

Endometrial Cells Derived From Donor Stem Cells in Bone Marrow Transplant Recipients

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DISORDERS OF THE UTERINE endometrium are common, leading to abnormal uterine bleeding, infertility, pregnancy complications, miscarriage, endometriosis, and cancer.¹⁻³ Although the generation and growth of the uterine endometrium is important in normal pregnancy and disease, it is poorly understood. The uterine endometrium is one of the most dynamic human tissues and consists of a glandular epithelium and stroma that are completely renewed in each monthly menstrual cycle. Endometrial stem cells are thought to reside in the basalis layer and serve as a source of cells that differentiate to form the endometrium. These endogenous stem cells allow the rapid regeneration of the endometrium necessary to support pregnancy.

Stem cells of bone marrow origin have the ability to transdifferentiate into multiple nonhematopoietic cell lineages. Adult bone marrow stem cells differentiate into hepatocytes, skin, muscle, lung, and neuronal cells in rodents.¹⁻⁹ Human bone marrow-derived circulating stem cells have been reported to differentiate into normal endothelial cells, hepatocytes,^{10,11} neurons,¹² cardiomyocytes,^{13,14} skin, and gastrointestinal epithelium.^{13,15} To date, however, there have been no reports that circulating stem cells of extrauterine origin can differentiate into human endometrial tissue and contribute to endometrial regeneration.

See also pp 75 and 104.

Context Regeneration of the endometrium in each menstrual cycle is required for reproduction. Endogenous endometrial stem cells reside in the basalis layer and serve as a source of cells that differentiate to form the endometrium. Bone marrow-derived cells have been shown to take on functions outside the hematopoietic system.

Objective To investigate the possibility that cells of extrauterine origin could repopulate the endometrium.

Design, Setting, and Patients Endometrium from 4 HLA-mismatched bone marrow transplant recipients (1998-2002) was evaluated for donor HLA expression. Each recipient had a bone marrow donor with an HLA type that enabled determination of the origin of any cell. Endometrial biopsies also were obtained from 4 healthy control women.

Main Outcome Measure HLA type was determined by immunohistochemistry and by reverse transcription-polymerase chain reaction.

Results Donor-derived endometrial cells were detected in endometrial biopsy samples from all bone marrow recipients and accounted for 0.2% to 48% of epithelial cells and 0.3% to 52% of stromal cells. None of the controls demonstrated HLA mismatch in endometrial samples.

Conclusion These findings demonstrate that endometrial cells can originate from donor-derived bone marrow cells and suggest that nonuterine stem cells contribute to the regeneration of endometrial tissue.

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METHODS

Study Participants and Endometrial Biopsies

We studied 4 female allogeneic bone marrow transplant recipients who received marrow from a single-antigen mismatched related donor and therefore had an HLA type that allowed determination of the origin of any cell. To avoid the possibility that men may not harbor female reproductive tract stem cells, each recipient had received marrow from female donors. All bone marrow transplants were performed for leukemia treatment. All women were of reproductive age (28-43 years) and had received total body irradiation and chemotherapy with cyclophosphamide and in 2 cases also cytarabine or busulfan at least 2 years before biopsy.

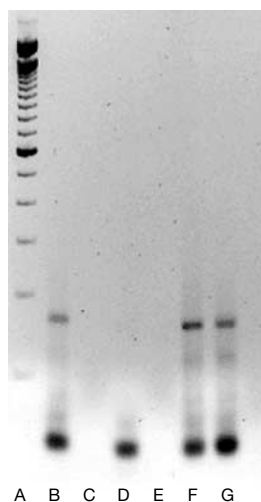
Endometrial biopsies were performed for clinical indications; 1 was

performed for the evaluation of infertility after sex steroid treatment and the other 3 for abnormal vaginal bleeding. All endometrial biopsies obtained from HLA-mismatched recipients and identified by the investigator in 1998-2002 were included. An equal number of endometrial biopsies were obtained from healthy nontransplanted control women of reproductive age (32-41 years) as part of fertility evaluation or for abnormal vaginal bleeding. Endometrial biopsies were obtained under an approved human investigation committee protocol. Written informed consent was

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Figure 1. Gel Electrophoresis of Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Amplification Products



Lane A, Molecular weight markers. Lane B, Positive control (actin). Lanes C–G show RT-PCR amplification products using HLA-A11 exon 3 primers and varying other components: lane C, reverse transcriptase and no endometrial RNA; lane D, reverse transcriptase and endometrial RNA from an HLA-A11–negative control; lane E, no reverse transcriptase and endometrial RNA from an HLA-A11–positive control; lane F, reverse transcriptase and endometrial RNA from an HLA-A11–negative transplant recipient (patient 1) who received HLA-A11–positive bone marrow; and lane G, reverse transcriptase and endometrial RNA from an HLA-A11–positive control.

obtained from each patient at the time of endometrial biopsy from Yale University School of Medicine, New Haven, Conn.

