Journal of Reproduction and Development, Vol. 62, No 3, 2016

—Original Article—

Growth factor induced proliferation, migration, and lumen formation of rat endometrial epithelial cells *in vitro*

Md. Rashedul ISLAM[1, 2], Kazuki YAMAGAMI[1], Yuka YOSHII[1] and Nobuhiko YAMAUCHI[1]

[1]*Department of Animal and Marine Bioresource Sciences, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan*

[2]*Department of Genetics and Animal Breeding, Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200, Bangladesh*

Abstract. Endometrial modulation is essential for the preservation of normal uterine physiology, and this modulation is driven by a number of growth factors. The present study investigated the mitogenic, motogenic, and morphogenic effects of epidermal growth factor (EGF) and hepatocyte growth factor (HGF) on rat endometrial epithelial (REE) cells. The REE cells were isolated and cultured and then characterized based on their morphology and their expression of epithelial cell markers. The MTT assay revealed that EGF and HGF induce proliferation of REE cells. Consistent with increased proliferation, we found that the cell cycle regulatory factor Cyclin D1 was also upregulated upon EGF and HGF addition. REE cell migration was prompted by EGF, as observed with the Oris Cell Migration Assay. The morphogenic impact of growth factors on REE cells was studied in a three-dimensional BD Matrigel cell culture system, wherein these growth factors also increased the frequency of lumen formation. In summary, we show that EGF and HGF have a stimulatory effect on REE cells, promoting proliferation, cell migration, and lumen formation. Our findings provide important insights that further the understanding of endometrial regeneration and its regulation.

Key words: Endometrial epithelial cells, Growth factors, Lumen formation, Migration, Proliferation, Rat

(**J. Reprod. Dev. 62:** 271–278, 2016)

The endometrium is composed of luminal and glandular epithelial cells, stromal components, and a closely associated extracellular matrix [1]. Endometrial cells, especially the luminal and glandular epithelial cells, deserve special attention due to their role in modulating the native physiology of the uterus [2]. The mitogenic, motogenic, and morphogenic regulation of endometrial epithelial cells is vital for preserving normal uterine physiology [3]. Although endometrial proliferation is estrogen driven, it is also mediated by a number of growth factors through autocrine and/or paracrine signaling [4]. Furthermore, the estrogen-induced proliferation of endometrial epithelial cells is poorly understood. Previous studies suggest that a number of growth factors control the proliferation of the endometrium [5]. For example, epidermal growth factor (EGF) and hepatocyte growth factor (HGF) are known as potent stimulators of proliferation in many cell types, namely, fibroblasts, keratinocytes and epithelial cells [6]. Epidermal growth factor receptors (EGFR) and hepatocyte growth factor receptors (c-MET) in the endometria of rats [3], humans [5], and mice [4], dimerize upon ligand binding, and trigger several signaling pathways [7]. When activated, these signaling pathways promote the transition of cells from G0 to G1, and to a lesser extent from G1 to S phase, resulting in epithelial cell proliferation and survival [3, 5].

Previous studies found that the migration of endometrial epithelial cells was also induced by growth factors. For example, HGF, a pleiotropic, mesenchymal-cell derived growth factor, has a motogenic effect on epithelial cells through regulating their interaction with mesenchymal cells [8, 9]. It was also reported that the motogenic effects of HGF on epithelial cells included disruption and scattering of epithelial colonies, as well as increased cell motility [10]. Furthermore, HGF induced migration of human endometrial epithelial cells has also been observed [5]. Like HGF, EGF also has a motogenic effect on human keratinocytes and rat intestinal epithelial cells [11–13].

Growth factors are indispensable for repair and morphogenesis in the tissues that produce them [14]. For example, HGF appears to play a critical role in restoration of the liver and kidneys [15–17]. HGF also stimulates the formation of epithelial tubules *in vitro* [18], and triggers lumen formation in human endometrial epithelial cells [5]. On the other hand, endometrial epithelial cells were reported to produce EGF and EGF receptors, and therefore EGF may have a morphogenic effect on epithelial cells [3–5].

Due to the impracticalities of studying the human endometrium *in vivo*, a number of animal models, especially rodent models, are employed to study the molecular events underlying endometrial functions. Fortunately, although there are abundant disparities among species, the self-governing nature of endometrial modulation is widely conserved. At present, most of the studies of human endometrial function are based on commercially available cell lines. Therefore, the uses of rat endometrial epithelial cells can potentially further our understanding of endometrial functions. It is now well documented that EGF, HGF and their receptors (EGFR and c-MET) are expressed

Received: December 1, 2015
Accepted: February 13, 2016
Published online in J-STAGE: March 4, 2016

Correspondence: N Yamauchi (e-mail: ny0531@agr.kyushu-u.ac.jp)

and temporally regulated in response to mitogenic, morphogenic, and motogenic stimulation of epithelial cells [3–5]. Earlier studies suggested that a combination of EGFR and c-MET activation resulted in signaling by multiple receptor tyrosine kinases (RTKs) and that these signaling pathways could be initiated by each receptor or the combined activation of both receptors [7]. Both EGFR and c-MET are expressed in endometrial epithelial cells [3–5], and both play vital roles in endometrial function. Therefore, we investigated the effect of EGF, HGF, and a combination of EGF and HGF, on the proliferation, migration, and lumen formation capacity of rat endometrial epithelial cells.

Materials and Methods

Animals

Wistar strain rats aged 10 to 12 weeks (200–250 g) were raised at the Laboratory of Reproductive Physiology and Biotechnology, Department of Animal and Marine Bioresource Sciences, Graduate School of Agriculture, Kyushu University, Japan. The rats were housed under temperature- and light-controlled conditions (lights on at 0800 h, off at 2000 h) with free access to food and water. The stages of the estrus cycles in each rat were determined by vaginal smear. Adult female rats were mated with males, and the day on which spermatozoa were found on the vaginal smear was designated as 0.5 days post coitus (dpc). Finally, female rats were used for endometrial epithelial cell isolation, as well as uterine tissue analysis, at 1.5 dpc. All animal experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals (Graduate School of Agriculture, Kyushu University, Japan) with the approval of the Kyushu University Laboratory Animal Care and Use Committee.

