doi:10.4149/neo_2009_01_13

The inhibitory effect of human embryonic germ cells on ovarian cancer

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Received June 27, 2008

Cancer cells share numerous characteristics with pluripotent stem cells which develop normally, contributing to the tumor cell plasticity. So we investigated the effect of human embryonic germ cells (hEGCs) on SKOV3 cells. We examined the efficacy of hEGCs through coculturing SKOV3 with hEGCs, detecting tunel apoptosis and caspase-9 activity by immunocytochemistry, and measuring the expression of AKT by real-time PCR and western blot. Further a xenograft model in SCID mouse to examine the effect of hEGCs on SKOV3 in vivo was used. Our results showed that there was a 1.5-fold growth reduction for SKOV3 in the coculture group. HEGCs induced apoptosis via caspase-9 activation and AKT down-regulation in SKOV3. This tumor cell inhibition was demonstrated also in the used animal tumor model. Taken together, our observations demonstrated that the hEGCs could inhibit the growth of SKOV3 by inducing apoptosis by inhibiting AKT pathway.

Key words: embryonic germ cells; ovarian cancer; apoptosis

Aggressive tumor cells have numerous comparability with pluripotent stem cells. The relationship between cancer cells and stem cells has recently gained popularity due to the identification of several types of cancer stem cells [1]. It is hypothesized that there are cancer stem cells in the tumor which may arise from the mutation of normal stem cells [2–3]. This hypothesis brings new perspectives for the treatment of tumors [4]. Pluripotent stem cells demonstrate self-renewal and display the ability to differentiate into a variety of normal cell types based on different microenvironments. This comparison evokes our question: do the human pluripotent stem cells, that follow this normal developmental process, have the potential to keep the growth of tumor cells under control?

The development of tumor cells including differentiation, growth, and invasion correlates with the surrounding microenvironment [5]. It is important to note that certain molecular milieus may be used as potential treatment modalities. There is some evidence that embryonic stem (ES) cells may inhibit tumor cell growth. Lightfoot et al. reported that mouse ES cells could inhibit the growth of human pancreatic carcinoma cells *in vitro* [6]. A recent study by Hendrix et al. demonstrated that metastatic melanoma cells could be reverted to a normal, skin cell-like type under the microenvironments of two hESC lines [7].

The human embryonic germ cell (hEGC) belongs to the pluripotent stem cell and comes from primordial germ cells that have similar characteristics as ES cells. Our current study investigated the potential effect of hEGCs on a human ovarian cancer cell line (SKOV3) both *in vitro* and *in vivo*.

Materials and methods

Embryo collection, Cell culture and identification. With the approval of embryo donors in accordance with the guidelines of Chinese national ethics committee, human embryos at 5-9 weeks postconception were collected at termination of pregnancy. The routine derivation of primordial germ cells (PGCs) was done [8]. Dissociated primordial germ cells (PGCs) were plated onto the feeder layer in DMEM/F-12 (Gibco) containing 20% ES-cell-tested fetal bovine serum (HyClone), 1 mM L-glutamin (Gibco), 0.1 mM β-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate (Sigma), 10 μM forskolin

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(Upstate), 4 ng/ml human recombinant basic fibroblast growth factor (bFGF; Peprotech), 100 U/ml penicillin, and 100 μg/ml streptomycin. About seven days later, human embryonic germ (hEG) colonies were isolated, mechanically digested and passaged onto new feeder layer. Samples were taken for identification and differentiation. HEG clumps were fixed in 4% paraformaldehyde in PBS. Alkaline phosphatase (AP) activity was detected. Antibodies for stage-specific embryonic antigens (SSEA-1, 3, 4), OCT-4, TRA-1-60, TRA-1-81 were purchased from CHEMICON, and were detected according to instructions. Secondary antibidies conjugated with fluorescin isothiocyanate (FITC; BeiJing ZhongShan Golden Bridge Biotechnology Co., Ltd.) were used.

Coculture of hEG cells with SKOV3 cells, Hela cells, HELF cells and MEF. Human epithelial ovarian cancer cell line (SKOV3), human cervical cancer cell line (Hela) and human normal lung cell line (HELF) was obtained from Basic Medicine Research Institute, Qilu hospital, Shandong University PR China. Mouse embryonic fibrocyte (MEF) was obstained from 13d fetus mouse. All the cells were incubated in 37 °C, 5% CO2, 95% humidity. The four kinds of cells were plated onto the 6-well culture plates as 2×10^4 /well. After 12 h, we put EG clones (10 clones per well) on some wells of 6-well culture plates as the experimental groups, and changed the medium with EG culture medium. The other wells as the control groups were only changed with EG culture medium. After coculturing for 48 h, hEG clones in the experimental groups were taken out and correspondingly the cells of the control groups in the same area were also scraped. The four kinds of cells in the two groups were collected respectively for cell counting.