Histology and Immunohistochemistry

Formalin-fixed paraffin-embedded biopsy specimens were cut into serial sections 6 μ m thick, placed on coated slides, and deparaffinized through a series of xylene and ethanol washes. Immunohistochemistry was performed by using monoclonal antibodies against human HLA antigens A11, A3, and B7 (One Lambda; Pel Freez, Rogers, Ark); calcitonin (Neomarkers, Cambridgeshire, England); CD45 (Neomarkers); and β galactosidase (Vector, Burlingame, Calif). Slides were incubated with primary antibodies, followed by biotin-conjugated appropriate secondary antibodies. 3-3'-Diaminobenzidine or Nova

red (Vector) was used as the substrate for the peroxidase reaction with Vectastain (Vector). Secondary antibodies used for immunofluorescence were conjugated to fluorescein isothiocyanate or tetra-rhodamine isothiocyanate. Controls consisted of tissue sections obtained from nontransplanted women undergoing endometrial biopsies and immunostaining of transplanted patients' sections without primary or without secondary antibodies.

More than 200 high-power fields were counted for each patient. Three observers assessed each of the samples. The interobserver and intraobserver reliability were 6% and 4%, respectively. Each observer counted 5 identical slides twice; error was defined as differences in counts between observers or repeated counts by each observer.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from endometrial tissue by using TriZol reagent in accordance with the manufacturer's guidelines (Life Technologies Inc, Gaithersburg, Md). The reverse transcription (RT) step was done by using the bulk first-strand reaction mix (Amersham/Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's direction. Briefly, 1 μ g of extracted RNA was diluted in 20 μ L of RNase-free water, heated to 65°C for 10 minutes, and then chilled on ice. For first-test-strand complementary DNA synthesis, heat-denatured RNA solution, along with deoxynucleotide triphosphates and 200-mM deoxynucleoside dithiothreitol, was added to 11 μ L of reagent, followed by incubation at 37°C for 1 hour, and then heated to 90°C for 5 minutes and chilled on ice.

Primers used for polymerase chain reaction (PCR) are as described for clinical use in HLA typing for bone marrow transplantation.^{16,17} To amplify the 164–base-pair region of HLA-A11 (FIGURE 1), the following primers were used: A11N, which consists of base pairs 381 through 399 of exon 3; and AL1, which consists of base pairs 527

through 544 of the same exon.¹⁸ Polymerase chain reaction was performed as follows: 2 minutes at 95°C, 35 cycles of 95°C for 1 minute, 52°C for 30 seconds, 72°C for 2 minutes, and extension at 72°C for 8 minutes.

RESULTS

The characteristics of the bone marrow transplant recipients are shown in the TABLE. Donor-derived endometrial cells were detected in endometrial samples of bone marrow recipients. Endometrial samples subjected to RT-PCR demonstrated the expression of messenger RNA (mRNA) for a donor-derived HLA type. Endometrial samples from nontransplanted controls did not demonstrate expression of mRNA for a discrepant HLA type (Figure 1). Because this mRNA may have derived from migratory bone marrow–derived leukocytes, immunohistochemistry was performed to determine cell identity.

Immunohistochemistry revealed endometrial epithelial cells and stromal cells of donor origin in the uterus of women who were bone marrow transplant recipients (FIGURE 2). Endometrial sections from nontransplanted controls did not demonstrate cells with a discordant HLA type. The percentage of bone marrow–derived cells was greater in stroma than glandular epithelium and was localized in focal areas, suggesting local proliferation of donor-derived stem cells. In one patient, the endometrium consisted of more than 50% donor-derived cells (Figure 2C). Others consisted of approximately 0.3%, 4%, and 11% donor-derived cells (Table). Although most glands consisted of entirely host or entirely donor-derived cells, some glands consisted of a fraction of cells of each origin or contained only a few cells of donor origin (Figure 2D, E). Large numbers of transient mature leukocytes of donor origin were observed. Some epithelial cells of donor origin demonstrated cilia characteristic of functionally differentiated secretory endometrium (Figure 2E).

