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Clonogenicity of Human Endometrial Epithelial and Stromal Cells[1]

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ABSTRACT

The human endometrium regenerates from the lower basalis layer, a germinal compartment that persists after menstruation to give rise to the new upper functionalis layer. Because adult stem cells are present in tissues that undergo regeneration, we hypothesized that human endometrium contains small populations of epithelial and stromal stem cells responsible for cyclical regeneration of endometrial glands and stroma and that these cells would exhibit clonogenicity, a stem-cell property. The aims of this study were to determine 1) the clonogenic activity of human endometrial epithelial and stromal cells, 2) which growth factors support this clonogenic activity, and 3) determine the cellular phenotypes of the clones. Endometrial tissue was obtained from women undergoing hysterectomy. Purified single-cell suspensions of epithelial and stromal cells were cultured at cloning density (300–500/cm^2) in serum medium or in serum-free medium supplemented with one of eight growth factors. Small numbers of epithelial (0.22%) and stromal cells (1.25%) initiated colonies in serum-containing medium. The majority of colonies were small, containing large, loosely arranged cells, and 37% of epithelial and 1 in 60 of stromal colonies were classified as large, comprising small, densely packed cells. In serum-free medium, transforming growth factor-α (TGFα), epidermal growth factor (EGF), platelet-derived growth factor-BB (PDGF-BB) strongly supported clonogenicity of epithelial cells, while leukemia-inhibitory factor (LIF), hepatocyte growth factor (HGF), stem-cell factor (SCF), insulin-like growth factor-I (IGF-I) were weakly supportive, and basic fibroblast growth factor (bFGF) was without effect. TGFα, EGF, PDGF-BB, and bFGF supported stromal cell clonogenicity, while HGF, SCF, LIF, and IGF-I were without effect. Small epithelial colonies expressed three epithelial markers but not stromal markers; however, large epithelial colonies showed little reactivity for all markers except α_6-integrin. All stromal colonies contained fibroblasts, expressing stromal markers, and in some colonies, myofibroblasts were also identified. This analysis of human endometrium has demonstrated the presence of rare clonogenic epithelial and stromal cells with high proliferative potential, providing the first evidence for the existence of putative endometrial epithelial and stromal stem cells.

female reproductive tract, growth factors, menstrual cycle, uterus

INTRODUCTION

The human endometrium is the mucosal layer of the uterus comprising luminal and glandular epithelial cells, stromal fibroblasts, vascular smooth muscle cells, endothelial cells, and leukocytes. Epithelial cells form the surface epithelium and tubular glands that extend from the surface to the endometrial-myometrial interface. The endometrium is divided into two functional layers, the upper functionalis, which contains glands loosely held together by supportive stroma, and the lower basalis, consisting of branching glands and dense stroma [1, 2]. Both functionalis and basalis have been further subdivided into two morphologically distinct layers [1, 3, 4], although others consider the distinctions between the four layers less obvious based on protein expression and proliferative activity, and favor the concept of polarized microenvironments [2, 5].

The human endometrium has remarkable regenerative capacity, able to grow from an initial 0.5–1 mm following menstruation to 5–7 mm in thickness [3]. It is characterized by cyclical processes of cellular proliferation, differentiation, and breakdown as part of each menstrual cycle throughout the reproductive years. The growth of the new endometrial functionalis begins in the proliferative phase in response to rising levels of circulating estrogen [6]. Rising progesterone during the secretory phase blocks epithelial mitoses, and cells commence differentiation [3]. Stromal proliferation also reduces [6], and predecidualization begins around blood vessels and extends to the stroma beneath the surface epithelium. Upon implantation, endometrial stromal cells complete decidualization and terminally differentiate. In the absence of implantation, the functionalis regresses and is shed as menstruation proceeds, then a new cycle commences [3].

Regeneration commences during menstruation as the surface of the endometrium is rapidly covered by epithelial cells, which is complete within 48 h after shedding [3, 7–9]. Several theories have been put forward regarding the source of cells for human endometrial regeneration; however, specific mechanisms have yet to be elucidated. Stromal metaplasia as the source of cells for the new surface epithelium was suggested [10], but it has now been demonstrated that regeneration occurs from protruding stumps of basal glands [7], although glands from occasional patches of remnant lower functionalis also appear to contribute [3], thus supporting the basalis as the most likely source of cells for regenerating the new functionalis [1, 9]. Further evidence includes regrowth of endometrium able to support pregnancy in humans and primates following almost complete removal of the endometrium [1, 11].

Cycles of endometrial growth, differentiation, and regression are under tight regulation of ovarian sex steroid

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hormones. A role for estrogen has been demonstrated in postmenopausal women given estrogen replacement therapy, resulting in the regeneration of their atrophic endometrium [12]. Estrogen and progesterone also interact with locally produced growth factors in an autocrine and/or paracrine manner [13]. Endometrial epithelial cells synthesize and secrete several growth factors, including epidermal growth factor (EGF), transforming growth factor-α (TGFα), and insulin-like growth factor-I (IGF-I) [13]. Also, TGFα, IGF-I, basic fibroblast growth factor (bFGF), epidermal growth factor receptor (EGFR), and platelet derived growth factor-β (PDGFβ) receptor expression are increased in epithelial cells during rapid growth associated with the proliferative stage [13–15]. Hepatocyte growth factor (HGF), leukemia inhibitory factor (LIF), and stem-cell factor (SCF) also play important roles in endometrial cell proliferation, angiogenesis, and implantation [13, 16, 17] and may have roles in endometrial regeneration. These growth factors may also modulate the effects of estrogen or progesterone or each other by altering receptor expression [13, 18].

At present, it is becoming increasingly clear that stem cells exist in many, if not all, adult tissues [19]. In regenerative adult tissues such as bone marrow, intestine, and skin, rare populations of primitive, undifferentiated adult stem cells have been identified. Epithelial stem cells have been demonstrated in many epithelial tissues, where they have a role in normal tissue renewal or regeneration following damage, both in rapidly dividing tissues and in those with slow turnover such as liver, prostate, and pancreas [20]. Stromal or mesenchymal stem cells have been identified in bone marrow and dental pulp [21–23]. Adult stem cells are small populations of quiescent cells with the potential to regenerate the entire tissue in which they reside. They differ from embryonic stem cells in that they are not pluripotential because they lack the ability to produce all cell types present in the embryo and express different markers [24]. Adult stem cells are committed to a more specific lineage, although they still possess high proliferative potential and can persist for the lifetime of the individual in many human tissues. Adult stem cells undergo asymmetric cell divisions, which enable them to maintain their own population of undifferentiated cells (self-renewal), while also producing more differentiated progenitor daughter cells that are capable of rapid proliferation [25]. The progenitors and their daughter cells proliferate, producing progressively more differentiated and mature progeny, characterized by the expression of more surface markers [19]. These more mature cells, also known as transit amplifying cells (TA cells) are characterized by their limited proliferative potential and inability to self-renew but are readily observed in tissue sections by expression of proliferation markers [19]. The primary function of a stem cell is to produce large numbers of mature and functional cells that regenerate the tissue in which they reside. Adult stem cells are either unipotent, bipotent, or multipotent and able to reconstitute any part of a tissue by producing one or more of the component cells. For example, bone marrow stromal stem cells give rise to cartilage, bone, adipose tissue, and fibrous and myelosupportive tissue given the appropriate conditions [22].

Some years ago, it was proposed that human endometrium may contain a population of stem cells that are responsible for its remarkable regenerative ability [1, 9]. While adult stem cells have been discovered in a growing list of human tissues, there are no studies to date that have identified adult stem cells in endometrium. Adult stem cells are difficult to identify in tissues because they constitute very small populations of cells and possess few distinguishing surface markers. Alternate ways of identifying adult stem cells are to demonstrate their functional properties [26, 27]. Classically, adult stem cells are identified by their clonogenic activity, defined as the ability of a single cell to produce a colony when seeded at extremely low densities. This well-characterized functional activity of adult stem cells in vitro has been used to demonstrate their existence in various tissues [23, 28–32]. We hypothesized that a small population of both epithelial and stromal stem cells residing in the basalis layer of human endometrium would exhibit clonogenic activity in vitro. The aims of this study were to 1) examine the clonogenic activity of human endometrial epithelial and stromal cells, 2) identify the growth factors that support this clonogenic activity, and 3) determine the cellular phenotypes of the clones.

MATERIALS AND METHODS

Human Tissues

Endometrial tissues (n = 17) were collected from ovulating women aged 34–51 yr undergoing hysterectomy for fibroids or adenomyosis, who had not taken exogenous hormones for 3 mo before surgery. Informed written consent was obtained from each patient and ethics approval was obtained from the Monash Medical Centre Human Research and Ethics Committee B. The stage of the menstrual cycle was categorized into proliferative (n = 10) and secretory (n = 7) by experienced histopathologists, who assessed hematoxylin-eosin-stained endometrial sections using the criteria of Noyes et al. [33].