Apoptosis assay and immunocytochemistry. SKOV3 cells were cocultured with hEG cells for 48 h as decribed above and then were fixed for apoptosis assay. We used tunel assay and rabbit anti-caspase-9 antibody (KeyGEN Inc.) to detect the apoptosis according to manufacturer's instructions. Immunocytochemical staining was performed according to standard procedures and horseradish peroxidase (HRP)-conjugated goat-anti-rabbit secondary antibody (KeyGEN Inc) was used. Staining was visualized using diaminobezidin (DAB) system (BeiJing ZhongShan Golden Bridge Biotechnology Co., Ltd.). The positive cells were counted by IPWIN51C 5.1.0.20 (Media Cybermetics.Inc).

Quantitative real time reverse transcription-PCR. SKOV3 cells were collected as cell counting. Total RNA was extracted with the RNeasy Kit and reverse transcribed. The primers used were human AKT1 sense 5'-ATG AGC GAC GTG GCT ATT GTG AAG-3' and anti-sense 5'-GAG GCC GTC AGC CAC AGT CTG GAT G-3', human AKT2 sense 5'-ATG AAT GAG GTG TCT GTC ATC AAA GAA GGC-3' and anti-sense 5'-TGC TTG AGG CTG TTG GCG ACC-3', human AKT3 sense 5'-CAG TCT GTC TGC TAC AGC CTG GAT A-3' and anti-sense 5'-ATG AGC GAT GTT ACC ATT GT-3', and β-actin sense 5'-GAT TCC TAT GTG GGC GAC GAG-3' and anti-sense 5'-CCA TCT CTT GCT CGA AGT CC-3' [8]. For quantitative mRNA expression analysis, a real-time RT-PCR protocol was applied using a 7500 Real Time PCR System (AB Applied Biosystems) and SYBR^R Green Realtime PCR Master Mix (TOYOBO CO., LTD. Biochemical Operations Department OSAKA. JAPAN.). Cycling conditions were as follows: initial enzyme activation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, 58 °C for 5 s, and 72 °C for 10 s [9]. Quantitative measurements of each mRNA expression were performed twelve times. The data was analysed by Sequence Detection Software Version 1.4.0.25 (AB Applied Biosystems).

Western blot. The collected SKOV3 cells were lysed in 50 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 0.1% sodium dodecyl sulfate, and proteinase inhibitor (Jingmei Biotech Co., Ltd.). The total protein was extracted and was separated on a 12% SDS-PAGE, blotted onto nitrocellulose membranes. The membranes were washed in PBS, and blocked in buffer (3% bovine serum albumin) for 1 h at room temperature. Then the membranes were incubated overnight at 4 °C with a polyclonal goat anti-AKT1/2 antibody (Santa Cruz) and rabbit anti-phospho AKT antibody (KangChen Bio-Tech) diluted 1:1000 in blocking buffer, followed by incubation with HRP-conjugated goat-anti-rabbit secondary antibody and HRP-conjugated rabbit-anti-goat secondary antibody (KeyGEN Inc) separately. Bands were visualized using DAB system. Examination of each protein was performed three times.

In vivo studies. The SKOV3 cells were harvested with 0.25% trypsin from tissue culture flasks and washed twice with PBS. Forty SCID mice (SLACCAS INC. ShangHai) were divided into two groups: EG group (n=20) and control group (n=20). First, a total of 1×10^7 cells in 0.2 ml PBS were subcutaneously (s.c.) injected into the right flank of all the SCID mice. Seven days after SKOV3 cells injection, 2×10^5 EG cells in 0.1ml PBS were injected into the tumors in EG groups, only 0.1ml PBS was injected into the tumors in control groups. Tumor volume was assessed by measuring two axes (R_1, R_2) and calculated using the formula: $V = 1/6\pi R_1^2 R_2$. The mice were sacrificed respectively at day 14, 24, 35. Tumor tissues were fixed in 10% formaldehyde, and embedded in paraffin wax, then cut into 6-μm-thick sections. The sections were also taken for tunel apoptosis assay.

Statistical analysis. Data are presented as mean ± SD in text and figures. Differences on the proliferation of SKOV3 cells and the positive cell counting of immunocytochemistry were evaluated statistically by the two-sided t-test. A *p*-value of 0.05 was adopted for determining statistically significant differences.