To distinguish CD45-positive leukocytes from endometrial cells, serial immunohistochemistry was per-

formed on each slide. CD45 is present on all human cells of hematopoietic origin, except erythroid cells and platelets. Identification of leukocytes with CD45 antibody demonstrated the existence of endometrial cells that were not CD45 positive (FIGURE 3), which confirmed that the immunohistochemical staining was identifying endometrial cells of donor origin rather than migratory mature bone marrow-derived cells.

Immunofluorescence was used to colocalize the discrepant HLA type with calcitonin, which identifies endometrial epithelial cells in the secretory phase of the menstrual cycle.¹⁹ Colocalization of the HLA-discrepant epithelial cells and calcitonin expression demonstrated that cells of donor ori-

gin expressed this endometrial cell-type specific marker and were capable of expressing a marker of functional differentiation (FIGURE 4). Staining with 4,6-diaminidino-2-phenylindole, which forms fluorescent complexes with natural double-stranded DNA, did not reveal any evidence of double signal intensity (data not shown), indicating a low likelihood of cell fusion.

COMMENT

In human or murine bone marrow transplant recipients, donor-derived cells can differentiate into numerous cell types of mesodermal, ectodermal, and endodermal origin. This preliminary study suggests that bone marrow-derived cells can differentiate into human uterine endometrium.

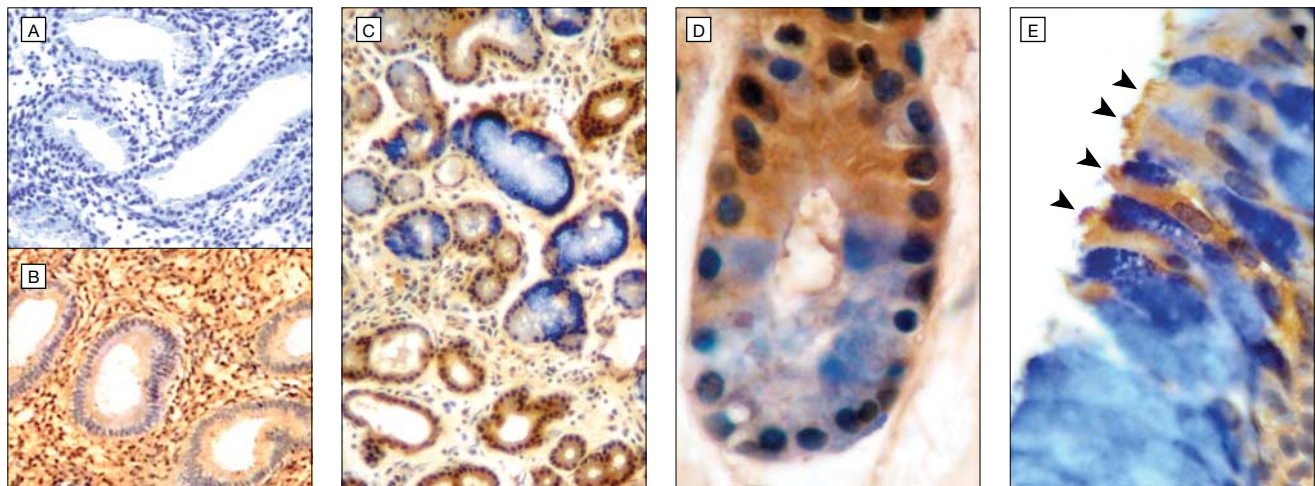
Transient infiltration of the endometrium by bone marrow-derived cells (primarily macrophages, neutrophils, and eosinophils) occurs predictably in each cycle; however, these are thought to have a role in inflammation and immunomodulation rather than to serve as a source of stem cells.²⁰ Transient infiltration could be distinguished from endometrial cells by CD45 immunoperoxidase staining. In this study, transient CD45-positive cells were distinct from glandular epithelium or stroma. Local clusters of donor-derived cells suggest that single progenitor cells underwent clonal expansion and differentiation. However, although some endometrial glands appeared to be completely composed of donor-derived cells, others contained

Table. Bone Marrow Transplant Recipients

Patient	Age, y	Conditioning Regimen	Time From Transplantation to Biopsy, mo	Positive Cells/Total No. of Cells (%)	
				Donor-Derived Epithelial Cells	Donor-Derived Stromal Cells
1	43	TBI, cyclophosphamide, cytarabine	157	4950/10 400 (48)	96 704/187 000 (52)
2	25	Cyclophosphamide, busulfan	129	1203/12 300 (10)	20 941/193 600 (11)
3	32	TBI, cyclophosphamide	35	684/17 300 (4)	6832/156 200 (4)
4	28	TBI, cyclophosphamide	24	45/19 500 (0.2)	675/210 000 (0.3)

Abbreviation: TBI, total body irradiation.