Tissue samples comprising endometrium attached to 5 mm myometrium were collected in HEPES-buffered Dulbecco modified Eagle medium/Hams F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA) with 1% antibiotic-antimycotic solution (final concentrations: 100 μg/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B; Invitrogen), and 5% newborn calf serum (NCS; CSL Ltd., Parkville, VIC, Australia), stored at 4°C, and processed within 2–18 h.

Preparation of Single Purified Cell Suspensions of Human Endometrial Epithelial and Stromal Cells

A single-cell suspension of endometrial cells was obtained using enzymatic digestion and mechanical means adapted from Gargett et al. [34]. The endometrium was scraped off the underlying myometrium, diced finely, and dissociated in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS, pH 7.4) containing 300 μg/ml collagenase type III (Worthington Biochemical Corporation, Freehold, NJ) and 40 μg/ml deoxyribonuclease type I (Roche Diagnostics, Mannheim, Germany) in a shaking incubator (Bioline 4700; Edwards Instrument Company, Narellan, NSW, Australia) rotating at 150 rpm at 37°C. At 15-min intervals, the digests were pipetted vigorously and dissociation was monitored microscopically. A fine slice of myometrium that forms the endometrial-myometrial junction was also digested separately to ensure that any penetrating endometrial glands and stromal tissue were also dissociated. After 45 min, the cell suspensions were filtered using a 40-μm sieve (Becton Dickinson Labware, Franklin Lakes, NJ) to separate single cells from debris and undigested myometrial tissue fragments. Further dissociation of the filtrate was prevented by the addition of HEPES-buffered DMEM/F-12/5%NCS. The sieves were backwashed to obtain myometrial and glandular fragments, which were further dissociated for 45–60 min as described above, to produce single-cell suspensions. Myometrial fragments were checked microscopically at 15-min intervals until all obvious penetrating glands were dissociated. Cell suspensions were filtered as above and combined. To remove erythrocytes, the cells were resuspended in 10 ml HEPES-buffered DMEM/F-12/5%NCS, underlaid with 2.5 ml Ficoll-Paque (Pharmacia Biotechnology, Uppsala, Sweden) and centrifuged for 8–10 min at 390 × *g*. Endometrial cells were removed from the Ficoll-Paque-medium interface, washed, and resuspended in a 1-ml volume HEPES-buffered DMEM/F-12/1%NCS.

Endometrial epithelial cells were obtained by positive selection using BerEP4-coated magnetic Dynabeads (Dynal Biotech, Oslo, Norway); the BerEP4 antibody shows specificity for both luminal and glandular epithelium in full thickness endometrium (see Fig. 7, A and B). The required number of BerEP4 beads was calculated assuming 50% of the cell suspension was epithelial and four beads/epithelial cell were incubated with

the cell suspension for 30 min at 4°C with end-over-end rotation. The beaded epithelial cells were recovered and washed several times in HEPES-buffered DMEM/F-12/1%NCS using a magnetic particle collector (Dynal Biotech) and the supernatant containing stromal cells and leukocytes also collected. The stromal cells were then negatively selected using anti-CD45 antibody-coated Dynabeads (Dynal Biotech) for removal of leukocytes as described above, assuming 50% of the remaining cells were leukocytes.

Endometrial Epithelial and Stromal Cell Clonal Culture

Beaded endometrial epithelial cells and purified endometrial stromal cells were seeded in triplicate at clonal density, 500 and 300 cells/cm^2, respectively, into 60-mm Petri dishes (Becton Dickinson Labware, Bedford, MA) coated with gelatin (Sigma-Aldrich, St. Louis, MO) for epithelial cells or fibronectin (10 μg/ml; Becton Dickinson Biosciences) for stromal cells and cultured in serum medium (SM), containing bicarbonate-buffered DMEM/F-12 medium, 10% FCS (CSL Ltd.), 2 mM glutamine (Invitrogen), and antibiotic-antimycotic. Endothelial cell growth factor, 20 μg/ml (Roche Diagnostics) was also included for epithelial cell culture in SM.

Epithelial and stromal cells were also cultured at clonal density in growth factor supplemented serum-free bicarbonate buffered DMEM/F-12 medium (SFM) containing 0.5% BSA (Invitrogen), insulin-transferrin-selenium-A (final concentrations: 6.7 ng/ml sodium selenite, 11 μg/ml sodium pyruvate, 10 μg/ml insulin, 5.5 μg/ml transferrin; Invitrogen), 50 μM 2-mercaptoethanol (Sigma-Aldrich), 100 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich), 100 μg/ml heparin (Sigma-Aldrich), 10 nM linoleic acid (Sigma-Aldrich), 2 mM glutamine, and antibiotic-antimycotic solution [35], with one of the following growth factors: PDGF-BB (PeproTech, Rocky Hill, NJ), longform EGF (GroPep, Adelaide, SA, Australia), bFGF (PeproTech), LIF (Chemicon, Temecula, CA), SCF (Chemicon), HGF (PeproTech), longform IGF-I (GroPep), and TGFα (PeproTech), all at 10 ng/ml final concentration. Epithelial cells were cultured on mitotically arrested mouse fibroblast feeder layers (see below) and stromal cells on fibronectin-coated dishes. All cultures were incubated for 15 days in a humidified CO_2 incubator at 37°C in 5% CO_2:95% air, and medium was changed every 3–4 days for epithelial cells and 6–7 days for stromal cells. Colonies were monitored microscopically on a daily basis to ensure that they were derived from single cells.

Mouse Fibroblast Feeder Layer for Clonal Epithelial Cell Culture in Serum-Free Medium

The mouse 3T3 fibroblast cell line obtained from American Type Culture Collection (ATCC, Manassas, VA) was cultured on gelatin-coated flasks for 7–10 days in $NaHCO_3$-buffered DMEM/F-12 high glucose medium containing 4 mM glutamine, 1% antibiotics, and 10% FCS. When 80% confluence was reached, 3T3 cells were treated with mitomycin C (10 μg/ml; Sigma-Aldrich) for 3–4 h at 37°C, washed, harvested with trypsin (Sigma-Aldrich), and seeded onto 20-cm^2 gelatin-coated culture dishes at 4×10^5 cells/plate. Feeder layers were incubated for 1 h at 37°C before seeding with human endometrial epithelial cells.

Cloning Efficiency

Cultures were terminated at 15 days by fixation in 10% formalin for 10 min after a PBS wash, then stained with Harris hematoxylin (Amber Scientific, Belmont, WA, Australia) for 5 min, washed with tap water followed by Scotts tap water, and dried. Clusters of cells were considered colonies when they were visible macroscopically and contained greater than 50 cells. Colonies were counted and the cloning efficiency (CE) determined from the formula CE (%) = (number of colonies/number of cells seeded) × 100. The number of cells per colony was determined using a 10 × 10 grid on an eyepiece graticule. A representative number of cells contained within the grid were counted from the center of the colony and also for the edges of the colony, where cells were loosely packed, then the number of grids/colony counted and multiplied by cell number/grid to give a total cell count per colony.

Immunohistochemistry

Endometrial BerEP4-beaded epithelial and stromal cells were seeded at 500 and 300 cells/cm^2, respectively, onto 25-mm fibronectin-coated Thermanox coverslips (Nalge Nunc International, Naperville, IL) in six-well dishes and cultured in SM or SFM for 15 days as described above. Coverslips were washed with PBS, cells fixed in acetone for 2 min, and then incubated with 0.3% hydrogen peroxide (Orion Laboratories Pty. Ltd., Welshpool, WA, Australia) to quench endogenous peroxidase, followed by protein blocking agent (Immunon Thermo Shandon, Pittsburgh, PA) for 10 min each at room temperature (RT). Primary antibodies were diluted in 0.1% BSA/PBS and incubated for 1 h at 37°C. Coverslips were then washed with PBS, followed by sequential 15-min incubations at RT with DAKO LSAB+ biotinylated secondary antibody and streptavidin horseradish peroxidase conjugate (DAKO Corporation, Glostrup, Denmark) and 5 min at RT with chromogen, AEC (Zymed, San Francisco, CA), or DAB (Sigma-Aldrich). Cells were counterstained with Mayer hematoxylin (Amber Scientific) for 30 sec. Coverslips were examined under a Zeiss microscope (Axioskop; Carl Zeiss, Oberkochen, Germany) and images were captured using a digital video camera (Fujix; Fuji, Tokyo, Japan).