Results

Derivation and Characterization of hEG cells. We successfully isolated and cultured the hEG cells growing as tightly

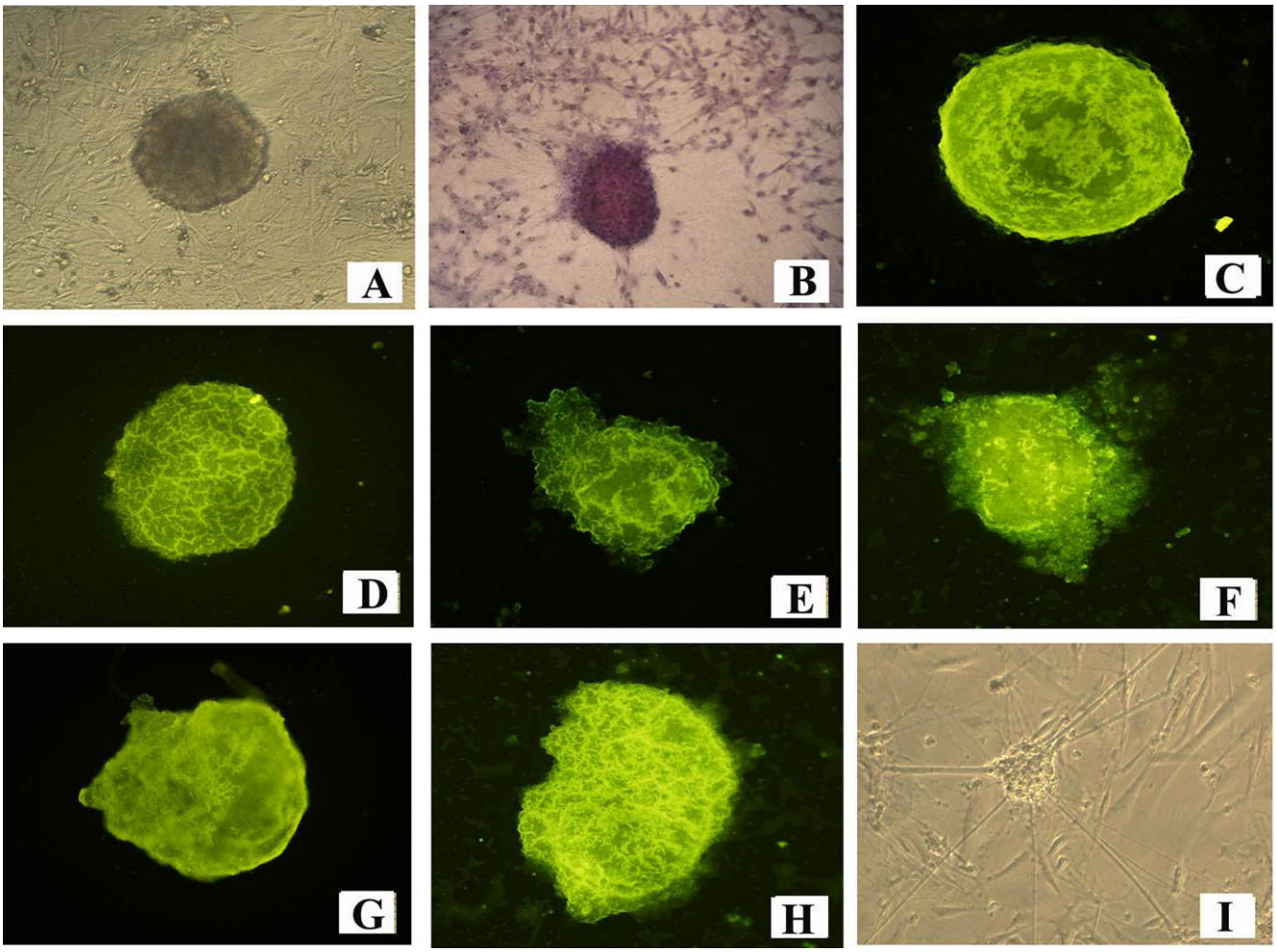

Figure 1. The culture and identification of hEG cells. HEG clones grew on the feeder (A 40×). Human EG cells are positive for the enzyme alkaline phosphatase (B 40×), the stage-specific embryonic antigens SSEA-1 (C 200×), SSEA-3 (D 100×) and SSEA-4 (E 100×), the tumor rejection antigens TRA-1-60 (F 100×) and TRA-1-81 (G 100×), as well as OCT-4 (H 200×). HEG cells can spontaneously differentiate to nerve-sphere liked clumps (I 40×).

packed cells with distinct cell borders (Figure 1A). The HEG cells were passaged every 4-5 days and spontaneously-differentiated clones were eliminated during routine passage. Identification was performed on hEG clones at passage 1, 2, 3. The hEG clones showed high level of AP activity and were characterized by a range of cell surface markers including: SSEA-1, SSEA-3, SSEA-4, OCT-4, TRA-1-60, TRA-1-81 (Figure 1B-H). When we replaced the conditioned culture medium with DMEM/F-12 and 10% heat-inactivated fetal calf serum, hEG cells spontaneously differentiated into cell clumps like nerve-sphere (Figure 1I).