Figure 2. Donor-Derived Cells in the Endometrium of an HLA-A11–Mismatched Bone Marrow Transplant Recipient (Patient 1)



Immunohistochemistry using anti-HLA-A11 monoclonal antibody, biotin peroxidase detection system, diaminobenzidine as the chromogen (brown), and hematoxylin counterstain. A, HLA-A11–negative control (original magnification $\times 200$). B, HLA-A11 immunopositivity (brown) in an HLA-A11–positive control (original magnification $\times 200$). C, HLA-A11 immunopositivity (brown) in patient 1. More than 50% of the cells were of donor origin (original magnification $\times 100$). D, Endometrial glands partially derived from cells of donor origin (brown; original magnification $\times 400$). E, Rare cells of donor origin (brown) in an endometrial gland. Functional differentiation is noted by characteristic cilia. Arrowheads identify the ciliated epithelial surface (original magnification $\times 600$).

fractions of donor-derived cells or only a small number of individual cells of donor origin, which implies that not all glands are clonal in origin. The greater number of donor-derived stromal cells compared with glandular epithelial cells suggests differential cell-type-specific rates of transdifferentiation.

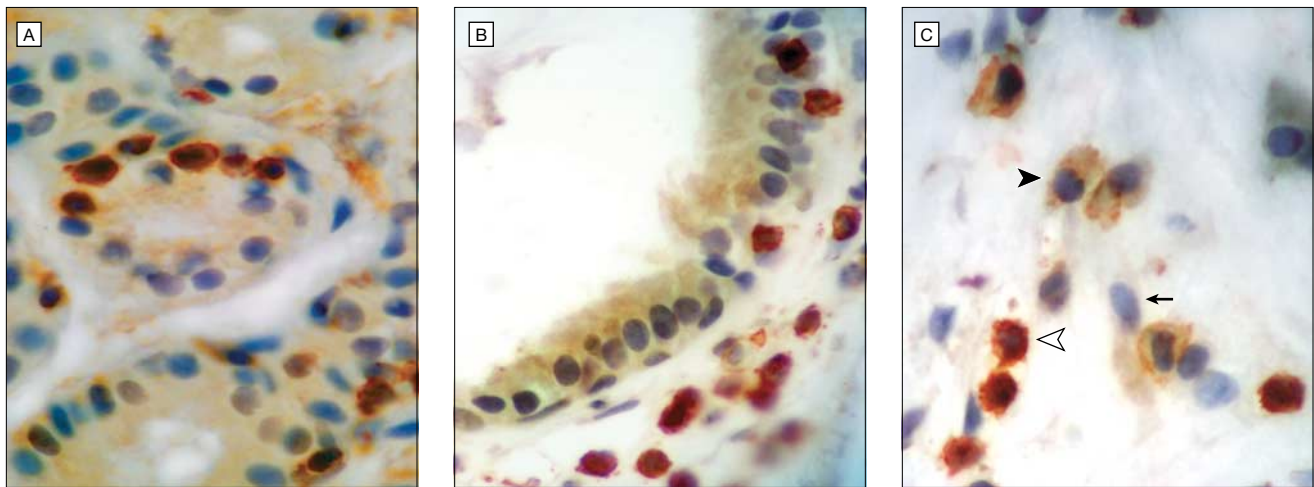
The considerable variation in the percentage of cells of donor origin likely depends on the length of time from treatment to biopsy, as well as mul-

tip factors that reflect tissue damage, loss of endogenous stem cells, and tissue repair by recruitment of bone marrow-derived cells; here, these factors include chemotherapy regimen, radiation dosage, graft-vs-host disease, and other endometrial conditions unrelated to the transplant. The woman whose endometrial sample demonstrated the most bone marrow-derived cells had received dual-agent chemotherapy and total body irradiation and showed evidence of moderate graft-vs-host disease.

