Human amniotic epithelial cells inhibit growth of epithelial ovarian cancer cells via TGF-β1-mediated cell cycle arrest

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Abstract. It is reported that human amniotic epithelial cells (hAECs) endow intrinsic antitumor effects on certain kinds of cancer. This research was designed to evaluate whether hAECs endowed potential anticancer properties on epithelial ovarian cancer (EOC) cells in vivo and in vitro, which has not been reported before. In this study, we established a xenografted BALB/c nude mouse model by subcutaneously co-injecting ovarian cancer cell line, SK-OV-3, and hAECs for 28 days. In ex vivo experiments, CCK-8 cell viability assay, real-time PCR, cell counting assay, cell cycle analysis and immunohistochemistry (IHC) assay were used to detect the effects of hAEC-secreted factors on the proliferation and cell cycle progression of EOC cells. A cytokine array was conducted to detect anticancer-related cytokines released from hAECs. Human recombinant TGF-β1 and TGF-β1 antibody were used to treat EOC cells and analyzed whether TGF-β1 contributed to the cell cycle arrest. Results from in vivo and ex vivo experiments showed that hAEC-secreted factors and rhTGF-β1 decreased proliferation of EOC cells and induced G0/G1 cell cycle arrest in cancer cells, which could be partially reversed by excess TGF-β1 antibody. These data indicate that hAECs endow potential anticancer properties on epithelial ovarian cancer in vivo and in vitro which is partially mediated by hAEC-secreted TGF-β1-induced cell cycle arrest. This study suggests a potential application of hAEC-based therapy against epithelial ovarian cancer.

Introduction

The amniotic membrane is the innermost layer of fetal membrane that encloses the amniotic fluid and the fetus during pregnancy. Human amniotic epithelial cells (hAECs) can be easily obtained from amnion membrane with enzymatic digestion after being separated from chorion (1). hAECs are reported to have the ability to differentiate into all three germ layers such as treating primary ovarian insufficiency (4), alveolar defect (5), skin regeneration (6) and so on.

Some unique traits of hAECs have attracted increasing attention about the potential antitumor properties of hAECs, such as induction apoptosis in lymphocytes and inhibition angiogenesis in a rat dorsal skinfold chamber model (7). Niknejad et al reported that hAEC-conditioned medium (hAEC-CM) could induce apoptosis in HeLa cells and MDA-MB-231 cells in vitro (8). Kang et al showed that hAECs displayed anticancer activity in a breast cancer-bearing nude mouse model through both cell-to-cell contact and paracrine way (9). However, Mamede et al revealed adverse effects of amniotic membrane-extracted proteins on human cancer cell lines tested by MTT assay in vitro (10).

The effects of hAECs on human epithelial ovarian cancer (EOC) have not been reported before. To characterize whether hAECs have innate antitumor effects on EOC cells in vivo, we established an SK-OV-3/hAECs co-injected xenografted BALB/c nude mouse model. In addition, we used hAEC-CM or Transwell co-culture system to detect the influences of hAEC-secreted factors on proliferation and cell cycle progression of EOC cells in vitro. Immunohistochemistry (IHC)
was used to test the effects of hAECs on regulators of cell cycle progression, including p16INK4A, p21, phospho-c-Jun N-terminal kinases (JNK) and phospho-pRB (Ser807) (phospho-retinoblastoma protein) in tumor tissues. Cytokine array was conducted to discover anticancer-related cytokines released from hAECs, which showed that TGF-β1 was the most abundant cell cycle-regulatory cytokine secreted from hAECs. Recombinant human TGF-β1 (rhTGF-β1) was used to treat EOC cells and then cellular viability and cell cycle were analyzed. TGF-β1 antibody was added in the Transwell system to neutralize hAEC-secreted TGF-β1 and then cell cycle of EOC cells was analyzed.

Materials and methods

Isolation and culture of human amniotic epithelial cells. Human placentas were obtained at term pregnancy during uncomplicated Caesarean sections with written and informed consent from women who were negative for HIV-I, and hepatitis B and C. This study has been approved by the Institutional Ethics Committee of the International Peace Maternity and Child Health Hospital, and written informed consent was obtained from all participants. The protocol of hAECs isolation was described previously (11). hAECs was cultured in DMEM/F12 (Thermo Fisher Scientific) medium supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), streptomycin (100 U/ml; Thermo Fisher Scientific), penicillin (100 U/ml; Thermo Fisher Scientific), 1X non-essential amino acids (Thermo Fisher Scientific), and 1 mM of sodium pyruvate (Thermo Fisher Scientific), and incubated at 37°C in an incubator containing 5% CO₂. Once the density of cells reached 80-90% confluency, cells were collected for subsequent experiments. hAECs (5x10⁶) were seeded in 100 mm diameter dishes (Corning, USA) in complete culture medium for 72 h. The hAEC-CM were then collected, 0.22 mm filtered, diameter dishes (Corning, USA) in complete culture medium and hAEC-CM at 1:1 ratio or different concentration of rhTGF-β1 for 48 h.