Isolation and culture of rat endometrial epithelial (REE) cells

According to the protocol previously developed in our laboratory [19], rat endometrial epithelial (REE) cells were isolated from uterine horns at 1.5 dpc. The uterine lumens were filled with phosphate buffered saline (Dulbecco's PBS (–); Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% collagenase (Worthington Biochemical Corporation, Lakewood, NJ) and incubated at 37°C for 45 min in a shaking water bath. The dissociated cells, including both rat endometrial epithelial (REE) cells and rat endometrial stromal (RES) cells, were washed with the basic culture medium Phenol red-free Dulbecco's modified Eagles medium with Hams F-12, 1:1 (v/v) (DMEM/Hams F-12; Nacalai Tesque, Kyoto, Japan) containing 10% charcoal-stripped fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA), and 1% Antibiotic-Antimycotic Mixed Stock Solution (Nacalai Tesque). Then, the cell suspension was plated onto 35 mm culture dishes, and allowed 1 hour of pre-incubation in a humidified atmosphere of 5% CO_2 at 37°C. After pre-incubation, non-attached REE cells were collected and counted using a hemocytometer. Then, 1×10^4 cells were seeded in each well of 96-well dishes (Corning, Corning, NY, USA) coated with BD Matrigel (BD Biosciences, San Jose, CA, USA). Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Culture medium was changed every two days.

Immunocytochemistry and immunohistochemistry

Cultured REE cells were characterized according to their morphology (by phase contrast microscopy) and by an indirect immunofluorescence staining method [20]. Cultured cells were fixed for 5 min in neutral buffered formalin (NBF); after a PBS wash, they were subjected to cold methanol (at –20°C) treatment for 10 min. After another PBS wash, nonspecific antibody binding was blocked by incubating cells in 2% (v/v) goat serum in PBS (blocking buffer) for 30 min. Cells were incubated at 4°C overnight with mouse anti-Cytokeratin antibody (C2931, Sigma-Aldrich, St. Louis, Missouri, USA), rabbit anti-Vimentin antibody (V6630, Sigma-Aldrich), rabbit anti-Desmin antibody (AM31980PU-S, Acris Antibodies, San Diego, USA), and mouse anti-Von Willebrand Factor (VWF) antibody (AM08419PU-N, Acris Antibodies), each diluted 1:200 in blocking buffer. The specificity of the immunofluorescence staining was confirmed by staining with secondary antibodies in the absence of primary antibodies. After a PBS wash, cells were incubated for 1 h at room temperature with the secondary goat anti-mouse IgG (H+L), F (ab) 2 fragment (Alexa Fluor 488 conjugated) antibody (1:200; Cell Signalling Technology) and Alexa Fluor 594 conjugated goat anti-rabbit IgG (H+L) antibody (1:200; Invitrogen, Carlsbad, CA) diluted in blocking buffer. Nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI; EX013, DOJINDO, Tokyo, Japan). Cells were subsequently washed in PBS and immunostaining was detected using a Nikon Ti-U inverted fluorescence microscope (Nikon, Tokyo, Japan).

For immunohistochemistry, uterine tissues were collected from the uterine horns of rats at 1.5 dpc, embedded in an optimum cutting temperature (OCT) compound (Sakura Finetek Japan, Tokyo, Japan), and frozen immediately in liquid nitrogen. The samples were cut into 7 μm sections with a Leica CM1950 cryostat (Leica Biosystems, Nussloch, Germany) and placed onto MAS coated glass slides (Matsunami Glass, Osaka, Japan). After air-drying, the sections were subjected to immunostaining, following the procedure described earlier in this section, with the exception that methanol treatment was not performed.

Total RNA isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from REE cells using an RNeasy Mini Kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions and a previously published protocol [20]. RNA quality was assessed by spectrophotometric UV absorbance at 260/280 nm using a BMe-spect2 (BM Equipment, Tokyo, Japan). RNA samples were reverse transcribed and quantified by PCR (qPCR) with a GoTaq 2-step RT-qPCR System (Promega, Madison, WI, USA) using an oligo-dT primer and a random primer. The cDNA was synthesized according to customized reaction conditions (i.e., annealing for 5 min at 25°C, extension for 1 h at 42°C, and heat inactivation). All the reagents for reverse transcription were purchased from Promega, unless otherwise specified. The expression of epidermal growth factor receptor (*EGFR*) and hepatocyte growth factor receptor (*c-Met*) in REE cells was examined using RT-PCR. The PCR reaction mix was heated to 94ºC for 2 min, then subjected to 39 cycles of 94ºC for 30 sec, 60ºC for 30 sec, and 68ºC for 40 sec. The last cycle of the PCR reaction included an additional 3 min at 68ºC. *GAPDH* amplification was used as a reference. Then, the PCR products were analyzed by electrophoresis in 1.5% agarose gel and the images were captured.

Table 1. List of primers used for reverse transcription PCR (RT-PCR) and real time quantitative PCR (RT-qPCR)

Name	Nucleotide sequences (5'–3')	Length (bp)	GenBank accession no.
Gapdh	F-AACCTGCCAAGTATGATGACATCA	111	NM_017008.3
	R-TGTTGAAGCCGCAGGAGACAACCT		
Egfr	F-GATTAATCCCGGAGAGCCAGAG	415	NM_031507.1
	R-AGTTGGACAGGACGGCTAAG		
C-Met	F-AGTCCTACATTGATGTCCTGGG	315	NM_031517.2
	R-GGCTGGCCCCTATTTGCTTA		
Cyclin D1	F-CTCTTCGCACTTCTGCTCCTC	109	NM_171992.4
	R-GCCCTCCGTTTCTTACTTCAA		

F, Forward; R, Reverse.

All reagents for PCR were purchased from Promega, and all primers from Sigma-Aldrich, unless otherwise specified. The details of the primers used in this study are presented in Table 1.

Proliferation assay

The biological effects of EGF and HGF on rat endometrial epithelial cell proliferation were determined using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) based cell growth determination kit (CGD-1; Sigma-Aldrich) according to the protocol of Sugawara *et al*. [5]. REE cells were isolated, and 1×10^4 cells were seeded in each well of 96-well dishes coated with BD Matrigel. After 24 h of pre-incubation, basic culture media was replaced by serum free media containing 1 ng/ml of recombinant rat EGF (PEPROTECH, Princeton Business Park, Rocky Hill, NJ, USA), 10 ng/ml of recombinant rat HGF (PEPROTECH), or a combination of 1 ng/ml of EGF with 10 ng/ml of HGF. After growth factor addition, the culture was continued for 120 hours with the culture medium being changed every two days. The MTT assay was subsequently initiated to investigate the proliferation of REE cells. The MTT assay measures the mitochondrial dehydrogenase activity of living cells as a proxy for cell viability. According the manufacturer's instructions, MTT solution was aseptically added to the culture in an amount equal to 10% of the culture volume, and incubated for 3 to 4 h. After the incubation period, MTT solvent was added to the culture in an amount equal to the original culture volume. Culture dishes were then gently stirred, and their absorbance was spectrophotometrically measured at a wavelength of 562 nm. Their background absorbance was measured at 630 nm and then subtracted. In this assay, the number of living cells is proportional to the amount of MTT cleaved into insoluble formazan, which was detected spectrophotometrically at 562 nm.