The effect of HEG cells on the growth of the cocultured cells. HEG clones growed like bird's nest, which were easy to be distinguished from the cocultured cells in modality. After two days of coculture, hEG clones areas were scraped out of the experimental and control groups as described

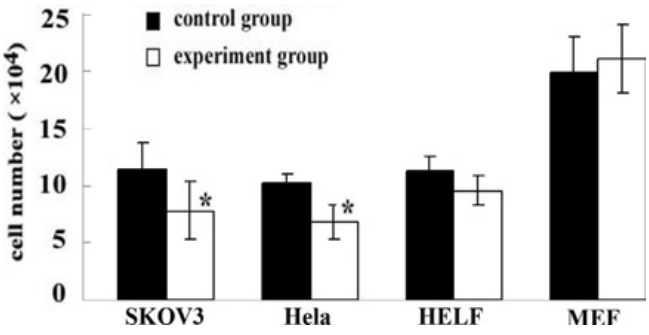

Figure 2. Cell counting of SKOV3, Hela, HELF and MEF cells. The number of three kinds of cells after 48hour coculture in the two groups are shown. The experiment was performed three times and mean & SD are shown (*: $p<0.05$).

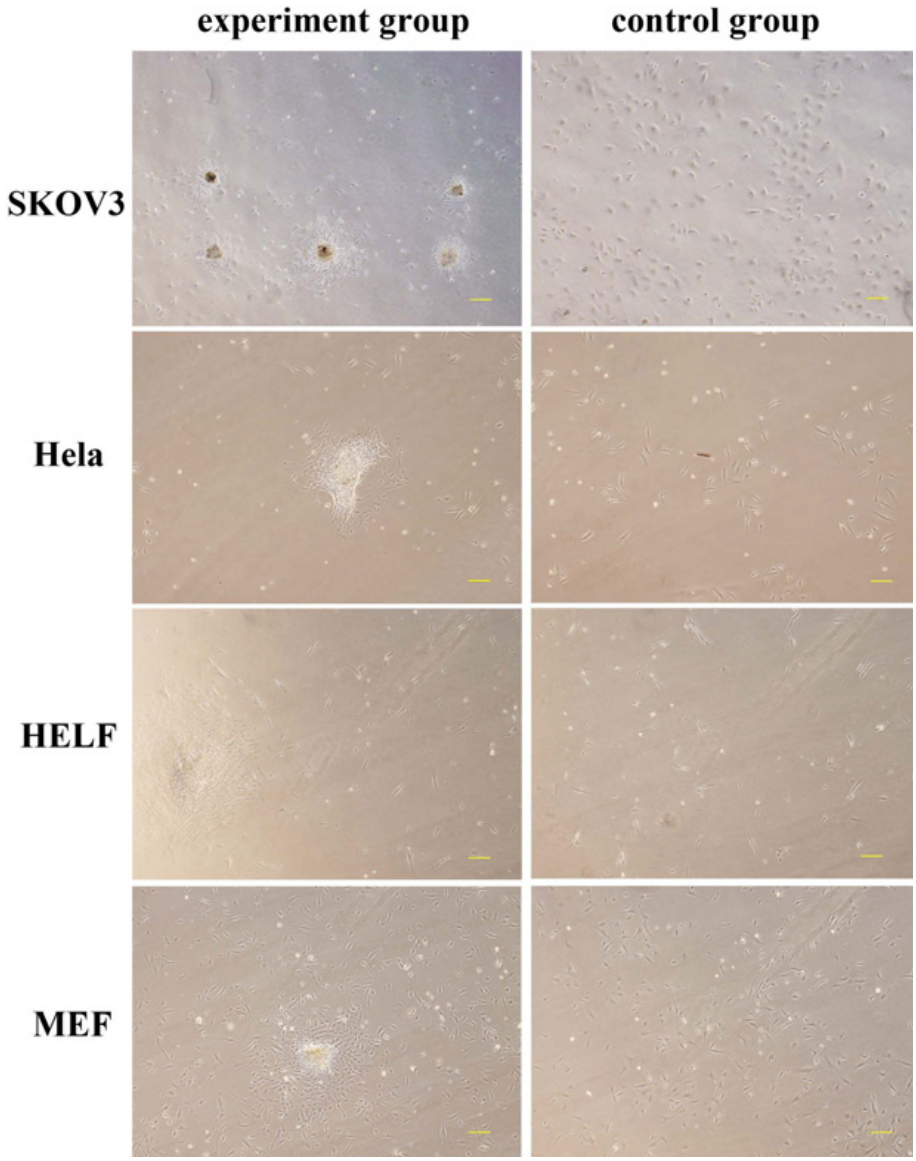

Figure 3. The growth state of four kinds of cells after 48h coculture in the two groups. SKOV3 and Hela cells around the hEG clones were sparser, while MEF cells were dense. (40×)

above. The residual cells in the two groups were trypsinized and collected for cell counting. The proliferation of SKOV3 and Hela cells was inhibited by hEG cells. There was an average of $(7.8\pm2.4)\times10^4$ SKOV3 cells in the experiment group versus $(11.5\pm2.3)\times10^4$ SKOV3 in the control group (p=0.0258) (Figure 2). For Hela cells, there was an average of $(7.5\pm1.0)\times10^4$ in the experiment group versus $(14\pm3)\times10^4$ in the control group (p=0.04). HEG cells could accelerate the growth of MEF and inhibit the growth of HELF, but had no statistical significance (Figure 2). Figure 3 showed that SKOV3 and Hela cells around the hEG clones were sparse, while MEF cells were dense.