Co-culturing ovarian cancer cell lines and hAECs. Transwell co-culture system (Corning) was used to evaluate the effects of hAEC-secreted factors on EOC cells. hAECs (1x10⁶ cells) were seeded on Transwell inserts and EOC cells (2x10⁶ cells) were cultured in 6-well culture plates at the lower compartment. Cells were cultured in DMEM/high glucose medium supplemented with 10% FBS, streptomycin (100 U/ml) and penicillin (100 U/ml). All groups were incubated at 37°C in a 5% CO₂ incubator for 4 days.

RNA extraction and real-time polymerase chain reaction (PCR). Total RNA was extracted from cultured EOC cells by TRizol (Thermo Fisher Scientific) according to the manufacturer's instructions. Real-time quantitative PCR reactions were performed in triplicate, using the SYBR Green Real-time PCR Master Mix (Takara, Japan). PCR primers were designed according to cDNA sequences in the NCBI database. The following primers were used: GAPDH, 5'-AGGTGCTGTGTAGGATTTG-3' and 5'-GGGGTCCTGTGGATGGCAACA-3'; PCNA, 5'-TCTCTGTTGCAAAAGGGAG-3' and 5'-TCAGTGGTAAGAAGTGGGAT-3'; Ki-67, 5'-GAGGCAATACTCCTGAAACC-3' and 5'-TTATTGCTGGCTCTTGGAGCC-3'. Cycling conditions for the PCR machine were as follows: 95°C 5 sec, 60°C 30 sec and 72°C 30 sec for 40 cycles. All reactions were performed in a 10-μl volume. Gene expression levels were evaluated using the ΔΔCt method, standardized to levels of GAPDH amplification.

Establishment of xenografted nude mouse models. Twelve female BALB/c nude mice (4-week-old) were obtained from Shanghai Jiao Tong University School of Medicine (Shanghai, China). All experimental protocols were approved by the Ethics Committee of the School of Medicine of Shanghai Jiao Tong University, and were in accordance with the approved guidelines set by the Institutional Animal Care and Use Committee. The mice were housed under a laminar flow hood in an isolated room and were maintained under pathogen-free conditions. Five mice per group were subcutaneously injected with 2x10⁶ SK-OV-3 or SK-OV-3/hAECs (2x10⁶:1x10⁶). Tumor volume was measured every two weeks and tumor weight was measured at the endpoint of the experiment. Animals were sacrificed by cervical dislocation under anesthetic status after 28 days. Tumor volume was calculated using the formula: tumor volume (mm³) = 0.52 x [width (mm)²] x [length (mm)] (12). To localize the hAECs in the tumor microenvironment, we transfected hAECs with green fluorescent protein (GFP) by lentivirus which was kindly provided by the Professor Lijian Hui (Chinese Academic of Sciences, Shanghai, China). Two mice were used to establish SK-OV-3/hAECsGFP (2x10⁶:1x10⁶) xenografts and were sacrificed after 28 days.

Immunofluorescence (IF) staining and immunohistochemistry (IHC) staining. Tissue sections were deparaffinized and dehydrated. The slides were then incubated in a 3% H₂O₂ solution...
to block the endogenous peroxidase (IF staining can skip this procedure), followed by rinsing in PBS. To retrieve the antigenicity, sections were then treated with heated antigen retrieval solution containing ethylene diamine tetraacetic acid (EDTA; Wuhan Goodbio Technology, Wuhan, China). After being incubated with 5% bull serum albumin for 30 min to block the non-specific antibody binding sites, the samples were then incubated with the following primary antibodies at 4˚C overnight: PCNA (1:6,000 dilution; CST, USA), Ki-67 (1:200 dilution; Arigo Biolaboratories, Taiwan, China), p16 INK4A (1:300 dilution; Wanleibio, China), p21 (1:300 dilution; Wanleibio), phospho-JNK (1:300 dilution; Wanleibio), phospho-pRB (Ser807) (1:500 dilution; Abcam, UK). For IHC analysis, horse-radish peroxidase (HRP)-labelled anti-mouse/rabbit second antibodies and diaminobenzidine were used according to the manufacturer's instructions (Wuhan Goodbio Technology). Slides were counterstained with hematoxylin, differentiated with 0.1% hydrochloric acid alcohol, and washed by water. They were then dehydrated through a graded ethanol series, immersed in xylene and mounted with permount™ mounting medium. For IF analysis for GFP, tissues were stained with secondary antibody conjugated with Alexa Fluor 488 (1:200; Thermo Fisher Scientific). Then tissues were counterstained with DAPI (Thermo Fisher Scientific) and examined under the fluorescence microscope (Leica).