Antibodies used were mouse anti-human cytokeratin (CK), 2 μg/ml (clone MNF116, specific for CK 5, 6, 8, 17, and 19; DAKO), mouse anti-human epithelial antigen, 4.7 μg/ml (clone BerEP4; DAKO), mouse anti-human epithelial membrane antigen, undiluted (EMA; clone GP1.4; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), and rat anti-human CD49f (α6 integrin), 10 μg/ml (clone GoH3; BD Pharmingen, San Diego, CA) to detect epithelial antigens; mouse anti-human fibroblast antibody, 3.1 μg/ml (clone 5B5; DAKO), mouse anti-human CD90, 4 μg/ml (Thy-1; clone 5E10; BD Pharmingen), and mouse anti-human collagen type I, 20 μg/ml (Chemicon) to detect fibroblast antigens; and mouse anti-human CD31, 6 μg/ml (clone JC70A; DAKO) and mouse anti-human α-smooth muscle actin (αSMA), 1.8 μg/ml (clone IA4; DAKO) to detect endothelial and smooth muscle cells, respectively, as well as mouse anti-human nuclei antibody, which reacts with proteins in the nuclei of all human cell types, diluted 1:50 (clone 235-1; Chemicon). For negative controls, primary antibodies were substituted with an isotype-matched IgG at the same concentration. Positive controls were cultured endometrial epithelial and stromal cells, myometrial microvascular endothelial cells, and smooth muscle cells.

Statistical Analysis

Cloning efficiency data were analyzed using GraphPad PRISM software (version 3.00; GraphPad Software Inc., San Diego, CA). Data was tested for homogeneity of variance using Bartlett test and was found significant, and therefore nonparametric tests were used. Kruskal-Wallis one-way analysis of variance followed by Dunn multiple comparison test was used for comparison of the growth factors. Wilcoxon signed rank test was performed to determine statistical significance between small and large colony cloning efficiencies. Data are presented as mean ± SEM. Results were considered statistically significant when $P < 0.05$.

RESULTS

Clonogenicity of Human Endometrial Epithelial and Stromal Cells in Serum Medium

When purified single-cell suspensions of human endometrial epithelial cells were seeded at cloning densities (500/cm^2) in SM, approximately 30% of the cells attached within the first 24 h of culture, distributing approximately 6–10 cells/field of view as observed by phase-contrast microscopy at 10× magnification. Some individual epithelial cells commenced proliferating after a lag period of 3–4 days, forming small clusters of 15–20 flat epithelial cells by 6–8 days in culture (Fig. 1C). Many of these colonies continued to expand over the 15-day culture period examined in this study. Figure 1A shows a typical cloning plate with randomly distributed and well-separated colonies after 15 days in culture. Two types of colonies were distinguishable: colonies small in size (Fig. 1D), comprising large, loosely associated cells (Fig. 1E); and large colonies, containing small, densely packed cells (Fig. 1, F and G). If cultured for longer periods, the large colonies would expand sufficiently to cover the plate. In addition to the individual cells that formed the large and small colonies, a significant proportion of attached epithelial cells formed small nests of 5–20 cells within 2–3 days of seeding that resembled epithelial cells when cultured at high densities

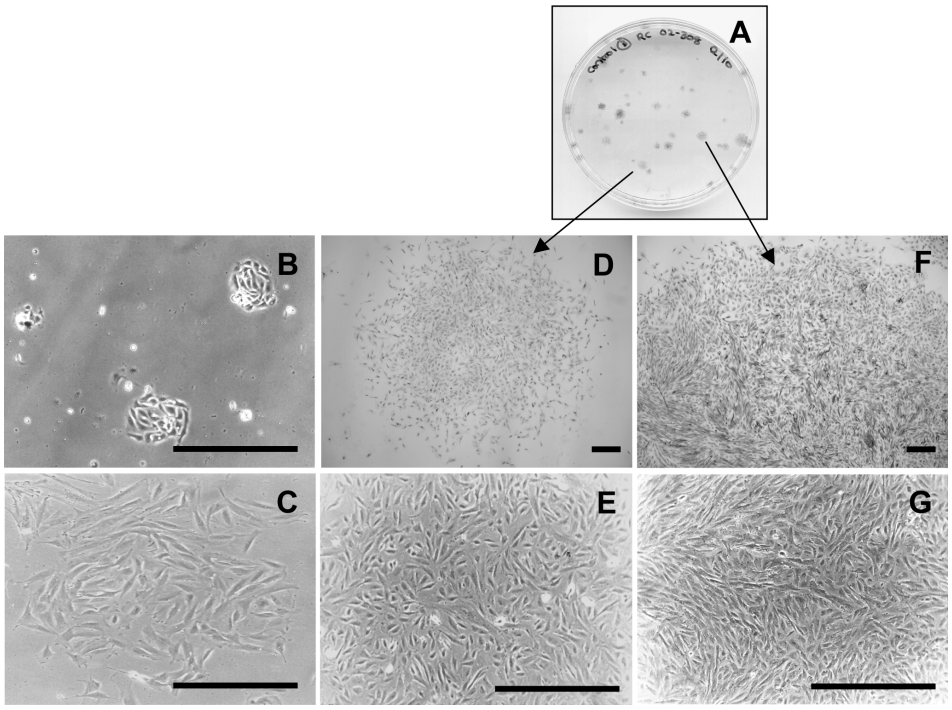

FIG. 1. Colonies formed by human endometrial epithelial cells seeded at clonal density and cultured in serum medium. **A**) Culture dish displaying the distribution of colonies and variation in colony size after 15 days of culture. Phase-contrast photomicrographs of (**B**) small nests of mature epithelial cells on Day 3, (**C**) a typical epithelial colony by Day 7, (**D**) a typical small colony, and (**E**) a magnified view of its center on Day 15, and (**F**) a typical large colony and (**G**) a magnified view of its center on Day 15. Scale bars = 200 μm.

(Fig. 1B) [36]. Individual cells within these colonies assumed a basic polyhedral shape, but by Days 8–10 in culture, most had become apoptotic and had lifted off the plate. We believe these clusters of cells were formed by mature epithelial cells and, because they were no longer present after 15 days in culture, they were not included in the cloning efficiency data.

Similarly, approximately 20% of purified human endometrial stromal cells seeded at cloning density (300/cm^2) in SM attached within the first 24 h of culture, with approximately three to four cells observed per field of view (10× magnification). Of the attached stromal cells, only a small number actually initiated colonies. Figure 2A shows a typical cloning dish with colonies of varying sizes ranging in number from 12 to 96 per dish. Similar to epithelial cells, two colony morphologies were observed: small colonies of loosely packed cells (Fig. 2, B and D) and large colonies, containing a dense center of tightly packed cells (Fig. 2E) with an overall swirly appearance (Fig. 2C) that was different from epithelial colonies. For the first week, the growth rate for the two colony types was similar, with colonies generally comprising less than 100 cells after 7 days. However, after 12–15 days in culture, the small colonies had either stopped proliferating or were slowly increasing in size, while around Days 10–12, the growth of a minority of colonies increased dramatically, and by Day 15 contained as many as 10 000 cells. Large colonies were only observed in some experiments (8/13).

We next examined the size distribution of these epithelial (Fig. 3A) and stromal (Fig. 3B) colonies by scoring 111 colonies from seven samples and 88 colonies from five samples, respectively, and sorting them into numerical order. Figure 3 shows that colonies could be categorized into two groups: colonies containing less than 4000 cells, which corresponded to small colonies, and large colonies containing more than 4000 cells. The data show that small colonies predominated after 15 days in culture; while large colonies were less common for both epithelial and stromal cells, there was a greater proportion of large colonies for epithelial compared with stromal cells. Cloning efficiencies were determined for both large and small colonies and are reported in this manner for all future experiments. Figure 4 shows the total (large plus small colonies) CEs for epithelial and stromal cells cultured in SM. Overall, the cloning efficiency of human endometrial epithelial cells from ovulating women was 0.22% ± 0.07% (n = 16) with 0.08% ± 0.03% for large colonies and 0.13% ± 0.04% for small colonies. There was no difference between cloning efficiencies for large and small epithelial clones ($P = 0.11$). The mean total CE for human endometrial stromal cells was 1.25% ± 0.18% (n = 13), which was significantly higher than epithelial cells ($P < 0.0001$) (Fig. 4). The CE for small stromal colonies was 1.23% ± 0.18% (n = 13) and was significantly more than obtained for large stromal colonies (0.02% ± 0.01%, n = 13, $P = 0.0002$).

A large variation in total cloning efficiencies was observed between patient samples, ranging from 0.08% to 0.45% for epithelial and 0.27% to 2.54% for stromal cells, almost a 6-fold and 10-fold variation, respectively. The cloning efficiency for epithelial cells from proliferative-phase endometrium (0.20% ± 0.09%, n = 10) was slightly lower but not significantly different from that of secretory endometrium (0.25% ± 0.12%, n = 6). For stromal cells, the cloning efficiency of proliferative-phase endometrium (1.38% ± 0.32%, n = 6) was greater than secretory phase (1.11% ± 0.21%, n = 7), although this difference was not statistically significant.