HEG cells induced apoptosis of SKOV3 cells. Tunel and caspase-9 staining of SKOV3 were shown in Figure 4. It is apparent that SKOV3 cells were less populated near the EG clones when compared to those farther away from the EG clones. The SKOV3 cells cocultured with EG cells showed more positive staining of tunel and caspase-9, especially those near the EG clones. Numbers of positive cells in tunnel and caspase-9 staining were shown in Figure 4G and Figure 4H respectively.

HEG cells influenced AKT expression in ovarian cancer cells. The collection of SKOV3 cells in these assays was similar to that of cell counting. We determined the

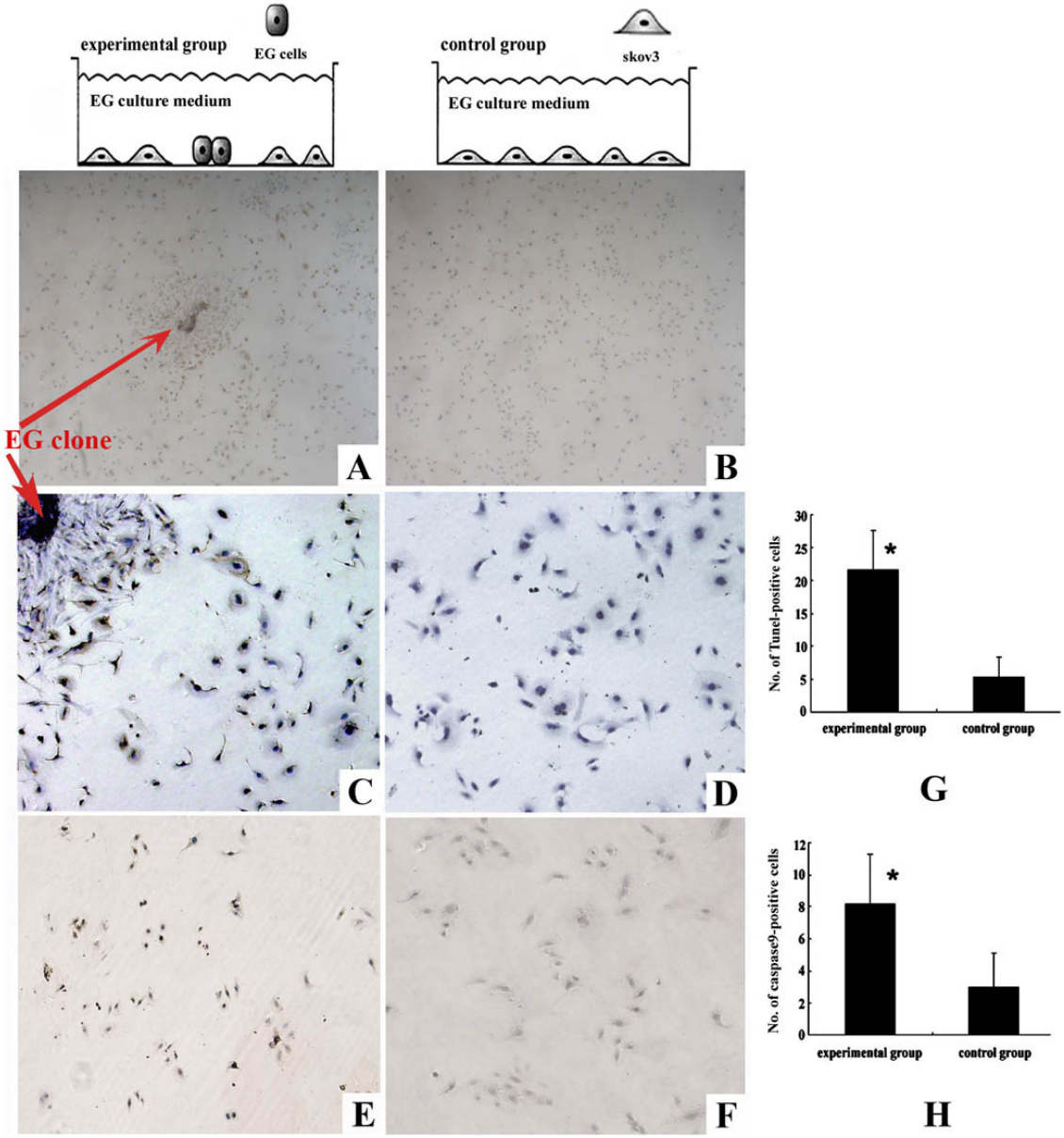

Figure 4. Apoptosis assay and immunocytochemistry in the in vitro study. The above two drawings are the sketch maps of coculture in vitro. (A, C) Staining of tunel apoptosis assay (brown) in the experiment group. (B, D) In contrast, the control group shows less staining of tunel. Figure 4G showed the positive cell counting of tunnel (*: p=0.006, n=8). (E) Positive immunostaining of caspase-9 (brown) in the experiment group. (F) The control group has less immunostaining of caspase-9. Figure 4H showed the positive cell counting of caspase-9 (*: p=0.018, n=6). Figure 4C and 4D are a magnification of 4A and 4B, respectively. Figure 4C, 4D, 4E, 4F are amplified 100×.