For the semi-quantitative evaluation of p21, p16 INK4A and phospho-JNK expressions, we used a scoring method described by Budwit-Novotny et al (13). The evaluation of PCNA, Ki-67 and phospho-pRB (Ser807) were measured by the percentage of cells with positive signals in the nucleus.

Cytokine array. A human antibody array 1000 (a combination of human L-507 and human L-493) (RayBiotech, Inc., USA) was performed to detect the expression of anticancer-associated cytokines in hAECs. Samples of hAEC-CM were collected as previously described. Fresh DMEM/F12 culture medium
was used as the control. The cytokine array was performed according to the instructions. The intensities of signals were quantified by densitometry.

**Cell cycle analysis.** EOC cells were cultured alone or in the presence of hAEC-CM. After 48 h, cancer cells were harvested by 0.25% trypsin/ethylene diamine tetraacetic acid, then being washed with phosphate-buffered solution, and fixed with 70% cold-ethanol for 24 h at -20°C. The cells were treated with 50 µg/ml RNase A (Tiangen, China) for 30 min at 37°C and then stained with 100 µg/ml propidium iodide (PI; Thermo Fisher Scientific) for 30 min at 4°C. The cells

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Figure 2. hAECs inhibit growth of SK-OV-3 cells in vivo. (A) Gross observation of subcutaneous xenografts obtained from SK-OV-3 injected group and SK-OV-3/hAECs co-injected group (scale bar, 1 cm). (B) The average tumor weight of SK-OV-3 injected group was significantly higher than that of SK-OV-3/hAECs co-injected group at day 28 (n=10). (C) The average tumor volume of SK-OV-3 injected group was significantly bigger than that of SK-OV-3/hAECs co-injected group at day 14 and day 28 (n=10). (D) (a) Representative image of GFP-transfected hAECs (scale bar, 200 µm). (b) We did not find positive signal of hAEC-GFP in the negative control (scale bar, 100 µm). (c) The positive signals were found in the xenografted tumor tissues obtained from SK-OV-3/hAECsGFP- injected group (scale bar, 100 µm). (E and F) Proliferation of cancer cells were tested by IHC using antibodies against PCNA and Ki-67 in SK-OV-3 and SK-OV-3/hAECs tumor tissues (scale bar, 100 µm; n=5). Data were analyzed by Mann-Whitney U test and were represented as means ± SEM. *p<0.05, **p<0.01 and ***p<0.001.
were analyzed using a Cytomics™ FC500 flow cytometer (Beckman Coulter, Brea, CA, USA). Data were analyzed using Beckman Coulter CxP software.

**Enzyme-linked immunosorbent assay (ELISA).** The ELISA kit was used to test the efficiency of TGF-β1 antibody added to Transwell system to neutralize hAEC-secreted TGF-β1 at 48 h (R&D Systems, USA) and was conducted according to the manufacturer's instructions.

**Statistical analysis.** Results from three independent experiments are reported as the mean ± SEM. Kolmogorov-Smirnov test was used to test whether data accorded with Gaussian distribution. Homogeneity of variance test was conducted by Bartlett's test. Two-tail Student's t-test or ordinary one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test or Mann-Whitney U test (non-parametric test) or Kruskal-Wallis test (non-parametric test) was used to evaluate statistical differences via GraphPad Prism version 6 (GraphPad Software). Differences were considered significant at p-value <0.05.

**Results**

**hAECs inhibit proliferation of EOC cells in vitro in a paracrine manner.** The hAECs represented a cobblestone-like morphology. These cells were positive for epithelial marker CK7, and negative for mesenchymal marker vimentin using IF analysis (Fig. 1A).

