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Effects of epidermal growth factor on the proliferation and cell cycle regulation of cultured human amnion epithelial cells

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Human amnion epithelial cells (HAECs) hold great promise in tissue engineering for regenerative medicine. Large numbers of HAECs are required for this purpose. Hence, exogenous growth factor is added to the culture medium to improve epithelial cells proliferation. The aim of the present study was to determine the effects of epidermal growth factor (EGF) on the proliferation and cell cycle regulation of cultured HAECs. HAECs at P1 were cultured for 7 days in medium containing an equal volume mix of HAM's F12: Dulbecco's Modified Eagles Medium (1:1) supplemented with different concentrations of EGF (0, 5, 10, 20, 30 and 50 ng/ml EGF) in reduced serum. Morphology, growth kinetics and cell cycle analysis using flow cytometry were assessed. Quantitative gene expression for cell cycle control genes, pluripotent transcription factors, epithelial genes and neuronal genes were also determined. EGF enhanced HAECs proliferation with optimal concentration at 10 ng/ml EGF. EGF significantly increased the proportion of HAECs at S- and G2/M-phase of the cell cycle compared to the control. At the end of culture, HAECs remained as diploid cells under cell cycle analysis. EGF significantly decreased the mRNA expression of p21, pRb, p53 and GADD45 in cultured HAECs. EGF also significantly decreased the pluripotent genes expression: Oct-3/4, Sox2 and Nanog; epithelial genes expression: CK14, p63, CK1 and Involucrin; and neuronal gene expression: NSE, NF-M and MAP 2. The results suggested that EGF is a strong mitogen that promotes the proliferation of HAECs through cell cycle regulation. EGF did not promote HAECs differentiation or pluripotent genes expression.

[**Key words:** Epidermal growth factor; Human amnion; Epithelial; Pluripotent; Cell cycle; Tissue engineering]

Amnion contains two different types of cells namely, the amnion epithelial cells, which form a continuous monolayer that is in contact with the amniotic fluid, and the amnion mesenchymal cells, which are sparsely distributed within the collagenous stroma that underlies the amnion epithelium (1). Recently, attention is given to human amnion epithelial cells (HAECs) as they characteristically make an excellent alternative for embryonic stem cells. HAECs express surface markers associated with embryonic stem cells, e.g., Stage-specific embryonic antigen 3 (SSEA-3) SSEA-4, tumor rejection antigen 1-60 (TRA-1-60) and TRA-1-80. Additionally, they also showed that HAECs express the pluripotent and multipotent markers such as Oct-3/4, Sox2, Nanog, FGF-4, Rex-1, FZD-9, BST-1 and ABCG2 as well as neural stem cell markers such as nestin, vimentin and neuron specific enolase (NSE) (2–5). Their characterization and differentiation potentials into three germ layers (ectodermal-, mesodermal- and endodermal-lineages) have been thoroughly carried out (2,3). Although they have pluripotent characteristics, they do not form teratoma upon transplantation into the testes of the severe combined immunodeficiency (SCID) mice (2). They have low immunogenicity which reduces the risks of rejection upon transplantation (6). Furthermore, they are easily accessible and highly abundant since amnion is normally discarded after birth.

However several limitations remain before it can be accepted as a stem cell source for effective transplantation, as large numbers of HAECs are required which is crucial for its application in cell transplantation and epithelial tissue engineering. One of the limitations is our yield of only 5.09×10^7 HAECs per amnion in average using enzymatic trypsin-EDTA digestion (3) and they proliferate poorly in culture. Even when HAECs are cultured in growth factor supplemented medium containing platelet derived growth factor (PDGF), fibroblast growth factor (FGF) and/or hydrocortisone, they still do not achieve a considerable increase in growth rate (7,8). HAECs synthesize epidermal growth factor (EGF) protein but supplementation of culture medium with exogenous EGF to promote HAECs proliferation in culture only supported growth for 6–8 passages (7,9). Nevertheless, how EGF regulates the HAECs proliferation and cell cycle progression, expression on pluripotent genes, epithelial genes and neuronal genes are still not known till today.

EGF is a monomeric peptide that promotes mitogenesis in tissues of endodermal, mesodermal and ectodermal origin (10). EGF plays an important role in cell proliferation, migration and

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locomotion (11–13). It induces the proliferation of different types of cells, for instance, the growth of keratinocytes of the skin and oropharynx, and retinal pigment epithelial cells, without inducing differentiation (11,12,14,15). EGF and its related peptides have been implicated in the promotion of cell proliferation in wound healing such as in re-epithelialization (12). After EGF binds to its receptors (EGFR), the modulatory effects exerted by EGF had been associated with the differentiation retardation and proliferation enhancement via the cell cycle regulating genes (11,16–18).