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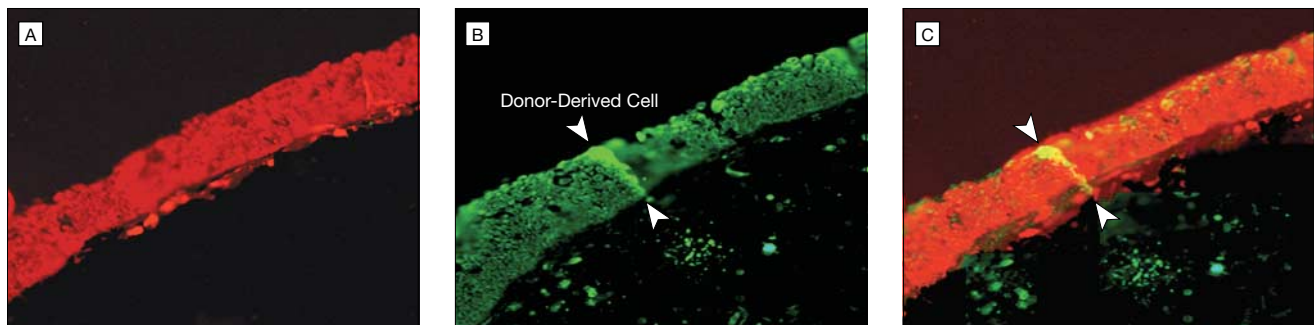
Endometrial ablative techniques in women are fraught with a high long-term failure rate and rarely result in complete long-term absence of endometrial tissue or abnormal endometrial bleeding; this treatment failure occurs despite apparent initial destruction or removal of all endometrial tissue. Conversely, repair of a damaged uterine cavity with little remaining endo-

Figure 3. Donor-Derived Endometrial Cells and Differentiation From Transient Leukocytes



Immunohistochemistry for HLA-A11 using anti-HLA-A11 monoclonal antibody, biotin peroxidase detection system, and diaminobenzidine as the chromogen (brown), and for leukocyte common antigen (CD45) using anti-CD45 monoclonal antibody, biotin peroxidase detection system, and Nova red as the chromogen (red). Hematoxylin was used as the counterstain. A, Control showing concordant HLA-A11-positive epithelial cells (brown) and CD45-positive leukocytes (brown and red) in a nontransplanted individual (original magnification $\times 200$). B, Endometrial glands of HLA-A11-positive donor origin (brown) in HLA-A11-negative transplant recipient (patient 1) and surrounding leukocytes (red). C, Endometrial stromal cells in HLA-A11-negative transplant recipient (patient 1) showing endogenous stromal cells (HLA-A11 negative/CD45 negative; blue [small arrow]); stromal cells of HLA-A11-positive donor origin (HLA-A11 positive/CD45 negative; brown [black arrowhead]); and leukocytes of donor origin (HLA-A11 positive/CD45 positive; brown and red [white arrowhead]) (original magnification $\times 400$).

Figure 4. Differentiation Shown by Immunofluorescence in Donor-Derived Cell in Endometrial Epithelial Layer



Immunofluorescence using tetra-rhodamine isothiocyanate for calcitonin expression (red) and fluorescein isothiocyanate for donor-derived cells (green) in endometrial epithelial cells of HLA-A11-negative transplant recipient (patient 1) who received bone marrow from an HLA-A11-positive donor. A, Calcitonin expression in endometrial epithelium indicative of receptivity to blastocyst implantation. B, Mismatched HLA-A11-positive endometrial epithelial cell of donor origin (arrowheads). C, Merge demonstrating HLA-mismatched cell expressing calcitonin as a marker of functional differentiation (original magnification $\times 100$).

metrium (Asherman syndrome) has a high success rate.²¹ Both of these clinical observations suggest that bone marrow–derived regeneration of endometrium has clinical significance.

Ectopic location of the endometrium is characteristic of endometriosis. Endometriosis is a common disease, occurring in approximately 15% of women, causing infertility and pelvic pain.²² The predominant theory for the origin of endometriosis is retrograde menstruation through the fallopian tubes, with ectopic implantation; however, this theory cannot explain foci of endometriosis outside the peritoneal cavity.^{23,24} A nonendometrial cir-

culating source of stem cells that can result in endometrial cells suggests an alternative origin of some endometriosis. In some instances, endometriosis may arise by differentiation of bone marrow–derived cells into endometrium in ectopic locations. There is an association between endometriosis and immune disorders, perhaps indicating that a single disorder may be common to ectopic bone marrow–derived transdifferentiation of endometrium and other immune phenomena.²⁵⁻²⁷ This hypothesis warrants further investigation.

These preliminary findings suggest that bone marrow–derived cells can

generate endometrium, which may have clinical implications for establishing and maintaining pregnancy, treating uterine disorders, and therapeutically augmenting stem cell transdifferentiation into endometrium.

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