Clonogenicity of Human Endometrial Epithelial and Stromal Cells in Serum-Free Medium

Because culture medium containing FCS contains numerous unspecified components that may support cell growth or inhibit clonogenic activity [35], we undertook cloning assays in serum-free conditions to determine which growth factors supported clonogenic activity of human endometrial cells. Growth factors that have important roles in endometrial growth and function and/or have been suc-

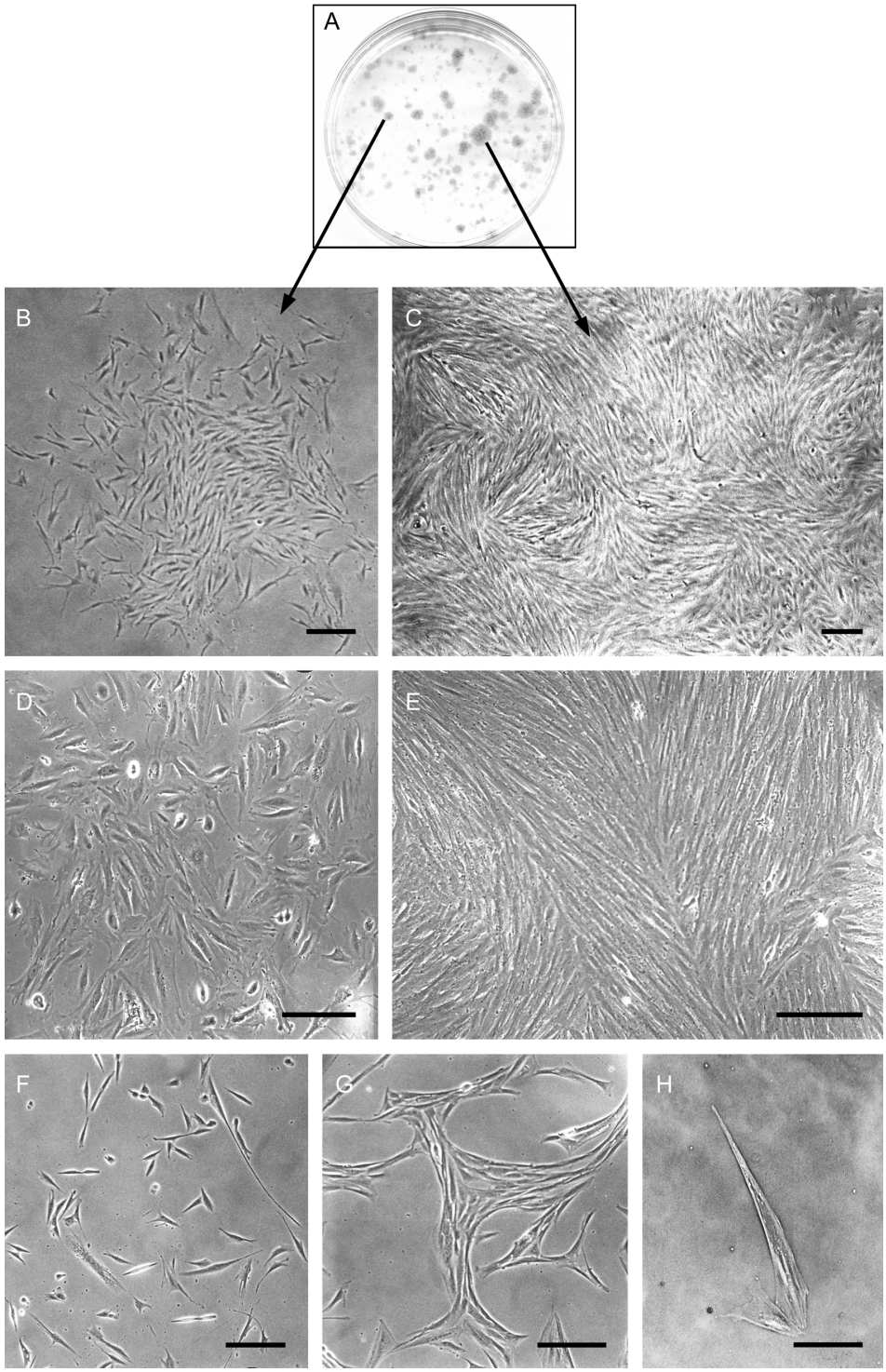

FIG. 2. Colonies formed by human endometrial stromal cells seeded at clonal density and cultured for 15 days. **A**) Typical appearance of a culture dish showing the distribution of colonies and colony size variation. Representative phase-contrast photomicrographs of a (**B**) small and (**C**) large colony, and a more magnified view showing the center of a (**D**) small and (**E**) large colony cultured in serum medium. Characteristic colony and cell morphology observed when cells were cultured in serum-free medium containing (**F**) PDGF, (**G**) TGFα, and (**H**) LIF. Scale bars = 200 μm.

cessfully used to expand other stem-cell populations [13, 35, 37] were chosen. Epithelial and stromal cells were seeded in triplicate in serum-free medium containing bFGF, PDGF-BB, TGFα, EGF, IGF-I, SCF, HGF, or LIF (each 10 ng/ml) and cloning efficiencies measured after 15 days in culture. Initially, epithelial and stromal cells were seeded on fibronectin-coated plates; however, epithelial cells did not survive in SFM and no colonies formed. Several investigators have previously shown that epithelial cells from other tissues only formed clones in SFM when cultured on fibroblast feeder layers, which provide stromal support and a more appropriate adult stem-cell niche in vitro [29, 30, 38]. This approach was adopted and human endometrial epithelial cells were cultured in SFM on mouse fibroblast feeder layers (Fig. 5A). Individual epithelial cells were difficult to locate during the first 6–8 days in clonal culture due to the sheet of feeder layer in the background, although attached Dynabeads aided their visualization. Some epithelial cells initiated colonies morphologically similar to those obtained in SM. The small colonies of this phenotype comprised of loosely packed cells with large cytoplasm and low nuclear:cytoplasmic ratio, forming a colony, which was generally circular in shape with a slightly irregular outline (Fig. 5, D and E). Large colonies formed a well-defined

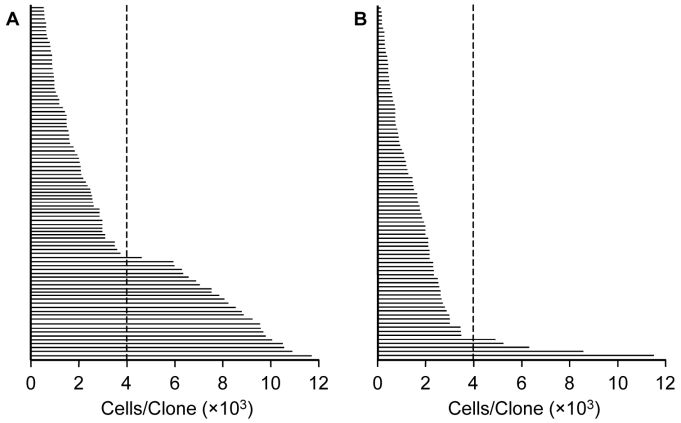

FIG. 3. Distribution of colony sizes of human endometrial (**A**) epithelial and (**B**) stromal cells cultured in serum medium for 15 days. The number of cells per colony from 111 epithelial colonies from seven samples and 88 stromal cell colonies from five samples were counted, with the data generated used to discriminate small and large colonies, as shown by the vertical line at 4000 cells.

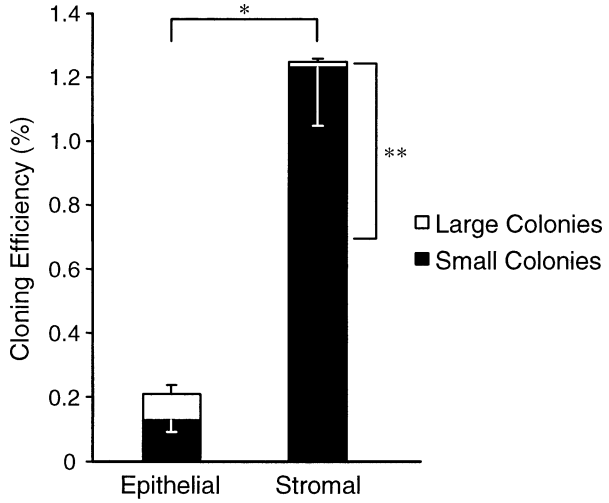

FIG. 4. The cloning efficiency of human endometrial epithelial and stromal cells cultured in serum medium. Each bar consists of the cloning efficiency for small (black bars) and large (white bars) colonies, which together represent the total cloning efficiency. Results shown are means ± SEM. Statistical significance between epithelial and stromal cloning efficiencies (* $P = 0.0001$) and between large and small stromal colonies (** $P = 0.0002$) is shown.

circular arrangement comprising small compact cells with a high nuclear:cytoplasmic ratio (Fig. 5, F and G). A third distinctive colony phenotype also formed. These resembled small colonies and comprised heterogeneous cell types, both large and small, and the colonies displayed a very irregular outline (Fig. 5B). In SFM, mature epithelial cells formed small, rosette-shaped clusters during the first 3 days in culture (Fig. 5C), which, unlike those in SM, persisted throughout the 15-day culture for all of the growth factors examined and even formed in the absence of growth factors. These clusters were not counted because they were too small (<50 cells), were not visible macroscopically, and were excluded from the cloning efficiency data.