Therefore, the aim of the present study was to determine the effects of EGF on the proliferation and cell cycle regulation of cultured HAECs. The effects of EGF on the stemness, epithelial genes and neuronal genes were also evaluated, as well as determination of the optimal concentration of the EGF in the culture medium that will promote the proliferation of HAECs for the purpose of tissue engineering. In this study, reduced serum has been used to minimize the influence of other cytokines present in the serum.

MATERIALS AND METHODS

This study was approved by the Universiti Kebangsaan Malaysia Research and Ethics Committee (Approval Project Code: FF-272-2007). Informed, written consent was obtained from donors prior to collection. Human term placentas were obtained from uncomplicated, elective caesarean section from healthy mothers of age ranging from 20-40 years.

Isolation and culture of cells The amnion layer was mechanically peeled off the chorion and washed several times with phosphate buffered saline (PBS, Invitrogen, Carlsbad, CA, USA) to remove the red blood cells. It was then cut into 2×2 cm and processed using the previous methods (3). Briefly, 10 ml of 0.05% trypsin containing 0.2 g/L of EDTA (Invitrogen, Gibco/BRL) was used to dissociate HAECs from 20 pieces of amnion (2×2 cm) and incubated at 37°C with constant agitation. Trypsin was inactivated by adding 10 ml of F12:DMEM (1:1) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen; FD+10% FBS). After the first 10 min of digestion, the cells were discarded to exclude cell debris. Subsequently, cells from the two repeated digestion of 30 min each were collected and washed with PBS. HAECs were then plated on 6-well plates at 20,000 cells/cm^2 seeding density, in culture medium F12:DMEM (1:1) supplemented with 10% FBS, 1% antibiotic-antimycotic (Invitrogen), 1% Glutamax (Invitrogen) and 1% vitamin C (Merck, Germany). The culture plates were incubated in a humidified incubator at 37°C with 5% CO_2.

HAECs from the initial culture (P0) were subcultured to passage 1 (P1) with a split ratio of 1:3 (seeding density at $20{,}000 \pm 3223$ cells per cm^2). Various concentrations of human recombinant epidermal growth factor, EGF (Peprotech, USA) (0 ng/mL EGF, 5 ng/mL EGF, 10 ng/mL EGF, 20 ng/mL EGF, 30 ng/mL EGF and 50 ng/mL EGF) were added to the medium F12:DMEM (1:1) supplemented with 2% FBS, 1% antibiotic-antimycotic, 1% Glutamax and 1% vitamin C. This culture medium was changed every 3 days. After 7 days, the cells were trypsinized using 0.125% trypsin-EDTA (Invitrogen). The cell viability and cell number of HAECs were determined by using hemocytometer and trypan blue dye exclusion (Invitrogen). The doubling time (DT) and growth rate (GR) were then calculated using the following equations,

$$\mathrm{DT\ (days)} = \mathrm{Log}\ (Ne/Ns)\ \mathrm{x}\ (1/\mathrm{Log2}) \quad (1)$$

$$\mathrm{GR\ (cells/days/cm^2)}: [(Ne\ \mathrm{e}\ Ns)/\mathrm{t}]/\mathrm{surface\ area} \quad (2)$$

where N_e is the number of cells at the end of culture, N_s is the number of cells at the start of culture, t is the duration of cell culture in days (19,20).

Cell cycle analysis using flow cytometry To determine the cell cycle distribution analysis, HAECs at passage 1 (P1) cultured in 0 ng/ml EGF, 10 ng/ml EGF and 50 ng/ml EGF were trypsinized after 7 days of culture. A total number of 5×10^5 cells were used for the analysis. HAECs were processed using CycleTEST PLUS DNA Reagent Kit (Becton Dickinson) following manufacturers' instruction. The propidium iodide (PI) stained cells in suspension were analyzed using FACScan flowcytometers (Becton Dickinson). At least 20,000 events were evaluated. Raw data were collected using CELLQuest software (Becton Dickinson). These data were analyzed using Modfit Cell Cycle Analysis Software (Verity House Software, Topsham, ME, USA).