CCK-8 assay was conducted to test whether hAEC-secreted factors could influence the viability of EOC cells. Results showed that significant vitality inhibition in EOC cells was induced by hAEC-CM in different concentration compared with the control group at 48 h (n=6). Moreover, the growth inhibition induced by hAEC-CM on EOC cells was significantly dose-dependent (Fig. 1B).

Results from real-time PCR showed that the expression levels of PCNA and Ki-67, two genes relating to cellular proliferation, were significantly downregulated within EOC cells co-cultured with hAECs compared with the control group (Fig. 1C; n=3).

**hAECs inhibit growth of SK-OV-3 cells in a tumor-bearing nude mouse model.** To explore the effects of hAECs on EOC cells in vivo, we established a tumor-bearing nude mouse model by subcutaneously co-injecting SK-OV-3 cells and hAECs at 2:1 ratio (2x10⁶:1x10⁶ cells; five mouse per group) at both sides of the scapular region. Four weeks after injection, we observed that hAECs significantly decreased the average weight and average volume of xenografted tumors compared with the control group (Fig. 2A-C; n=10). To further trace the location of hAECs in the tumor tissues, we labelled hAECs with GFP (Fig. 2D-a) and established a xenografted mouse model by subcutaneously co-injecting SK-OV-3 and hAEC-GFP+ cells at 2:1 ratio (2x10⁶:1x10⁶ cells; two mice were used) at both sides of the scapular region. After 28 days, hAEC-GFP+ cells were found in the stromal area of xenografted tumor tissues and no positive signal was observed in the negative control using IF assay (Fig. 2D-b and -c).

Results from IHC assay showed that hAECs significantly decreased the positive rates of both PCNA and Ki-67 in the SK-OV-3/hAECs co-injected tumor tissues compared with the...
that in the only SK-OV-3 injected tumor tissues (Fig. 2E and F; n=5), which was consistent with the ex vivo results.

**hAECs induce G0/G1 cell cycle arrest in EOC cells in a paracrine manner.** Cell count assay showed that hAEC-CM significantly decreased the cell numbers of both SK-OV-3 and A2780 cells at 48 h, indicating hAEC-secreted factors influenced the division of EOC cells (Fig. 3A; n=3).

By using Transwell system, we observed that hAECs significantly induced G0/G1 cell cycle arrest in both SK-OV-3 and A2780 cells with a significant decrease in S phase using flow cytometry analysis (Fig. 3B; n=3).

**hAECs influence expression of cell cycle-regulatory proteins in vivo.** We further tested the effects of hAECs on the expression levels of three negative regulators of cell cycle progression, p16\[^{INK4A}\] and p21, and phospho-JNK, which was associated with TGF-β1-induced growth inhibition, in the tumor tissues (14-16). IHC assay revealed that SK-OV-3/ hAECs co-injected tumor tissues exhibited significantly

![Figure 4](image)
Figure 5. TGF-β1 was enriched in hAEC-released cell cycle-regulatory cytokines and induced G0/G1 cell cycle arrest in EOC cells. (A) (a) Selective map of the human antibody array 1000. hAECs released multiple cell cycle-regulatory cytokines, including TGF-β1, GM-CSF, IL-8, IFN-γ, IL-6, IL-1, TNF-α, TGF-β2 and Smad 7. (b) The intensities of the signals were quantified by densitometry and the expression of Smad 7 was regarded as control. (B) CCK-8 cell viability assay used to test the effects of rhTGF-β1 in different concentrations on the viability of EOC cells at 48 h (n=6; performed in triplicate). Data were analyzed by Mann-Whitney U test. (C) After being treated with rhTGF-β1, cell cycle of EOC cells was analyzed by flow cytometry. (D) ELISA was used to test the efficiency of TGF-β1 antibody added to neutralize the function of hAEC-secreted TGF-β1. Data were analyzed by Kruskal-Wallis test. (E) After being treated with TGF-β1 antibody in the Transwell system, cell cycle of EOC cells was analyzed by flow cytometry. Data are presented as means ± SEM. *p<0.05, **p<0.01 and ***p<0.001.
higher expressions of p16INK4A, p21 and phospho-JNK in association with TGF-β1 protein, when compared with xenograft tissues obtained from xenografts compared with control group (Fig. 4D; n=5).