expression of total and phosphorylated AKT in the coculture system as well as in the control group. As shown in Figure 5, expression of AKT and phosphorylated AKT was found in the two groups, but the p-AKT protein was considerably higher in the control group. Because of the high homology of the three AKT isoforms, we therefore, examined the expression of the different AKT isoforms in SKOV3 cells both of experiment and control group by real time RT-PCR. Expression of AKT1, AKT2 and AKT3 mRNA was observed in the two groups. The mRNA levels of AKT1, AKT2, AKT3 in the experimental group were 0.67, 0.08, 0.49 times of that in the control group, respectively (Figure 5).

In vivo effect of EG cells to SKOV3 cells. SKOV3 cells were subcutaneously injected into the right flank of SCID mice. Seven days after injection, the primary tumor became

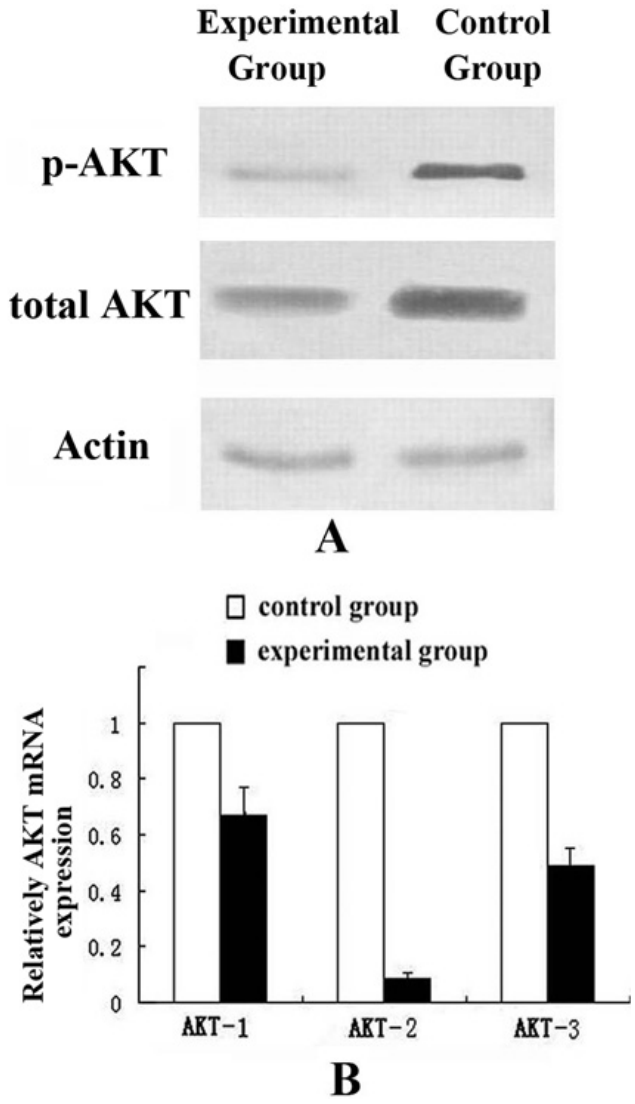

Figure 5. Expression of AKT in SKOV3 cells of two groups. As seen in A, expression of AKT1/2, phospho-AKT and β-actin protein in the two groups was investigated by western blot and performed three times. B shows the result of real-time RT-PCR for quantification of AKT1, AKT2 and AKT3 mRNA expression. As a control, mRNA expression levels were normalized against β-actin mRNA expression. Quantitative measurements of each mRNA expression were performed twelve times.

palpable, the average diameter of tumor was about 0.5mm. The hEG cells were injected into the tumor then. The time-course of tumor volume change is shown in Figure 6. The tumor volumes at day 14, 21, 28, 35 were compared respectively with the tumor volume at day 7 to index the change (Table 1). It is noteworthy that the tumors in the EG group grew dramatically slower than those in the control group. More positive signal of the tunel apoptosis was detected in EG group (Figure 6).

