

# Embryonic Stem Cell-Preconditioned Microenvironment Effects on Epidermoid Carcinoma

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## ABSTRACT

Embryonic stem cell-preconditioned microenvironment is important for cancer cells properties by change cell morphology and proliferation. This microenvironment induces cancer cell reprogramming and results in a change in cancer cell properties such as differentiation and migration. The cancer microenvironment affects cancer cell proliferation and growth. However, the mechanism has not been clarified yet. Using the ES-preconditioned 3-D microenvironment model, we provide evidence showing that the ES microenvironment inhibits proliferation and reduces oncogenic gene expression. But ES microenvironment has no effect on telomerase activity, cell viability, cellular senescence, and methylation on Oct4 promoter region. Furthermore, methylation of Nanog was increase on ES-preconditioned microenvironment and supports results that no difference on RNA expression levels. Taken together, these results demonstrated that in the ES-preconditioned 3-D microenvironment is a crucial role for cancer cell proliferation not senescence.

(Key words : ES cells, Microenvironment, 3D culture, Reprogramming)

## INTRODUCTION

Several groups published their study about the complex and enigmatic relationship between stem cells and their environment (9, 11). Cancer is interacts and controlled by its surrounding microenvironment (12). The challenge is to better understand the etiology of the plastic phenotype expressed by the most aggressive tumor cells in response to their environment and to develop therapeutic strategies that incapacitate target molecules and induce subsequent differentiation in cancer patients (9). A431 cells are model cell line for epidermoid carcinoma are used in studies of the cell cycle and cancer-associated cell signaling pathways since they express abnormally high levels of the epidermal growth factor receptor (EGFR) (2).

During embryonic development, the extracellular matrix (ECM) plays a critical role in regulating stem cell differentiation into various lineages, as well as in cell migration and proliferation (3, 6, 8, 10, 15, 23). Recent findings indicated a tumor suppressive effect of an embryonic zebrafish microenvironment on human metastatic melanoma cells (14) And we published that ES-preconditioned matrigel change cancer cell prosperity by Gremlin (11)

Based on this concept, we investigated the cancer cell behavior by three-dimensional (3-D) in vitro culture

model system, especially epidermoid carcinoma cells. Using an A431 cell line, we determined A431 cell growth, epigenetic change, and oncogenic gene expression.

## MATERIALS AND METHODS

### Cell Culture

A431, epidermoid carcinoma cells were maintained with 10% FBS -DMEM, respectively. The cells were carried out passage or split at 90% confluence and medium was changed every two day.

### Feeder Preparation and Embryonic Stem (ES) Cell Culture

To culture ES cell, primary CF-1 mouse embryonic fibroblast (MEF) cells were treated with 100  $\mu$ g/ml of mitomycin C for 2 h at 5% CO<sub>2</sub> incubator. The cells were trypsinized and  $7.5 \times 10^5 \sim 1 \times 10^6$  cells were seeded into T-25 cell culture flask, which pretreated with 0.1% gelatin for 2 hr at room temperature, and cultured at 5% CO<sub>2</sub> incubator for overnight. TT2 (27) mouse embryonic stem (ES) cells were thawed and seeded onto mitomycin C-treated feeder layer with ES medium (20% ES Cell qualified fetal bovine serum (Gibco, Grand Island, NY)-DMEM supplemented with  $10^3$  units/ml leukemia inhibitory factor (CHEMICON, ESGRO,

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ESG1107),  $10^{-4}$  M  $\beta$ -mercaptoethanol,  $1\times$  MEM nonessential amino acid (Gibco, Grand Island, NY) and 100 mM of sodium pyruvate (Gibco, Grand Island, NY) for 5 h. The ES medium was changed with fresh ES medium and medium was changed every day. The ES cells were passaged before ES cell colonies are contacting each other.