Discussion

hAECs show potent value in regenerative medicine. We have reported that both hAECs and hAEC-CM endowed ability of restoring fertility in mouse models of chemotherapy-induced POI/POF (premature ovarian failure and insufficiency) via inhibiting apoptosis in granular cells (11), and enhancing angiogenesis (4). Before further clinical research and application, it is necessary to evaluate the safety of hAEC-based therapy in malignant ovarian cancer. hAECs are often used as a source for cell-based therapy for cancer, because of their ability to induce apoptosis (8) and stimulating cell cycle arrest (19). It is known that proliferation of cancer cells depends in part on cell cycle progression (20). hAEC-secreted factors have been shown to have the ability to induce cell cycle arrest in several cancer cells (19,21). However, the specific factors and mechanisms responsible for the cell cycle-regulatory roles of hAECs on cancer cells still remain unclear. In this study, results from a cytokine array revealed that hAECs released various cytokines which are able to induce G0/G1 cell cycle arrest, including TGF-β1 (22), GM-CSF (23), IL-8 (24), IL-6 (25), IL-1 (26), IFN-γ, TNF-α (27), TGF-β2 (28) and Smad 7 (29). Our results showed that the secretion of TGF-β1 derived from hAECs was abundant, this is consistent with the report of Kang et al showing the amount of TGF-β1 ranked first among those anticaner-associated factors (9). We used recombinant human TGF-β1 to imitate the function of hAEC-secreted TGF-β1 on EOC cells. We observed that rhTGF-β1 could inhibit the viability of EOC cells dose-dependently and induced G0/G1 cell cycle arrest in both cell lines. In addition, after confirming the concentration of TGF-β1 antibody used to neutralize hAEC-secreted TGF-β1 in the supernatant via ELISA, we found that hAEC-secreted factors-induced G0/G1 cell cycle arrest in Transwell system could be partially reversed by adding excess neutralizing TGF-β1 antibody, indicating that hAEC-secreted TGF-β1 play an important role in the induction of cell cycle arrest in EOC cells.

The cell cycle progression is well-organized by cyclin-dependent kinase (CDK) and CDK inhibitors. CDK activity is regulated by two families of inhibitors: INK4 proteins, including p16INK4A, p15INK4B, p18INK4C and p19INK4D and the Cip and Kip family, consisting of p21, p27 and p57 (30). Many studies reported that TGF-β1 could increase the expression of p16INK4A (14) and p21 (15), which functioned as negative regulators of cell cycle progression and might be associated with TGF-β1-mediated G0/G1 cell cycle arrest. Schlosshauer et al reported that the loss of p16INK4A might attribute to tumor progression from ovarian borderline tumors to micropapillary tumors and low-grade carcinomas through overcoming the senescence barrier and/or indirect p53 downregulation (31). Kudoh et al suggested that deletion of p16INK4A and/or p18INK4C was a potential indicator for poor chemotherapy response and adverse prognosis in ovarian cancer patients (32). Consistently, we found that lower expression of p16INK4A was observed in larger xenografts obtained from SK-OV-3 injected group compared with tumor tissues obtained from SK-OV-3/hAECs co-injected group. In addition, our results showed that hAECs upregulated expression of p21 in tumor tissues and p21 overexpression was considered to indicate a better prognostic factor in epithelial ovarian cancer (33,34). JNKs coordinate cell responses to stress and influence regulation of cell growth and transformation, whose phosphorylation is associated with TGF-β1-induced growth inhibition (35) and protein stabilization of p21 (16). Herein our result was consistent with it and showed that hAECs upregulated level of phospho-JNK in xenografted model.

TGF-β is a growth suppressing cytokine which maintains pocket proteins (p107, p130 and pRB) in an active, hypophosphorylated state through directly inducing expression of CDK inhibitor proteins (36), including p16INK4A and p21. By using a Rb1 knockout mouse model, Francis et al proved that hypophosphorylated pRB is required to inhibit expression of activator E2Fs (E2F1, E2F2 and E2F3) target genes, including positive regulators of cell cycle (PCNA and cyclin E1) (37).
In this study, we found that hAECs decreased level of phospho-pRB (Ser807) in tumor tissues with lower expression of PCNA and higher levels of p21 and phospho-JNK, indicating hAECs attenuated the malignant progression of EOC cells through halting cell cycle arrest in EOC cells. In conclusion, we demonstrated the inhibitory effects of hAECs on epithelial ovarian cancer cells in vivo and in vitro. TGF-β1 secreted from hAECs plays an important role in hAEC-mediated growth inhibition in epithelial ovarian cancer cells through inducing G0/G1 cell cycle arrest in cancer cells. This study provides a potential application of hAEC-based strategies against epithelial ovarian cancer. In addition, it suggests that hAEC-based therapy may be safe, but still needs further studies.

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