Migration assay

Rat endometrial epithelial cell migration was investigated using an Oris^{TM} Cell Migration Assay kit (Platypus Technologies, Madison, WI, USA). REE cells were isolated, seeded in specially designed 96-well dishes (1×10^4 cells per well), and analyzed according to the manufacturer's instructions. Briefly, after equilibration of plates at room temperature, Oris^{TM} cell seeding stoppers were placed in, and firmly sealed to, the 96-well plate. The Oris^{TM} migration mask was then applied to the bottom of the 96-well plate. The cell suspension (1×10^4 cells in basic culture media) was then pipetted into each well of the 96-well plate, and the plate was incubated for 16 h in a humidified atmosphere with 5% CO_2 at 37°C to permit cell attachment. After incubation, Oris^{TM} cell seeding stoppers were removed (except for a reference well) using the Oris^{TM} stopper removal tool. The culture medium was then replaced with fresh culture media containing 1 ng/ml of EGF, 10 ng/ml of HGF, or a combination of 1 ng/ml of EGF with 10 ng/ml of HGF, and the plates were incubated for 3 h in a humidified atmosphere with 5% CO_2 at 37°C. The cells were then stained using the indirect immunofluorescence staining method described earlier, and counted and imaged using a Nikon Ti-U inverted fluorescence-microscope. The migration was compared to a reference well where no cell migration occurred because the Oris^{TM} cell seeding stopper was not removed.

Lumen formation

To test the effect of growth factors on lumen formation by REE cells, a three-dimensional cell culture system was established according to a previously published protocol (with little modification) [5]. Briefly, REE cells were suspended in BD Matrigel and culture media containing 1 ng/ml of EGF, 10 ng/ml of HGF, a combination of 1 ng/ml of EGF with 10 ng/ml of HGF, or no additional growth factors. Cells (1×10^4 cells/well) were seeded in each well of a 96-well plate coated with BD Matrigel, and were allowed to grow in culture for at least 3 weeks in a humidified atmosphere of 5% CO_2 at 37°C. The morphology of cells within each culture was analyzed by phase-contrast microscopy using a Nikon light microscope (Nikon, Tokyo, Japan). The number of lumens formed under each condition was quantified, and phase-contrast images were taken at 10 × magnification. Experimental cell cultures with EGF and/or HGF added were compared to control cell cultures with no EGF or HGF added.

Cell recovery and Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

Cultured REE cells were recovered from the 96-well BD Matrigel plates using BD cell recovery solution (BD Biosciences, San Jose, CA). Briefly, cultures were washed with ice cold PBS and incubated on ice with BD cell recovery solution. The cell/gel layer was then removed from the plates by scraping. The resulting cell suspension was transferred to an Eppendorf tube, and cells were rinsed 2 or 3 times by repeated centrifugation (× 300 *g* for 5 min) and resuspension in fresh BD cell recovery solution (150 µl). The rinsed cells were

finally resuspended in PBS, and their total RNA was purified for quantification and reverse transcription as described earlier.

The expression of the cell cycle regulatory gene Cyclin D1 (*Ccnd1*) was analyzed following growth factor addition by reverse transcription and quantitative polymerase chain reaction (RT-qPCR). Specific primer sequences and the sizes of resulting PCR products for the reference and target gene are shown in Table 1. Quantitative real-time PCR was performed using the GoTaq qPCR Master Mix (Promega) and an Mx3000P qPCR System (Agilent Technologies, Santa Clara, CA, USA). PCR amplification was conducted with an initial 2 min step at 95°C, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. The fluorescent SYBR Green signal was measured immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold. GAPDH served as an internal control and was used to normalize for differences in each sample. All the reagents used for qPCR were purchased from Promega.

Statistical analysis

Each experiment was repeated at least four times. In each case, the mean of the control was compared with the mean of the experimental condition using a paired Student's *t*-test, and a P-value less than 0.05 ($P < 0.05$) was considered significant.

Results

Morphological and immunological characterization of rat endometrial epithelial cells

In the present study, rat endometrial epithelial (REE) cells were isolated and cultured on BD Matrigel. The REE cells in culture were predominantly polygonal in shape, as observed by phase-contrast microscopy (Fig. 1A). Furthermore, REE cells formed follicles in culture that featured cobblestone morphology (Fig. 1B). The cultured REE cells were further characterized by immunocytochemistry using an indirect immunofluorescence method (Fig. 1). An epithelial-cell specific mouse anti-Cytokeratin antibody produced clear labeling of the cytoskeleton of the REE cells (Fig. 1C), but neither rabbit anti-Desmin antibodies (Fig. 1E) nor mouse anti-Von Willebrand Factor antibodies (Fig. 1F) labeled these cells. Surprisingly, these cells expressed Vimentin, which was detected by a rabbit anti-Vimentin antibody (Fig. 1D). In support of the immunocytochemistry results, we further performed immunohistochemistry of *in vivo* rat uterine sections (1.5 dpc) using an indirect immunofluorescence method to validate the observed labeling of the cultured REE cells (Fig. 1), as well as to characterize the different compartments of the rat uterus. Immunohistochemistry revealed that the epithelial cell specific mouse anti-Cytokeratin antibody only labeled luminal and glandular epithelial cells (Fig. 1G). On the other hand, the rabbit anti-Vimentin antibody, rabbit anti-Desmin antibody, and mouse anti-Von Willebrand Factor antibody labeled the stroma (Fig. 1H), myometrium and perimetrium (Fig. 1I), and blood vessels (Fig. 1J), respectively. For all our experiments, the specificity of the antibodies was confirmed by control staining with secondary antibody in the absence of primary antibodies (data not shown).