Discussion

Pluripotent stem cells can be kept in an undifferentiated and well-balanced state via conditioned medium. They can also differentiate into normal phenotypic cells regulated by environmental parameters [10]. Therefore these stem cells represent an important source for potential transplantation therapy. As hEG cells belong to pluripotent stem cells, we derived human EG cells from the primary gonadal ridges of abortion fetus about 5-7 weeks. The cultured hEG cells showed abundant AP ability and expressed OCT-4 and other characteristic pluripotent cell surface markers, including SSEA-1, SSEA-3, SSEA-4, etc. These observations proved our cells accorded with the character of EG cells [11–12]. Some studies have reported that hEG cells, as a type of pluripotent stem cell, also have the ability to differentiate to other cell types and treat diseases as do ES cells [13–16].

Ovarian carcinoma has the highest mortality rate among gynecological malignancies. We studied the effect of hEG cells on the growth of human ovarian cancer cell lines. In the *in vitro* study, the data of cell counting showed the growth of SKOV3 cells was inhibited by EG cells (Figure 2). Furthermore SKOV3 cells near the EG clones were sparser than those farther away, but this phenomenon was not be observed in the feeder layer cells of hEG cells. This suggests that the inhibitory effect of EG cells to the growth of SKOV3 cells became more prominent when SKOV3 cells approached near EG cells. To see how specific the inhibition is, we also observed the effect of hEG clones on the growth of Hela, HELF and MEF at the same time. The results showed hEG cells could inhibit the growth of Hela and promote the growth of MEF, but had no evident effect on the growth of HELF. It was also observed that Hela cells near EG clones were sparser than those in the control groups. So we supposed that hEG cells could inhibit the growth of ovarian and cervical cancer cells and have no significant effect on the normal cells, which conformed to our initial supposition.

In the culture of hEG cells, MEF supported the growth of hEG cells and no toxic effect from hEG cells on MEF was observed. Comparing the growth of the four kinds of

Table 1 The change index of tumor volume at day 14, 21, 28, 35 compared with day 7

	Day 14/Day 7	Day 21/Day 7	Day 28/Day7	Day 35/Day7
Experiment Groups	1.49±0.35	3.88±1.22	7.82±1.13*	12.68±0.81*
Control Groups	1.85±0.35	4.85±1.04	13.08±2.7	27.78±7.24

*$p<0.05$ when day 28, day 35, the change indexes in the experiment groups compared with those in the control groups.