Total RNA extraction Total RNA was extracted from HAECs, after 7 days of cultured in 0 ng/ml EGF, 10 ng/ml EGF and 50 ng/ml EGF by using TRI-Reagent (Molecular Research Centre, USA) according to the manufacturer's instruction. 5 µl of polyacryl carrier (Molecular Research Centre) was added to precipitate the total RNA. The RNA pellet was washed with 75% ethanol and dried before dissolving in DNase- and RNase- free distilled water (Invitrogen). The total RNA yield and integrity was determined by spectrophotometer (Bio-Rad, USA). The extracted RNA was immediately stored at -80°C until further analysis.

TABLE 1. Average percentages of DNA content of HAECs in different stages of cell cycle after cultured in different concentration of EGF (0 ng/ml, 10 ng/ml and 50 ng/ml).

EGF Concentration/ Cell cycle phases	G0/G1	S	G2/M
0 ng/ml EGF	89.50 ± 0.7731	3.18 ± 0.4605	7.32 ± 0.8644
10 ng/ml EGF	79.69 ± 1.6518 *	7.39 ± 0.3438 **	11.06 ± 0.5903 ***
50 ng/ml EGF	79.68 ± 1.7909 *	7.94 ± 0.8558 **	10.73 ± 0.8220 ***

Significant differences when 10 ng/ml EGF, 50 ng/ml EGF were compared with the control (0 ng/ml EGF) at G0/G1 (*$p < 0.05$), S (**$p < 0.05$) and G2/M-phases (***$p < 0.05$), respectively.

cDNA synthesis Five micro liter of the total RNA was used to synthesize cDNA using Superscript™ III First-Strand Synthesis kit (Invitrogen) which contained reverse transcriptase enzyme mix, 2× RT reaction mix and *E. coli* RNase H. The reaction was performed according to the manufacturer's instruction. The cDNA was stored at -20°C until further analysis.

Quantitative gene expression evaluation by real-time polymerase chain reaction (PCR) The cDNA was subjected to quantitative PCR analysis using IQ™SYBR®Green Supermix (Bio-Rad) with the sets of primers (p21, p53, pRb, Cyclin D1, MKi67, GADD45, Oct-3/4, SOX2 Nanog, CK1, CK14, p63, Inv, NSE, NF-M, MAP 2

TABLE 2. Primers used in the quantitative polymerase chain reaction (PCR).

Gene	Accession no.	Primer sequences (5′……3′)	Product size (bp)
GAPDH	NM_002046	F: tcc ctg agc tga acg gga ag R: gga gga gtg ggt gtc gct gt	217
p21	NM_002577	F: gat ggc acc aga ggt ggt ta R: tcc cga aat att ggg gaa ag	198
p53	NM_000546	F: gga aga gaa tct ccg caa gaa R: agc tct cgg aac atc tcg aag	177
pRB	NM_000321	F: cag acc cag aag cca ttg aa R: ctg ggt gct cag aca gaa gg	115
GADD45	NM_052850	F: cca aga tgc cac aga tga ttg R: act cct tgg gtc cac ctg gta	140
CyclinD1	NM_053056	F: aga cct tcg ttg ccc tct gt R: cag tcc ggg tca cac ttg at	181
MKi67	NM_002417	F: gca caa aga ggc atc tca gg R: ctt tgt ctg cat ccg tgg tt	108
Oct-3/4	NM_002701	F: aag gat gtg gtc cga gtg tg R: gaa gtg agg gct ccc ata gc	180
Sox-2	NM_003106	F: tta cct ctt cct ccc act cca R: ggt agt gct ggg aca tgt gaa	132
Nanog	NM_024865	F: ctg tga ttt gtg ggc ctg aa R: tgt ttg cct ttg gga ctg gt	153
Cytokeratin 14	NM_000526	F: cga gga atg gtt ctt cac ca R: ttt cat gct gag ctg gga ct	151
P63	NM_003722	F: gaa acc aga gat ggg caa gtc R: gct tcg tac cat cac cgt tct	145
Involucrin	NM_005547	F: gaa aca gcc aac tcc act gc R: cat tct tgc tca ggc agt cc	123
Cytokeratin 1	NM_006121	F: ggg tgg tta tgg tcc tgt ctg R: gct ccc ttt ctc gag act tca	128
NSE (also known as Enolase 2)	NM_001975	F: ctg gag ttg gat ggg act ga R: cag gca gga tga ggt ctg ag	157
NF-M	NM_005382	F: gga aga gat cgc cga gta cc R: tag ctg ctg agg tcg tgg tt	135
MAP 2	NM_002374	F: gag gtt gcc agg agg aaa tc R: ttc gag ctg act gtc cgt tt	117