Growth factor effects on in vitro proliferation and cell cycle regulation

The effects of the growth factors EGF and HGF on *in vitro* proliferation, as well as the regulation of cell cycle regulatory factors, are summarized in Fig. 2. Initially the expression of *EGFR* and *c-Met* in REE cells was examined using RT-PCR followed by 1.5% agarose gel electrophoresis of the amplified products. The amplification yielded fragments consistent with the expected sizes of 415 bp for *EGFR* (Fig. 2A), 315 bp for *c-Met* (Fig. 2B), and 111 bp for the reference *GAPDH*. The mitogenic effects of EGF and HGF on cultured rat endometrial epithelial cells were then determined using an MTT assay. The assay revealed that a combination of EGF and HGF (1 ng/ml of EGF and 10 ng/ml of HGF) significantly ($P < 0.05$) increased the light absorption at 562 nm when compared with a control group without added growth factors (Fig. 2C). We also examined the levels of mRNA encoding Cyclin D1, an important regulator of cell cycle progression, using reverse-transcription and quantitative real-time PCR. Although the mRNA levels showed some changes upon treatment with 1 ng/ml of EGF or 10 ng/ml of HGF, the differences were not statistically significant when compared to the control. On the other hand, Cyclin D1 mRNA expression significantly increased ($P < 0.05$) upon simultaneous addition of 1 ng/ml of EGF and 10 ng/ml of HGF, compared with the untreated control group (Fig. 2D).

Effects of growth factors on in vitro migration of REE cells

The effects of EGF and HGF on REE cell migration were investigated using an Oris™ Cell Migration Assay kit (Fig. 3). It was observed that addition of 1 ng/ml of EGF significantly increased the number of cells that migrated into the center of the well ($P < 0.05$) compared to the control group without added growth factors. Although addition of 10 ng/ml of HGF, or a combination of EGF and HGF (1 ng/ml and 10 ng/ml, respectively), also had a tendency to increase REE cell migration, the differences were not statistically significant when compared with the control (Fig. 3A). Furthermore, immunocytochemistry revealed that the cells that had migrated were epithelial cells, based on labeling with an epithelial cell specific mouse anti-Cytokeratin antibody (merged image; Fig. 3B). On the other hand, no cells were observed in the center of the control wells following staining with mouse anti-Cytokeratin and DAPI (merged image; Fig. 3C).

Morphogenic effect of growth factors on REE cells

To examine the effects of EGF and HGF on the morphology and number of lumens formed in culture by REE cells, a three-dimensional BD Matrigel cell culture system was used. The changes in cell morphology were analyzed based on the parameters of cell clustering (Fig. 4A), and the number of lumen formed (Fig. 4B). The number of lumen formed under each growth factor treatment condition was compared with the number formed in the control condition without added growth factors. The data revealed that EGF and HGF each had stimulatory effects on lumen formation, and a combination of both significantly increased ($P < 0.05$) the number of lumen formed compared with the control. Although 1 ng/ml of EGF or 10 ng/ml of HGF individually had positive effects on the number of lumen formed, these were not statistically significant when compared to the control (Fig. 4C).

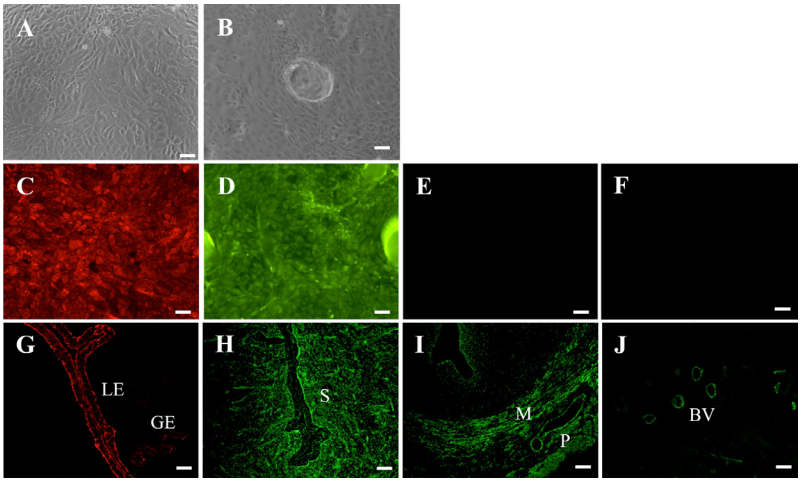

Fig. 1. Morphological and immunological characterization of rat endometrial epithelial (REE) cells. The purity of the isolated and cultured REE cells was determined by examining their morphology using phase-contrast microscopy, where these cells showed had a polygonal structure typical of epithelial cells (A). Additionally, REE cells formed follicles and displayed cobblestone structure (B) in culture. Cultured cells (C–F), and uterine sections as controls (G–J), were stained with mouse anti-Cytokeratin antibody (C, G), rabbit anti-Vimentin antibody (D, H), rabbit anti-Desmin antibody (E, I), or mouse anti-Von Willebrand Factor antibody (F, J). LE, luminal epithelium; GE, glandular epithelium; S, stroma; M, myometrium; P, perimetrium; BV, blood vessels. Scale bars indicate 50 μm.

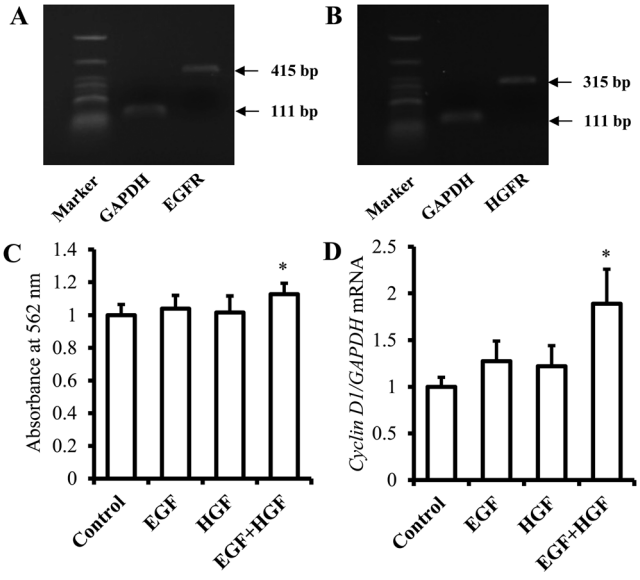

Fig. 2. Growth factor dependent *in vitro* proliferation of REE cells and regulation of Cyclin D1. Detection of *EGFR* (A) and *c-Met* (B) mRNA in REE cells by RT-PCR. The expected product sizes from *EGFR* and *c-MET* amplification were 415 bp and 315 bp, respectively. *GAPDH* (111 bp) was used as a reference. (C) The effect of growth factors on *in vitro* proliferation of REE cells. The absorbance was spectrophotometrically measured at a wavelength of 562 nm, and the background absorbance was measured at 630 nm and then subtracted. The absorbance was compared with the control, and expressed as mean ± SEM (n = 5). (D) Quantitative real-time PCR analysis of Cyclin D1 expression. The expression of the mRNA was normalized to the expression of *GAPDH* mRNA, measured from the same RNA preparation. The experimental concentrations of the growth factors were 1 ng/ml of EGF and 10 ng/ml of HGF, and the control did not have either growth factor added. The results are expressed as the mean ± SEM (n = 5) of each condition normalized against the control. Error bars show SEM. * indicates $P < 0.05$.