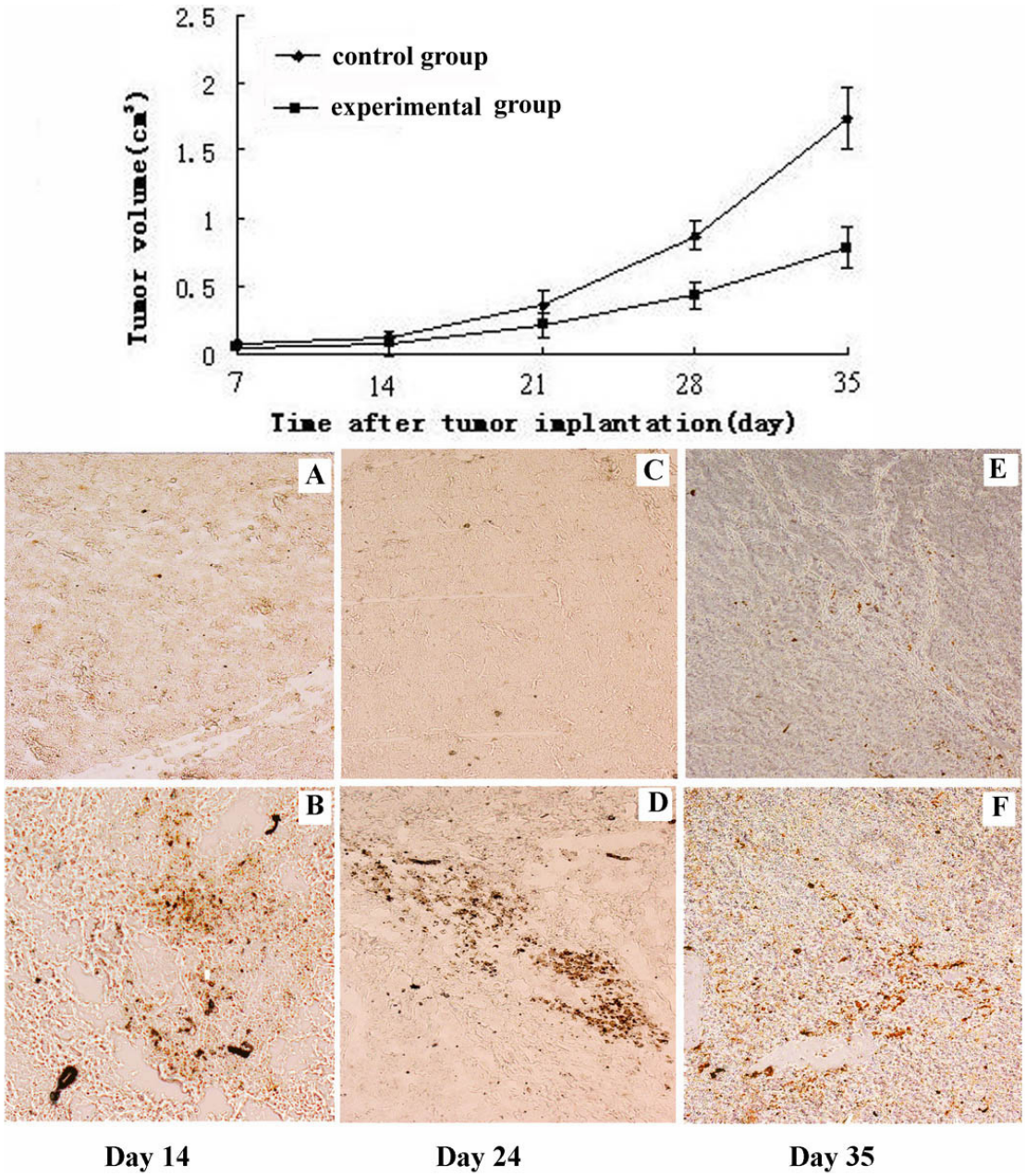

Figure 6. Tumor volumes change and tunel assay detection in the in vivo study. The tumor was detected after hEG cells or PBS was injected at day 7. The tunel assay was detected at day 14, 24, 35. As seen in Figure 6B, 6D, and 6F, positive tunel signals are shown in brown in the EG group. Figure 6A, 6C and 6E are the control groups. (40×)

cells, it showed the inhibitory effect may not be caused by some toxic compounds produced from hEG cells. Therefore, we examine the tunel apoptosis assay in SKOV3 cells. Apoptosis is caused by a cellular suicide programme. The TUNEL (the terminal transferase uridyl nick end labelling) assay developed by Gavrieli and colleagues is the widely used method to detect apoptosis. It visualized the DNA cleavage in situ by inserting a marker at the 3' nick end [17–18]. We discovered SKOV3 cells in the coculture group were rich in tunel positive signals, especially in the area around the EG clones. To further prove this phenomenon, we also detected caspase-9 antibody by immunocytochemistry. Caspase-9 (CASP-9), which belongs to the family of caspases, plays an important role in the apoptosis pathway and has close relation with the development and progression of cancer [19–20]. The result showed there were more

caspase-9 signals in the coculture group, which was consistent with the forenamed results. Therefore, it is likely that EG cells inhibit the growth of SKOV3 cells by inducing the apoptosis of SKOV3 cells.

To further examine the mechanism of this effect, we investigated the AKT signaling pathway in the coculture system. There is evidence that many signal pathways operate similarly in both normal stem cells and cancer cells [21–23]. The protein kinase AKT is involved in these members that are important for tumor development and progression, and caspase-9 is a downstream effector of AKT signaling pathway. SKOV3 ovarian carcinoma cells exhibit abnormally high level of AKT activity [24], whereas EG cells have a well-balanced AKT expression system [25–26]. Through real-time RT-PCR and western blotting, we discovered that the coculture with EG cells downregulated the expression of AKT in SKOV3 cells.

The protein kinase AKT/PKB family participates in the regulation of cell survival. AKT can be activated via phosphorylation. Subsequently, the p-AKT can promote cell proliferation and suppress apoptosis by phosphorylation of several downstream effector proteins (e.g. Caspase-9) that are involved in the regulation of some pro-apoptotic gene expression [8]. Therefore, negative regulation of PIP3/AKT may be the critical step for controlling the growth of tumor. As the phosphorylation of AKT protein was inhibited, suppressed caspase-9 was unlocked. Then activation of caspase-9 could activate the apoptosis of SKOV3 cells. So EG cells may influence the expression of AKT and p-AKT in SKOV3 cells in the coculture system and subsequently inhibit the growth of SKOV3 cells.

In addition, we investigated the inhibitory role of EG cells on ovarian cancer growth via a xenograft model in SCID mice. The *in vivo* study also indicated that transplantation of EG cells suppressed the growth of tumor. The tunel apopotosis signal could also be detected in this experiment. Therefore it proved EG cells may have the same effect *in vivo* as our *in vitro* studies have suggested.

In summary, our research demonstrated that hEG cells could induce the apoptosis of SKOV3 cells, and consequently inhibit the growth of SKOV3 cells *in vivo* and *in vitro* – via the AKT signal pathway. The deficiency of AKT and p-AKT in the experimental group may be attributed to the activation of caspase-9 which led to the apoptosis of cancer cells. Maybe hEG cells could inhibit the growth of other cancer cells. Our results may lead to a novel treatment application of pluripotent stem cells to cancer treatment. Certainly, the relationship between pluripotent stem cells and cancer cells is complex and further investigation is needed.

Acknowledgments. The authors are grateful to Rahul Parikh (The Cancer Institute of New Jersey, USA) for critical review of our manuscript. This work was supported by grants from National Natural Science Foundation of China (No. 30571953 & 30700163).

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