The morphology of stromal colonies cultured in SFM generally resembled those grown in SM, except for some colonies from all samples cultured in either PDGF-BB, EGF, or TGFα-supplemented SFM. Figure 2F shows one of these colonies, where the stromal cells were round and granular compared with the more usual flatter fibroblast-type cells observed in SM (Fig. 2D); the colony structure was also very loose (Fig. 2G), with only the occasional tight core of cells. In contrast, bFGF produced dense colonies similar to those found in SM.

Growth Factors Supporting Clonogenicity of Human Endometrial Epithelial and Stromal Cells

Figure 6A shows the clonogenic activity of endometrial epithelial cells cultured in SFM with the individual growth factors compared with that obtained in SM. Three growth factors, TGFα, EGF, and PDGF-BB, were found to strongly support epithelial clonogenic activity with higher, although not significantly different, cloning efficiencies compared with SM, and four, HGF, SCF, LIF, and IGF-I, were similar to SM. Basic FGF did not support clonogenic activity because the cloning efficiency was similar to that in SFM without added growth factor (data not shown). The proportion of large colonies in SFM for all growth factors, excluding bFGF, was slightly increased compared with SM, although not significantly, and constituted approximately 43% of the total colony number. There was a significant increase in average total CE for SFM supplemented with TGFα compared with bFGF ($P < 0.05$).

Four growth factors, bFGF, EGF, PDGF-BB, and TGFα, were found to support stromal colony growth, although not to the same extent as in SM (Fig. 6B). HGF, SCF, IGF-I, and LIF did not support stromal cell clonogenicity, with cloning efficiencies ranging between 0.01% and 0.02%. Surviving individual cells appeared senescent when cultured in media containing these growth factors (Fig. 2H) and similar to stromal cells cultured in SM in the absence of growth factors (data not shown). The CEs for the large colonies were varied, 0.024% ± 0.01% (n = 11) for bFGF, 0.018% ± 0.004% (n = 11) for EGF, 0.015% ± 0.01% (n = 10) for PDGF-BB, and 0.004% ± 0.002% (n = 12) for TGFα. No large colonies were observed in HGF-, SCF-, IGF-I-, and LIF-supplemented SFM. Figure 6B shows that clonogenic activity for SM and bFGF-supplemented SFM were statistically greater than for HGF, LIF, SCF, and IGF-I ($P < 0.05$). Statistical significance was also found between SM and TGFα ($P < 0.05$), between PDGF-BB and LIF ($P < 0.05$), and between EGF and both IGF-I and LIF ($P < 0.05$).

Cellular Phenotype of Human Endometrial Epithelial and Stromal Clones

The cellular compositions of endometrial epithelial and stromal clones were examined by immunostaining with cell-type-specific antibodies to determine the phenotype of the progeny. Epithelial cell markers examined were BerEP4 and EMA, which detect epithelial cell adhesion molecule (EpCAM), cytokeratin (CK), and CD49f (α_6-integrin), and stromal cell markers CD90 (Thy-1) [39] and collagen type I [40]. In SM, small epithelial colonies contained cells positive for all epithelial markers (Fig. 7, D and G; data not shown for EMA and CD49f). However, large epithelial clones were negative for BerEP4, CK (Fig. 7, E and H), and also for EMA (data not shown), while CD49f displayed weak staining (Fig. 7F). Stromal markers, CD90 (Fig. 7I) and collagen type I (data not shown), were negative for both large and small epithelial colonies. Human epithelial clones cultured on mouse feeder layers in SFM were identified with anti-human nuclei antibody (Fig. 7, L and O). Similar to SM, small colonies cultured on feeders were pos-

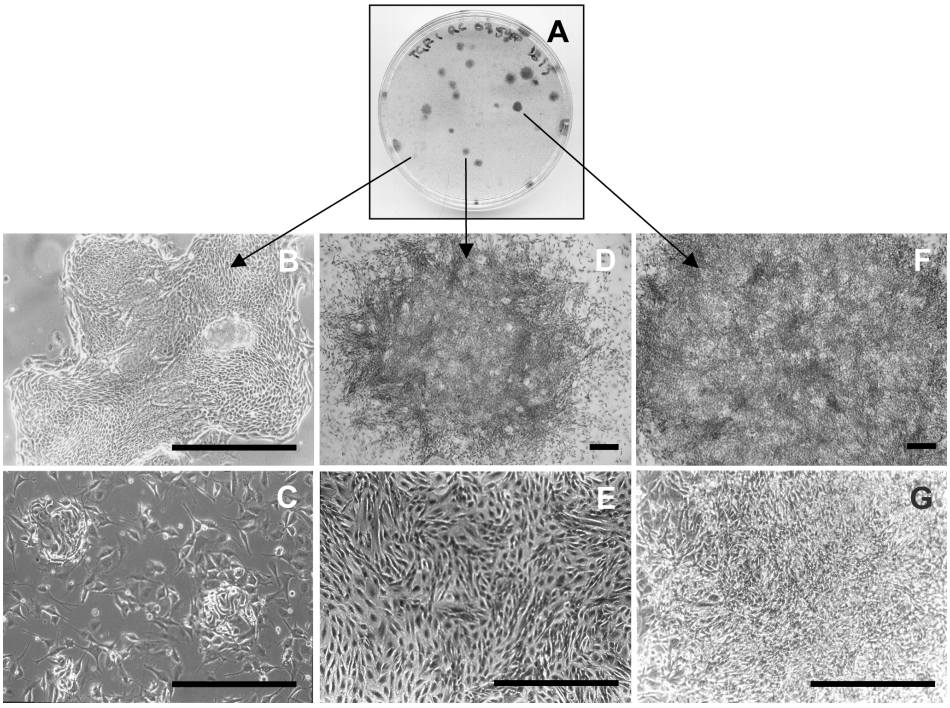

FIG. 5. Colony characteristics of human endometrial epithelial cells cultured for 15 days in serum-free medium on feeder layers. **A**) A culture dish displaying the distribution of colonies and variation in colony size, (**B**) a distinctive heterogeneous small epithelial colony comprising both large and small cells and found only in serum-free medium, (**C**) small, rosette-shaped clusters of mature epithelial cells, (**D**) a typical small colony and (**E**) a magnified view of the center showing large flat cells, (**F**) a typical large colony, and (**G**) a magnified view of the center showing small, dense cells. Scale bars = 200 μm.

itive for the epithelial markers BerEP4 (Fig. 7J) and CK (Fig. 7M), while large colonies did not react with BerEP4 (Fig. 7K) and were weakly stained with CK (Fig. 7N) or were negative (data not shown). In SFM, both small and large colonies were negative for the stromal markers.

In addition to the above markers, endometrial stromal colonies were examined for expression of markers for endothelial cells (CD31), smooth muscle cells, and myofibroblasts (αSMA), and fibroblasts (5B5, which has specificity for the beta subunit of prolyl 4-hydroxylase) [41]. All cells, from small (Fig. 8A) and large (Fig. 8B) stromal colonies in both SM and SFM stained strongly for the fibroblast markers 5B5, CD90 (Fig. 8E), and collagen 1 (results not shown). Some small (Fig. 8C) and large (Fig. 8D) stromal colonies contained αSMA-positive cells when cultured with some growth factors. The number of αSMA+ cells per colony ranged from 11.3% ± 2.3% for PDGF-BB to 46.1% ± 5.0% for EGF (Table 1). The percentage of αSMA-positive colonies varied from 17.1% for TGFα to 77.8% for PDGF-BB (Table 1). No stromal colonies immunostained with BerEP4 (Fig. 8F), cytokeratin (Fig. 8G), or CD49f (results not shown), except for the very occasional contaminating mature glandular epithelial cell not removed by the BerEP4 beads, which was positive for these markers. There was no CD31 staining (Fig. 8H).