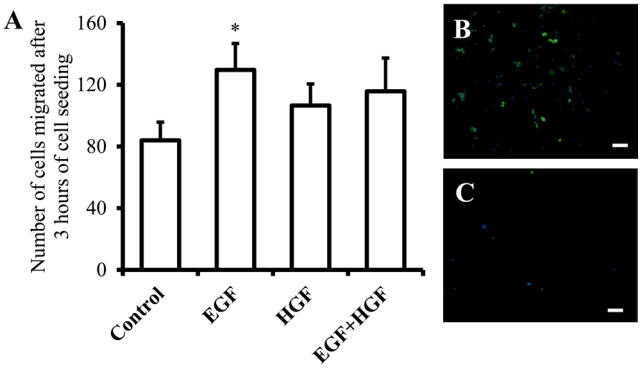

Fig. 3. Effect of EGF and HGF on the migration of REE cells *in vitro*. (A) The migration of each group was investigated using an Oris™ Cell Migration Assay kit. The experimental concentrations of the growth factors were 1 ng/ml of EGF, and 10 ng/ml of HGF, whereas the controls did not have added growth factors. The migration was compared with the reference (non-migrated) culture where the Oris™ cell seeding stoppers were not removed. The mean number of migrated REE cells under each condition was compared against the control (n = 4). Error bars show SEM. * indicates $P < 0.05$. Then the migrated and non-migrated cells were stained with an indirect immunofluorescence method using anti-Cytokeratin antibody (Green), and their nuclei were stained with DAPI (Blue). Representative merged images show anti-Cytokeratin antibody and DAPI staining of cells (B-migrated, C-non-migrated reference). Scale bar indicates 50 μm.

Discussion

In this study, REE cells were isolated, cultured, and characterized. The mitogenic, motogenic, and morphogenic effects of EGF and HGF on these cells were studied. REE cells were isolated and cultured on BD Matrigel, a commercially available basement membrane

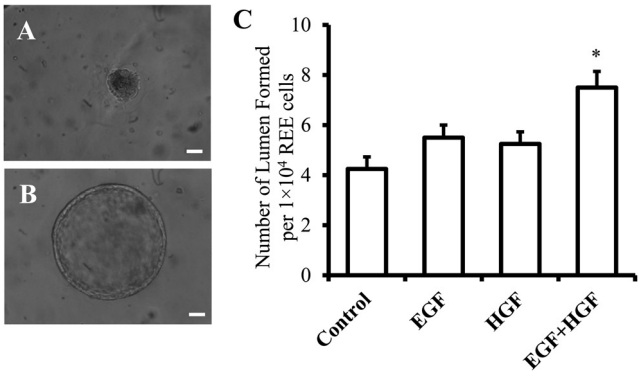

Fig. 4. Effect of growth factors on lumen formation by REE cells in a three dimensional BD Matrigel cell culture system. REE cells were suspended in BD Matrigel and culture media containing 1 ng/ml of EGF or 10 ng/ml of HGF, or a combination of 1 ng/ml of EGF and 10 ng/ml of HGF, or neither growth factor in the case of the control. Phase contrast microscopy was used to compare changes in morphology of the cells on the basis of cell clusters (A) followed by lumen formation (B) in each treatment. Representative images are shown. Scale bars indicate 50 µm. The number of lumens formed in each treatment was compared to the control (C). The results are expressed as the mean ± SEM (n = 4) for each condition. Error bars show SEM. * indicates $P < 0.05$.

comprised of Laminin, Collagen IV, matrix proteins, and a number of growth factors and proteases [21]. It is now well established that epithelial cell morphology, and tissue specific gene expression, are related to communication with a basement membrane *in vivo*. A number of studies revealed that REE cells were quite difficult to culture *in vitro* due to the lack of an appropriate basement membrane. However, the introduction of BD Matrigel made it possible to culture not only endometrial, but also breast [22, 23], and prostate [24] epithelial cells. In our study, cultured REE cells showed the expected appearance of epithelial cells, and assembled the characteristic multicellular structures. Thus, it was apparent that the cultured cells were specifically epithelial cells, in agreement with the findings of a previous study [19]. A typical epithelium is comprised of a sheet where individual epithelial cells are kept tightly bonded with their neighbors in an undeviating arrangement, and thus prevented from leaving the monolayer [25]. Furthermore, the expected cobblestone morphology of cultured REE cells was also observed in this study. This special feature of epithelial cells is not yet fully understood, but is observed frequently in the cultures of both animal and human epithelial cells [25, 26]. Once we had examined the morphology of the REE cells, we further characterized them using an indirect immunofluorescence staining method. Immunostaining revealed that Cytokeratin was expressed in these cells. This was further validated by immunostaining of rat uterine sections, where the luminal and glandular epithelial cells were also positive for Cytokeratin. It is now widely accepted that Cytokeratin proteins are markers of epithelial cells, and their extensive family consists of at least 20 members [27, 28]. Out of these 20 members, Cytokeratins 7, 8, 18, and 19 were detected in the endometrium during the proliferative phase [28]. Interestingly, cultured REE cells were also positively stained for the mesenchymal marker Vimentin, and this was likewise validated by staining of rat uterine sections. This phenomenon was not observed in our previous study [19] when REE cells were cultured, but this might be due to differences in culture conditions of our present investigation, in terms of number of the number of cells used and the times required for cultures to reach confluence. Based on the morphology of the cells, we conclude that our cultures only contained epithelial cells. In support of our conclusion, a number of other studies have shown that cultured epithelial cells express Vimentin, including cells of human, bovine, and ovine origin [29–32]. From these previous findings, and our current observations, it is obvious that this phenomenon of epithelial cells presenting mesenchymal features might be biologically important. Further study will be needed to understand the significance of this phenotype in human and animal endometrial epithelia.

