HLA-DR−, as assessed by single-cell FACS.

This study has shown significant changes in the frequencies of phenotypically defined stromal progenitors during development. It has not elucidated the hierarchy of stromal cell differentiation, in part because the technology of FACS is poorly suited for the analysis of adherent cell populations and in part because of an incomplete understanding of the phenotype of the most primitive stromal progenitor cell. Differentiation of pluripotent CD34+ hematopoietic progenitors is associated with the downregulation of CD34 expression. In stromal cell differentiation, both CD34+ and CD34− progenitors exist in fetal bone marrow; it is not clear whether the CD34+ cells precede the CD34− cells in ontogeny. The elucidation of the pathways of stromal cell differentiation will require the identification of additional markers that can define stromal cell lineages and new techniques of characterizing small numbers of adherent cells. It remains to be determined whether there is a hierarchy of (unidirectional) stromal cell differentiation similar to that described for the hematopoietic system or whether, as proposed by Maximow more than 70 years ago, a pluripotent stromal cell progenitor can arise either from a primitive mesenchymal stem cell or after the dedifferentiation of phenotypically mature stromal cells.

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