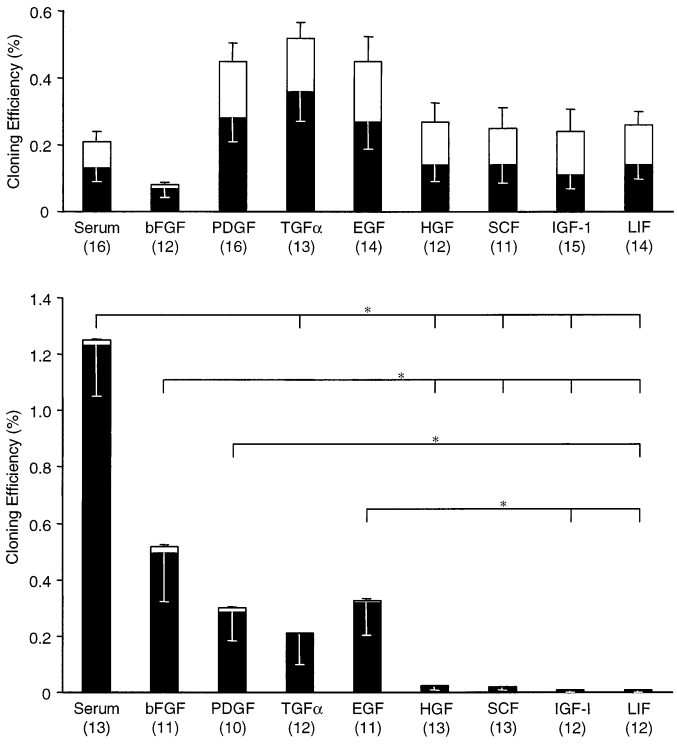

FIG. 6. Cloning efficiency of human endometrial (**A**) epithelial and (**B**) stromal cells cultured in serum-free medium containing one of eight growth factors. Results obtained in serum medium have been included for comparison. Results shown are means ± SEM. The numbers of samples for each culture condition are shown in parentheses. Statistical significance (* $P < 0.05$) between various culture conditions are shown.

DISCUSSION

Human Endometrial Epithelial and Stromal Cells Are Clonogenic In Vitro

The major finding of the present study was the discovery of a small population of human endometrial epithelial cells (0.22–0.52%) and stromal cells (1.25%) that possessed clonogenic activity in vitro. Two types of colonies were initiated by both epithelial and stromal cells: small, loosely arranged and large, densely packed. The majority of the colonies were small, and while approximately 37% of epithelial colonies were of the large phenotype, only 1 in 60 stromal colonies were large. A second major finding was that three growth factors, TGFα, EGF, and PDGF-BB, strongly supported both human endometrial epithelial and stromal cell clonogenicity. In addition, bFGF supported the greatest level of stromal cell clonogenic activity but was without effect on epithelial clonogenicity. Epithelial cells also required fibroblast feeders for clonogenic activity in

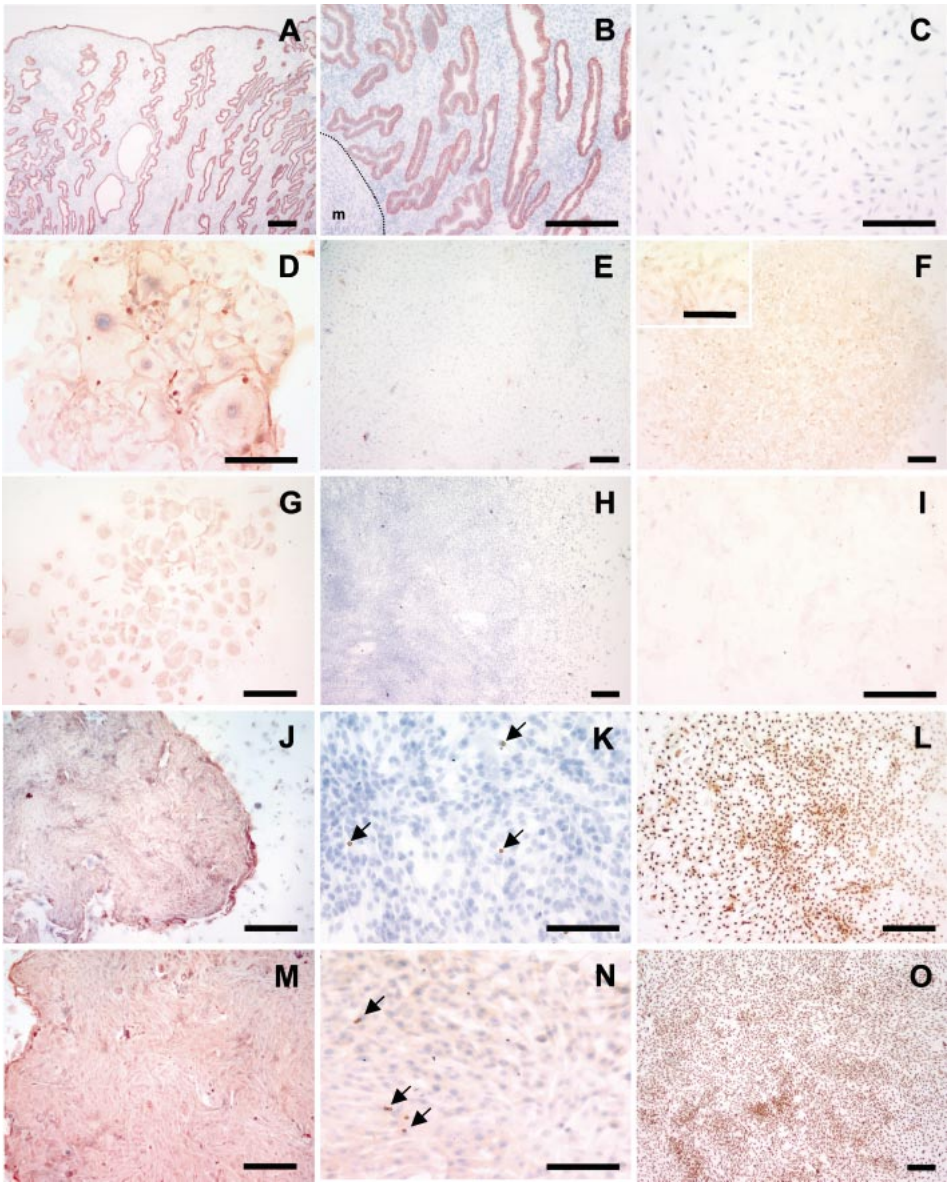

FIG. 7. Immunohistochemical phenotyping of the colony types initiated by human endometrial epithelial cells. Human endometrium in late secretory phase stained with the epithelial marker BerEP4 showing staining of (**A**) the surface epithelium and glands of the functionalis, and (**B**) glands in the basalis m, Myometrium. **C**) Typical isotype-matched negative control mouse IgG_1 of a small colony. Colonies cultured in serum medium were immunostained with epithelial markers BerEP4 on a (**D**) small and (**E**) large colony, (**F**) CD49f on a large colony, and cytokeratin on a (**G**) small and (**H**) large colony, and (**I**) fibroblast marker CD90 on a large colony. Colonies cultured in serum-free medium on feeder layer were immunostained with BerEP4 on a (**J**) small heterogeneous colony and (**K**) large colony, anti-human nuclei on a (**L**) small and (**O**) large colony, and cytokeratin on a (**M**) small heterogeneous and (**N**) large colony. The inset within **F** shows a magnified view of the center of the large colony. All colonies were counterstained with hematoxylin except **F**. Arrows indicate attached BerEP4 beads. Scale bars = 200 μm.

SFM. A third finding was that cloning efficiencies were highly variable between subjects for both epithelial and stromal cells. A fourth finding was that all small epithelial clones expressed epithelial markers; however, most large epithelial clones appeared to lose some epithelial phenotypic markers during in vitro culture. Both small and large stromal clones expressed fibroblast markers, and some large and small stromal colonies showed evidence of differentiation into myofibroblast lineage. Utilizing a powerful functional assay of stem-cell activity [26, 27] applied to many different tissues [21, 23, 29, 32, 42–44], our group is the first to establish epithelial and stromal clones from human endometrium, providing the first evidence for the existence of putative endometrial epithelial and stromal stem cells, some 20 yr after their existence was first postulated [1, 9, 10].

With no universal, specific markers available to distinguish adult stem cells from mature progeny and the paucity of stem cells in adult tissues, demonstration of functional stem-cell activity such as clonogenicity has typically been used to initially identify stem cells/progenitors in a wide range of adult tissues. Using this approach, the cloning efficiencies for epithelial cells from epidermis [30], prostate [45], and mammary gland [29] have been determined and are approximately 5%, which is 25 times higher than for human endometrial epithelial cells. However, a clonogenicity of 0.01% was observed in colon [46], much lower than endometrial epithelial cells, but similar to the percentage of large epithelial clones. Our data showed that 1 in 600–1250 endometrial epithelial cells formed large colonies with high proliferative potential. Although endometrial stromal cells produced a higher overall clonogenicity of 1.25%, only 1 in 1400–5000 formed large colonies, similar to endometrial epithelial cells. In comparison with the clonogenic activity of stromal cells in bone marrow, this is quite substantial, as only 1 in 10 000 are clonogenic [35]; however, this is less than dental pulp stromal cells, for which 1 in 140–500 form colonies. We are unaware of any studies examining clonogenicity or other stem-cell properties of stromal cells in tissues with a significant epithelial component. Our study of endometrium would suggest that these tissues may also have clonogenic stromal cells in their supporting stroma.