In this investigation, REE cells were treated with the growth factors EGF and HGF to uncover their effects on proliferation, migration, and lumen formation. EGF and HGF are thought to regulate many biological processes, namely growth, motility, wound healing, and differentiation, while also playing critical roles in safeguarding the epithelium [33]. Thus, progress has been made in identifying growth factor and receptor pairs that might regulate the function of epithelial cells. A number of studies have shown that c-Met and EGFR signaling can influence multiple biological processes, either when the receptors are independently activated, or when they are simultaneously activated to produce an integrated signal [7]. In our study, EGFR and c-Met were expressed in REE cells, in agreement with the findings of previous studies in the rat, human, and mouse endometrium [3–5]. These receptors were also found in human corneal epithelial cells [34], biliary epithelium [35], and mammary epithelial cells [7]. Upon binding with their target ligands, the receptors dimerize and activate a number of signaling pathways. The pathways they activate are reported to contribute to several biological processes, such as proliferation, survival, and motility [7]. Once expression of the receptors in REE cells was verified, the cells were treated with EGF and HGF, and their proliferation was measured in an MTT assay. The assay revealed that the growth factors both had a stimulatory effect on proliferation. Further, we observed that the effect of a combination of both growth factors was much greater than the effect of each individually. Previous studies using mouse and human endometrial and corneal epithelial cells suggested that individual growth factors had a significant, dose-dependent, proliferative effect [4, 5, 34]. It has also been reported that EGF mediates estrogen-induced proliferation of uterine epithelial cells [4], while other report has shown that transforming growth factor (TGF-α), insulin-like growth factor-I (IGF-I), and heparin-binding EGF-like growth factor (HB-EGF), are associated with the proliferation of the uterine epithelial cells [36]. Thus, the co-expression of EGF and HGF receptors in REE cells, and the increase in proliferation upon addition of both factors, suggests that the combined activation of multiple receptors may be important. This interpretation is also in agreement with a previous finding in mammary epithelial cells [7], and thus coordinated receptor co-activation might have significant effects on cell biology in multiple contexts. The proliferation of rat endometrial epithelial cells is affected by a combination of growth factors, whereas Madin-Darby canine kidney (MDCK) cells are insensitive to c-Met activation, indicating that not all cell lines that

express c-Met react similarly to HGF stimulation [37].

Growth factor receptor activation triggers a number of signaling pathways that control the rate of transition from G0 to G1, and the transition from G1 to S phase, resulting in epithelial cell survival and proliferation [3]. The cell cycle regulatory factor Cyclin D1 also plays a predominant role in the regulation of proliferation, connecting the extracellular signaling environment to cell cycle progression [38]. Consistent with this, in our study Cyclin D1 expression increased upon growth factor treatment, concurrent with the increased proliferation of REE cells. However, the regulation of Cyclin D1 expression is complicated and not completely understood [39]. Cyclin D1 expression levels are highly responsive to proliferative signals involving growth factor receptor activation, the resulting activation of Ras, and their downstream effectors. The levels of Cyclin D1 protein are also regulated by the rate of production, rate of translation, and stability of its mRNA. The Cyclin D1 protein is post-translationally regulated by degradation and localization. The expression level of Cyclin D1 increases after stimulation of dormant cells to re-enter the cell cycle, and it was also found to shuttle in and out of the nucleus during the cell cycle [40]. It has also been proposed that the expression level of Cyclin D1 is regulated by mitogens in the extracellular environment, which uncovers one probable relationship between mitogenic signaling and cell cycle progression. Thus, the increased proliferation of REE cells was likely directly influenced by the upregulation of Cyclin D1 upon EGF and HGF treatment, consistent with the known role of Cyclin D1 in regulating cell cycle progression and proliferation [39]. Previous study revealed that the overexpression of Cyclin D1 is associated with breast, hepatocellular, esophageal, head, neck, squamous cell, and thyroid carcinomas [40]. Other cyclins have also been implicated in tumorigenesis [41]. Thus, it is important to understand the regulation of Cyclin D1 in REE cells during proliferation, as this may further our understanding of the role of Cyclin D1 in epithelial cell cancers.

In our study, we examined several biological effects of EGF and HGF on cultured REE cells. In addition to proliferation, we examined migration using an Oris^{TM} Cell Migration assay kit. The assay revealed that EGF significantly increased migration by REE cells, in agreement with previous findings in human keratinocytes and rat intestinal epithelial cells [11, 12, 42]. Although HGF protein affected both the growth and motility of human endometrial epithelial cells in another study [5], we did not observe a significant effect of HGF on REE cell migration. It is well known that each growth factor induces specific signaling pathways that affect the migration of cells. For example, in a study of human gastric carcinoma cells lines, both EGF and HGF treatment affected cell migration significantly, but treatment with a combination of EGF and HGF did not [14]. Thus, the findings of this present study are in agreement with the findings in gastric carcinoma cell lines. Migration is important in many morphogenic processes, such as mammary gland development, which is also triggered by growth factors [43]. One study found that the EGF stimulation cooperated with HGF stimulation to induce migration in HC11 cells [43]. Migration of epithelial cells involves the movement of individual cells, or cell sheets or clusters from one place to another. This characteristic phenomenon has significance in various pathological and physiological processes including wound healing, cancer, inflammation, cell growth, and cell differentiation [44]. However, limited information is available regarding the migration of epithelial cells in the endometrium.