The present study has demonstrated the existence of two distinct types of endometrial epithelial and stromal colonies

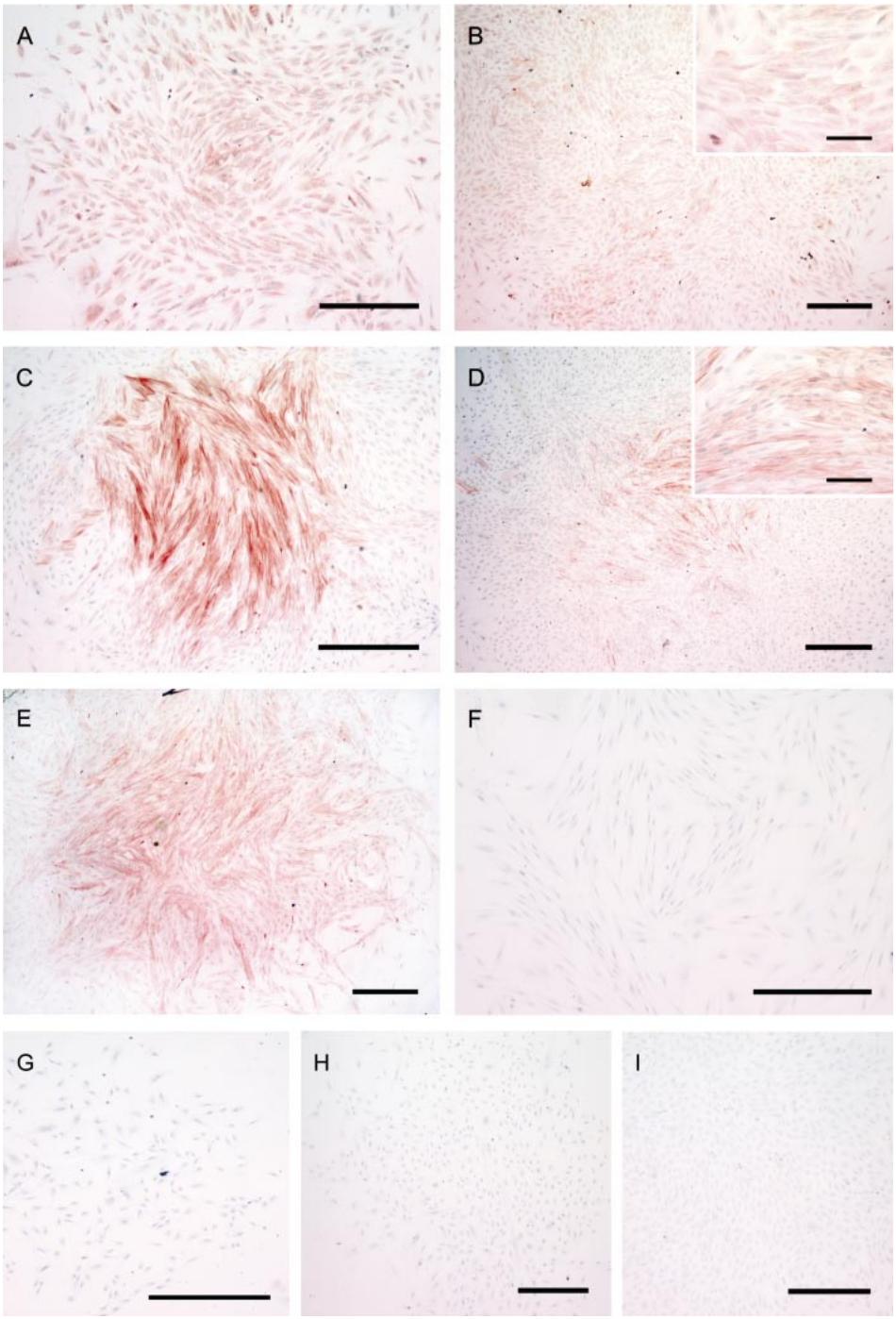

FIG. 8. Immunohistochemical phenotyping of large and small colonies initiated by human endometrial stromal cells. Colonies cultured in serum medium stained with fibroblast marker 5B5 on a (**A**) small and (**B**) large colony, smooth muscle cell marker αSMA on a (**C**) small and (**D**) large colony, (**E**) fibroblast marker CD90, (**G**) epithelial cell marker cytokeratin, (**H**) endothelial cell marker CD31, and (**I**) an isotype-matched negative control for αSMA mouse IgG_{2a}. **F**) A colony cultured in EGF-supplemented serum-free medium stained with an epithelial cell marker BerEP4. The insets within **B** and **D** show a magnified view of the center of the large colonies. Scale bars = 500 μm and 100 μm for insets.

in both SM and SFM. We speculate that the small, loosely arranged colonies of large-sized cells were initiated by putative endometrial epithelial or stromal TA cells, which are already committed to differentiate after a finite number of divisions [20] (Fig. 9). In contrast, we propose that the large, dense colonies of small cells, which were fewer in number and demonstrated high proliferation capacity, may have been initiated by a putative endometrial epithelial or stromal stem cell or committed progenitor, as shown in a diagrammatic representation of stem-cell hierarchy (Fig. 9). A common conceptual view of stem cells is their gradual loss of stem-cell properties (e.g., proliferative potential, clonogenicity) as they progress down the differentiation pathway (Fig. 9) [19]. Other evidence for the possible stem-cell nature of the endometrial epithelial and stromal cells initiating large colonies is their high nuclear:cytoplasmic ratio [47] and their ability to be recruited into the cell cycle when taken from their natural niche and placed into a favorable in vitro environment. The mouse feeder layer provided an optimal environment or appropriate stem-cell niche, as it appears that adjacent stromal cells assist with the proliferative function and survival of a range of epithelial stem cells [48]. Similarly, epithelial clones established from ocular epithelia and mammary tissue were facilitated by a feeder layer in SFM [29, 32], suggesting the importance of paracrine signaling [49] between putative epithelial stem and stromal niche cells in founding these large colonies that cannot be fully replicated in SM.

Cloning efficiencies varied greatly between individual samples for both epithelial and stromal cells. We hypothe-

TABLE 1. αSMA expression in stromal colonies from four endometrial samples.

Culture medium	Colonies with αSMA+ cells		αSMA+ cells per colony (% ± SEM)
	No. positive colonies/total	Percent (%)	
Serum	65/99	65.7	35.8 ± 4.0
bFGF	13/36	36.1	25.8 ± 7.9
PDGF	7/9	77.8	11.3 ± 2.3
TGFα	6/35	17.1	22.8 ± 15.7
EGF	35/71	49.3	46.1 ± 5.0

sized that cloning efficiencies may differ with stage of the menstrual cycle and thereby contribute to the overall variation observed. In particular, we postulated that the number of clonogenic endometrial cells would be highest in the proliferative phase, assuming that their number would be in steady state and remain constant throughout the menstrual cycle and they would be diluted by the increased number of cells present in a thicker secretory-phase endometrium. While stromal data demonstrated a trend for increased clonogenicity in the proliferative stage in both SM and SFM (unpublished results), the reverse was observed for epithelial cells. The lack of correlation between epithelial and stromal clonogenicity and menstrual cycle stage could be attributed to the broad classification of our samples into only two cycle stages due to low sample numbers and inherent individual variability of human tissues.

Identification of Growth Factors Supporting Human Endometrial Epithelial and Stromal Cell Clonogenicity

Three growth factors, TGFα, EGF, and PDGF-BB, provided major stimuli for epithelial and stromal clonogenic growth. TGFα and EGF bind to the EGFR [50] and significant levels are found in self-renewing epithelial tissues such as skin and gastrointestinal tract and endometrium [13]. Endometrial epithelial and stromal cells respond to both mitogens [13], but further work is required to determine whether EGFRs are expressed on clonogenic endometrial epithelial and stromal cells. EGF and PDGF regulate the proportion of quiescent G_0 cells entering the cell cycle [23]. The similar clonogenicity obtained for EGF, TGFα, and PDGF suggest these growth factors have equal capacity to recruit normally quiescent clonogenic endometrial epithelial and stromal cells into the cell cycle. However, we have yet to test growth factor combinations on clonogenic capacity of endometrial epithelial and stromal cells. EGF also supports the clonogenicity of breast, skin, and prostate epithelial cells [30, 31, 51] and bone marrow stromal stem cells [35], suggesting that EGF is a major mitogenic regulator for adult epithelial and stromal cell cloning activity. Clonogenicity of human endometrial stromal cells was greatest in bFGF containing SFM, which failed to support epithelial cell clonogenic activity. Basic FGF is predominately a mitogen for stromal cells because stromal but not epithelial cells express FGF-R1, suggesting that bFGF may act through this receptor to promote proliferation of clonogenic stromal cells.

Four growth factors, IGF-I, HGF, SCF, and LIF, were weakly supportive of clonogenic activity of endometrial epithelial cells but failed to support clonogenicity of endometrial stromal cells. While mature endometrial epithelial cells constitutively express receptors against each of these growth factors [17, 52–54], it is not known if they are expressed by epithelial colony-initiating cells. While IGF-I is

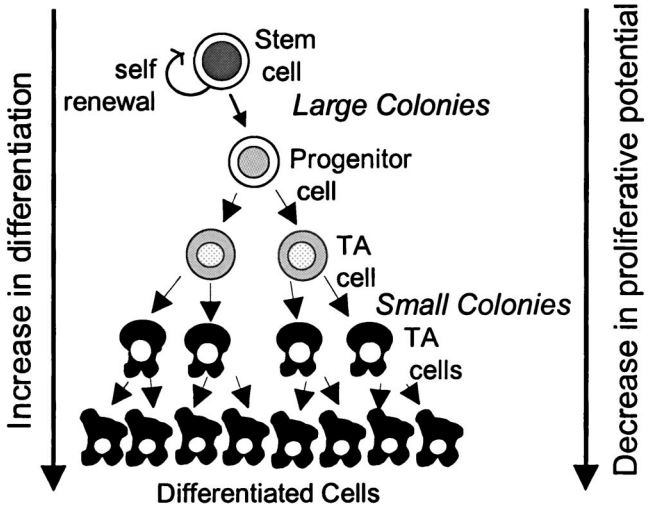

FIG. 9. Hierarchy of stem-cell differentiation showing possible relationship to large and small colonies initiated by epithelial and stromal cells. Stem cells are able to self-renew and replace themselves or differentiate to give rise to committed progenitors. These proliferate and give rise to more differentiated transit amplifying (TA) cells, which rapidly proliferate and finally differentiate to produce a large number of terminally differentiated functional cells with no capacity for proliferation. We postulate that the large colonies are initiated by putative stem/progenitor cells and the small colonies are initiated by putative TA cells.

a mitogen for many epithelial cell types [55], it appears less important for clonogenic endometrial epithelial cells, which may require exposure to a competence growth factor such as PDGF before IGF stimulation promotes movement through the cell cycle. IGF-I receptors may be lacking on clonogenic stromal cells because they are more ubiquitous on epithelial cells than on stromal cells [54]. HGF is important in modulating endometrial regeneration through stimulating proliferation of epithelial cells, which express c-Met, the HGF receptor [16]; but it is unknown whether clonogenic stromal or epithelial cells express c-Met, although the low cloning efficiencies in HGF medium suggest not. SCF is a hematopoietic stem-cell mitogen and LIF prevents mouse embryonic stem-cell differentiation, but both appear to function more in implantation [56] than in promoting the growth of clonogenic endometrial epithelial and stromal cells. The receptor for SCF, c-kit, is expressed on decidualized stroma [17], emphasizing its role in implantation and pregnancy.

Cellular Phenotype of Endometrial Epithelial Clones

The pseudostratified columnar epithelium of the endometrial surface, functionalis, and basalis all react with the epithelial markers BerEP4 (Fig. 7, A and B), CK [57], and CD49f [58]. The differing epithelial phenotypes of large and small clones suggest that the initiating cells have different properties. The progeny of cells initiating small colonies expressed the three epithelial markers, indicating their epithelial nature. The weak staining by CD49f and CK, together with the lack of BerEP4 expression in large colonies, suggest that these antigens are not expressed in undifferentiated cells in vitro [31]. We have observed that EpCAM detected by BerEP4 antibody appears to be lost during growth of the large epithelial colonies in culture (unpublished observations). An alternative explanation is that the large clones are stromal in origin. However, this is unlikely as there are five lines of evidence supporting the epithelial origin of the large BerEP4^{-}, CK$^{-/+}$, and CD49f^{low}

colonies. First, the clonogenicity of human endometrial stromal cells was extremely sensitive to prolonged collagenase exposure required to produce single epithelial cell suspensions, resulting in their loss of viability and failure to clone in culture. Second, we failed to reproduce similar colonies when stromal cells were cultured in SFM on feeder layers. Third, in SM, the clonogenicity for stromal cells was much higher (1.23% ± 0.18%) compared with epithelial cells and the proportion of large colonies was lower in both SM and SFM. Fourth, bFGF supported stromal but not epithelial clonogenicity. Finally, large epithelial clones did not express stromal phenotypic markers CD90 or collagen type I. Therefore, we conclude that these large colonies are not of a stromal origin. The loss of the epithelial markers on large clones requires further investigation because there is also the possibility that epithelial to mesenchymal transition has occurred, as shown for epithelial tumor cells of mammary tissues [59].

Cellular Phenotype of Endometrial Stromal Clones

Human endometrial stroma in both functionalis and basalis immunostains with fibroblast markers CD90 [39], 5B5 [41], and collagen type I [41]. These phenotypic markers confirmed that the progeny of cells initiating both large and small stromal colonies are predominantly composed of fibroblasts and suggests that some also differentiate into myofibroblasts, which express both fibroblast and smooth muscle cell markers [60]. Myofibroblasts are known to be present in the basalis [61]. Alternatively, these αSMA-expressing fibroblasts have differentiated into decidual stromal cells, which have a myofibroblast phenotype and contractile function [62]. The contractile function of myofibroblasts and decidual stromal cells is induced by PDGF and, in the present study, the greatest proportion of stromal colonies containing putative myofibroblasts were cultured in PDGF or serum-containing media. In either case, this suggests that clonogenic endometrial stromal cells can differentiate into another lineage, a property of multipotent adult stem cells. While we have demonstrated myofibroblast differentiation in some stromal colonies, further experimentation is required to determine whether clonogenic endometrial stromal cells have wider differentiation potential similar to that demonstrated for bone marrow and dental pulp stromal stem cells, which have the capacity to differentiate into chondrocytes, osteocytes, and adipocytes when cultured under certain conditions [21, 23, 35].

Having established that human endometrium contains a small number of clonogenic epithelial and stromal cells, exhibiting one stem-cell function in vitro, our next goal is to establish their stem-cell nature more definitively. It will be necessary to compare the proliferative potential of epithelial and stromal cells initiating large and small clones, as done for keratinocyte stem cells [63]. Clones established by limiting dilution will ensure they are initiated by single cells and these will be subject to serial cultivation until senescence is reached. We already have pilot data from limiting dilution analysis showing that small clones undergo up to 15–20 population doublings, while a large epithelial clone underwent 30, producing a total of 9×10^8 cells (unpublished observations). The capacity for self renewal is an important stem-cell property that needs to be examined in vitro by subcloning large and small clones as done for ocular epithelial cells [32]. Another key stem-cell property is the ability of putative stem-cell populations to reconstitute tissue in vivo, as has been done for intestinal epithelial cells [64]. The differential ability of large and small clones of epithelial and stromal cells to reconstitute endometrial glands and stromal tissue, respectively, when xenografted into immunocompromised mice would provide strong evidence that the large colony-initiating cells are adult stem cells in human endometrium. Only when the above criteria have been met can we definitively describe clonogenic endometrial epithelial and stromal cells as true endometrial stem cells.

Our original hypothesis was that the endometrial basalis contains a small population of epithelial and stromal stem cells and these would exhibit stem-cell activity. While this study has identified clonogenic cells in human endometrium, their location has not been determined. That the functionalis has higher proliferation indices for epithelial and stromal cells than the basalis [6, 65, 66] suggests that TA cells are possibly located in the functionalis and putative endometrial stem cells responsible for endometrial regeneration [1, 9] may be found in the relatively quiescent basalis. An alternative, less likely explanation is that the cytokine milieu of menstrual endometrium leads to dedifferentiation of epithelial and stromal cells in any remaining pockets of incompletely shed functionalis [3], which proliferate and form the new functionalis. The loss of differentiation molecules (EpCAM, CK) on epithelial cells in the large colonies and their high nuclear:cytoplasmic ratio may represent a form of dedifferentiation induced by in vitro culture, perhaps providing some support for the latter view.

The present study demonstrates that single-cell suspensions of endometrial cells generate colonies at very low seeding densities, as has been demonstrated in similar studies for a wide variety of tissues [23, 28, 29, 31, 32, 35, 43, 45, 51]. A more rigorous clonogenic assay is to perform limiting dilution analysis, which we undertook in pilot studies. Using this approach, we demonstrated that single cells initiated both epithelial and stromal colonies (unpublished observations). However, the low level of clonogenicity for endometrial cells and the large number of studies reported in the present study made the low seeding density a more feasible approach.

In conclusion, the present study is the first to demonstrate that human endometrium contains a small population of epithelial and stromal cells with clonogenic activity and the study has defined the isolation and culture conditions for their clonal analysis. We have demonstrated similarities and some differences in the properties of clonogenic endometrial epithelial and stromal cells; their incidence and their growth factor dependence for clonal growth. Our study suggests that signaling through the EGF and/or PDGF receptors is important in initiating proliferation of clonogenic epithelial cells, while EGF, FGF, and/or PDGF receptors have a role for clonogenic stromal cells. We have demonstrated that clonogenic stromal cells have multilineage differentiation potential, producing myofibroblasts and stromal fibroblasts. Together, the data herein provide the first evidence for the existence of putative epithelial and stromal stem cells in human endometrium.

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