Three-dimensional (3D) cultures are a valuable tool for better understanding tissue morphogenesis, as well as the pathogenesis of cancer [45]. Because 3D cultures mimic the normal environment of epithelial cells, they make it possible to examine the tissue or organ specific behaviors of these cells. Three-dimensional cultures of mouse endometrial epithelial cells have also been described, and in these cultures the cells adopt a morphology similar to their morphology *in vivo*. Beyond endometrial epithelial cells, most 3D cultures have been constructed using non-transformed but immortalized cell lines such as MDCK or MCF-10 [45]. In this study, to determine the morphogenic effects of EGF and HGF, a 3D BD Matrigel cell culture system was used. Under these conditions, the cultured cells first clustered and then formed lumens. This behavior was consistent with previous reports of human endometrial epithelial cells in culture [5]. We quantified the number of lumen formed under different conditions, and found that treatment with a combination of EGF and HGF caused cells to produce a significantly higher number of lumen than either growth factor alone. Although limited information is available regarding the morphogenic effects of growth factors on endometrial epithelial cells, one study on human endometrial epithelial cells showed that HGF had a significant effect on lumen formation in a dose dependent manner [5]. The study thus suggested that HGF might be an important mediator that triggered the reconstruction of endometrial glandular elements. However, the role of a combinatorial effect of growth factors still awaits definition in the context of endometrial epithelial cells. The role of lumen formation by epithelial cells in the endometrium is also not understood, although previous study suggested that this phenomenon might be associated with post-menstruation repair and reconstruction of an endometrium appropriate for implantation and pregnancy [5]. The mechanisms of endometrial lumen formation, and the role of the lumen in implantation and pregnancy, need further investigation.

In conclusion, our study demonstrates that both EGF and HGF stimulate proliferation, migration, and lumen formation by REE cells *in vitro*. Furthermore, we have shown that EGF and HGF trigger expression of the cell cycle regulatory factor Cyclin D1, which likely drives REE cell proliferation. Overall, our results provide new insights into mechanisms that may be critical for the regulation of endometrial regeneration, and these findings will inform future studies addressing this process.

Acknowledgements

This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (grant 25660214 and 26292141).

References

1. **Zhang YL, Davis DL.** Morphology of luminal and glandular epithelial cells from pig endometrium grown on plastic or extracellular matrices. *J Anim Sci* 2000; **78**: 131–138. [Medline]
2. **Davis DL, Blair RM.** Studies of uterine secretions and products of primary cultures of endometrial cells in pigs. *J Reprod Fertil Suppl* 1993; **48**: 143–155. [Medline]
3. **Haining RE, Cameron IT, van Papendorp C, Davenport AP, Prentice A, Thomas**

EJ, Smith SK. Epidermal growth factor in human endometrium: proliferative effects in culture and immunocytochemical localization in normal and endometriotic tissues. *Hum Reprod* 1991; **6**: 1200–1205. [Medline]

4. **Shiraga M, Komatsu N, Teshigawara K, Okada A, Takeuchi S, Fukamachi H, Takahashi S.** Epidermal growth factor stimulates proliferation of mouse uterine epithelial cells in primary culture. *Zoolog Sci* 2000; **17**: 661–666. [Medline] [CrossRef]
5. **Sugawara J, Fukaya T, Murakami T, Yoshida H, Yajima A.** Hepatocyte growth factor stimulated proliferation, migration, and lumen formation of human endometrial epithelial cells *in vitro*. *Biol Reprod* 1997; **57**: 936–942. [Medline] [CrossRef]
6. **Carpenter G, Cohen S.** Epidermal growth factor. *Annu Rev Biochem* 1979; **48**: 193–216. [Medline] [CrossRef]
7. **Accornero P, Miretti S, Starvaggi Cucuzza L, Martignani E, Baratta M.** Epidermal growth factor and hepatocyte growth factor cooperate to enhance cell proliferation, scatter, and invasion in murine mammary epithelial cells. *J Mol Endocrinol* 2010; **44**: 115–125. [Medline] [CrossRef]
8. **Nakamura T.** Structure and function of hepatocyte growth factor. *Prog Growth Factor Res* 1991; **3**: 67–85. [Medline] [CrossRef]
9. **Matsumoto K, Nakamura T.** 1993. Roles of HGF as a pleiotropic factor in organ regeneration. In: Golberg ID, Rosen EM (eds.), Hepatocyte Growth Factor-Scatter Factor (HGF-SF) and c-met Receptor. Basel: Birkhauser Verlag 1993; 225–249.
10. **Weidner KM, Arakaki N, Hartmann G, Vandekerckhove J, Weingart S, Rieder H, Fonatsch C, Tsubouchi H, Hishida T, Daikuhara Y, Birchmeier W.** Evidence for the identity of human scatter factor and human hepatocyte growth factor. *Proc Natl Acad Sci USA* 1991; **88**: 7001–7005. [Medline] [CrossRef]
11. **Blay J, Brown KD.** Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. *J Cell Physiol* 1985; **124**: 107–112. [Medline] [CrossRef]
12. **Barrandon Y, Green H.** Cell migration is essential for sustained growth of keratinocyte colonies: the roles of transforming growth factor-α and epidermal growth factor. *Cell* 1987; **50**: 1131–1137. [Medline] [CrossRef]
13. **Gherardi E, Stoker M.** Hepatocyte growth factor—scatter factor: mitogen, motogen, and met. *Cancer Cells* 1991; **3**: 227–232. [Medline]
14. **Takeuchi K, Shibamoto S, Hayakawa M, Hori T, Miyazawa K, Kitamura N, Ito F.** Hepatocyte growth factor (HGF)-induced cell migration is negatively modulated by epidermal growth factor through tyrosine phosphorylation of the HGF receptor. *Exp Cell Res* 1996; **223**: 420–425. [Medline] [CrossRef]
15. **Okajima A, Miyazawa K, Kitamura N.** Primary structure of rat hepatocyte growth factor and induction of its mRNA during liver regeneration following hepatic injury. *Eur J Biochem* 1990; **193**: 375–381. [Medline] [CrossRef]
16. **Kinoshita T, Hirao S, Matsumoto K, Nakamura T.** Possible endocrine control by hepatocyte growth factor of liver regeneration after partial hepatectomy. *Biochem Biophys Res Commun* 1991; **177**: 330–335. [Medline] [CrossRef]
17. **Igawa T, Matsumoto K, Kanda S, Saito Y, Nakamura T.** Hepatocyte growth factor may function as a renotropic factor for regeneration in rats with acute renal injury. *Am J Physiol* 1993; **265**: F61–F69. [Medline]
18. **Montesano R, Matsumoto K, Nakamura T, Orci L.** Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 1991; **67**: 901–908. [Medline] [CrossRef]
19. **Yamagami K, Yamauchi N, Kubota K, Nishimura S, Chowdhury VS, Yamanaka K, Takahashi M, Tabata S, Hattori MA.** Expression and regulation of Foxa2 in the rat uterus during early pregnancy. *J Reprod Dev* 2014; **60**: 468–475. [Medline] [CrossRef]
20. **Yamauchi K, Yamauchi N, Yamagami K, Nakamura N, Yamashita S, Islam MR, Tabata S, Yahiro K, Tamura T, Hashizume K, Hattori MA.** Development of an *in vitro* model for the analysis of bovine endometrium using simple techniques. *Anim Sci J* 2015; **86**: 523–531. [Medline] [CrossRef]
21. **Lang SH, Sharrard RM, Stark M, Villette JM, Maitland NJ.** Prostate epithelial cell lines form spheroids with evidence of glandular differentiation in three-dimensional Matrigel cultures. *Br J Cancer* 2001; **85**: 590–599. [Medline] [CrossRef]
22. **Chen LH, Bissell MJ.** A novel regulatory mechanism for whey acidic protein gene expression. *Cell Regul* 1989; **1**: 45–54. [Medline]
23. **Barcellos-Hoff MH, Aggeler J, Ram TG, Bissell MJ.** Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development* 1989; **105**: 223–235. [Medline]
24. **Webber MM, Bello D, Kleinman HK, Hoffman MP.** Acinar differentiation by non-malignant immortalized human prostatic epithelial cells and its loss by malignant cells. *Carcinogenesis* 1997; **18**: 1225–1231. [Medline] [CrossRef]
25. **Lee JM, Dedhar S, Kalluri R, Thompson EW.** The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 2006; **172**: 973–981. [Medline] [CrossRef]
26. **Zhang L, Rees MC, Bicknell R.** The isolation and long-term culture of normal human endometrial epithelium and stroma. Expression of mRNAs for angiogenic polypeptides basally and on oestrogen and progesterone challenges. *J Cell Sci* 1995; **108**: 323–331. [Medline]
27. **Moll R, Franke WW, Schiller DL, Geiger B, Krepler R.** The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. *Cell* 1982; **31**: 11–24. [Medline] [CrossRef]
28. **Moll R, Levy R, Czernobilsky B, Hohlweg-Majert P, Dallenbach-Hellweg G, Franke WW.** Cytokeratins of normal epithelia and some neoplasms of the female genital tract. *Lab Invest* 1983; **49**: 599–610. [Medline]
29. **Bergh J, Nilsson K, Dahl D, Andersson L, Virtanen I, Lehto VP.** Expression of intermediate filaments in established human lung cancer cell lines. An indicator of differentiation and derivation. *Lab Invest* 1984; **51**: 307–316. [Medline]
30. **Munson L, Chandler SK, Schlafer DH.** Cultivation of bovine fetal and adult endometrial epithelial cells. *J Tissue Cult Methods* 1988; **11**: 129–133. [CrossRef]
31. **Johnson GA, Burghardt RC, Newton GR, Bazer FW, Spencer TE.** Development and characterization of immortalized ovine endometrial cell lines. *Biol Reprod* 1999; **61**: 1324–1330. [Medline] [CrossRef]
32. **Yamauchi N, Yamada O, Takahashi T, Imai K, Sato T, Ito A, Hashizume K.** A three-dimensional cell culture model for bovine endometrium: regeneration of a multicellular spheroid using ascorbate. *Placenta* 2003; **24**: 258–269. [Medline] [CrossRef]
33. **Ottino P, Taheri F, Bazan HE.** Growth factor-induced proliferation in corneal epithelial cells is mediated by 12(S)-HETE. *Exp Eye Res* 2003; **76**: 613–622. [Medline] [CrossRef]
34. **Wilson SE, He YG, Weng J, Zieske JD, Jester JV, Schultz GS.** Effect of epidermal growth factor, hepatocyte growth factor, and keratinocyte growth factor, on proliferation, motility and differentiation of human corneal epithelial cells. *Exp Eye Res* 1994; **59**: 665–678. [Medline] [CrossRef]
35. **Limaye PB, Bowen WC, Orr AV, Luo J, Tseng GC, Michalopoulos GK.** Mechanisms of hepatocyte growth factor-mediated and epidermal growth factor-mediated signaling in transdifferentiation of rat hepatocytes to biliary epithelium. *Hepatology* 2008; **47**: 1702–1713. [Medline] [CrossRef]
36. **Komatsu N, Maekawa T, Takeuchi S, Takahashi S.** Epidermal growth factor and transforming growth factor-alpha stimulate the proliferation of mouse uterine stromal cells. *Zoolog Sci* 2003; **20**: 639–645. [Medline] [CrossRef]
37. **Karihaloo A, O'Rourke DA, Nickel C, Spokes K, Cantley LG.** Differential MAPK pathways utilized for HGF- and EGF-dependent renal epithelial morphogenesis. *J Biol Chem* 2001; **276**: 9166–9173. [Medline] [CrossRef]
38. **Sherr CJ.** D-type cyclins. *Trends Biochem Sci* 1995; **20**: 187–190. [Medline] [CrossRef]
39. **Graña X, Reddy EP.** Cell cycle control in mammalian cells: role of cyclins, cyclin dependent kinases (CDKs), growth suppressor genes and cyclin-dependent kinase inhibitors (CKIs). *Oncogene* 1995; **11**: 211–219. [Medline]
40. **Yang K, Hitomi M, Stacey DW.** Variations in cyclin D1 levels through the cell cycle determine the proliferative fate of a cell. *Cell Div* 2006; **1**: 32. [Medline] [CrossRef]
41. **Wang S, Lloyd RV, Hutzler MJ, Safran MS, Patwardhan NA, Khan A.** The role of cell cycle regulatory protein, cyclin D1, in the progression of thyroid cancer. *Mod Pathol* 2000; **13**: 882–887. [Medline] [CrossRef]
42. **Stoker M, Gherardi E.** Regulation of cell movement: the motogenic cytokines. *Biochim Biophys Acta* 1991; **1072**: 81–102. [Medline]
43. **Benvenuti S, Comoglio PM.** The MET receptor tyrosine kinase in invasion and metastasis. *J Cell Physiol* 2007; **213**: 316–325. [Medline] [CrossRef]
44. **Hulkower KI, Herber RL.** Cell migration and invasion assays as tools for drug discovery. *Pharmaceutics* 2011; **3**: 107–124. [Medline] [CrossRef]
45. **Eritja N, Llobet D, Domingo M, Santacana M, Yeramian A, Matias-Guiu X, Dolcet X.** A novel three-dimensional culture system of polarized epithelial cells to study endometrial carcinogenesis. *Am J Pathol* 2010; **176**: 2722–2731. [Medline] [CrossRef]