### Preparation of 3D-Conditioned Matrix and Reprogramming Induction

Approximately 100,000 TT2 ES cells in compact colonies were seeded onto a 3D matrix comprised of growth factor-reduced Matrigel (14 mg/ml; BD Biosciences) and were cultured in the presence of CF-1 feeder conditioned medium for 3 to 4 days. Subsequently, the TT2 ES cells were removed from their 3D matrix treatment of 20 mM  $\text{NH}_4\text{OH}$  followed by thorough washes with double-distilled  $\text{H}_2\text{O}$ , phosphate-buffered saline (PBS) and complete medium. Alternatively, CF-1 feeder conditioned medium without ES cells were used for preparation of control conditioned 3D matrix. To induce reprogramming of cancer cells, each  $1\times 10^5$  A431 were seeded onto 12-well ES- or control-conditioned 3D matrix with complete growth medium described as above. The cells were induced reprogramming by culturing at 5%  $\text{CO}_2$  incubator for 3~4 days. At the end point, the cells were subjected further analysis of reprogramming.

### Cell Viability Test

Cell viability was determined by trypan blue stain. Cells were washed with DPBS and then incubated with 0.2% trypan blue in PBS (150 mM NaCl in 5 mM Phosphate buffer, pH 7.4) for 5 min at room temperature. After the reaction, we removed the trypan blue solution and wash several times with PBS. Then, take a picture under the light stereomicroscope. For the cell counting, trypsinization, harvest the cells and then put the 0.2% trypanblue in PBS. Counting the number of total and calculated a percentage of the total cell number.

### Bisulfite Sequencing

Genomic DNA was isolated from A431 cells cultured for 4 days on ES-conditioned 3-D matrix using the All-Prep DNA/RNA/Protein extraction kit (Qiagen, GmbH, D-40724 Hilden, Germany) according to manufacturer's suggested protocols. Bisulfite conversion was analyzed using the CpGenome Fast DNA modification kit (Chemicon, Temecula, CA) according to the manufacturer's suggested protocols. Converted DNA was amplified by PCR using specific primers for human *Oct3/4* (-1,500~-1,000 of human *Oct3/4*): sense, 5'-AGAGGAAAGGAG-GAATTAGA TAT-3' and antisense, 5'-CTCCCTCTCA-ACCAAAACT-3'); and human *Nanog* (-800~-200 of the human *Nanog*: sense, 5'-AGAGTGAATGAAAGAG-GAAA-3' and antisense, 5'-AACTTTTAAATCAAAAAT-

ATAATT-3'). The PCR conditions were 1 cycle of 95°C for 5 min, 35 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min, and 1 cycle of 72°C for 15 min. The amplified PCR products were introduced into the pGEM-T easy vector (Promega, Madison, WI) and sequenced. The DNA sequences were compared with genomic DNA sequences from the cells that were not cultured on conditioned 3-D matrix and the NCBI human genomic DNA database.

### RNA Extraction and Real Time RT-PCR

Total RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's suggested protocol and 1  $\mu\text{g}$  of total RNA was used for reverse transcription with superscript II RNaseH- reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer oligonucleotides. Gene expressions of *Nanog*, *Oct4*, *c-Jun* and *c-Fos* were analyzed by real-time polymerase chain reaction (PCR) with gene specific primers (*Nanog*, Hs02387400 g1; *Oct4*, Hs01895061 ul; *c-Jun*, Hs00277190; *c-Fos*, Hs00170630 ml and *beta-actin*, Hs99999903 ml) and TaqMan gene expression human primer/probe sets using 7500 real-time PCR machine (Applied Biosystems, Foster, CA) and data analysis was performed by the Applied Biosystems Sequence Detection Software (Version 1.2.3). To validate gene expression, semiquantitative and cycle dependent RT-PCR was performed with same samples for the real-time PCR at the 1 cycle of 95°C for 5 min, 40 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec, and 1 cycle of 72°C for 15 min.  $\beta$ -actin was used for normalization of gene expression as internal control.

### 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium Assay

A431 cells ( $1\times 10^3$ ) exposed with conditioned 3D matrix for 4 days were trypsinized, replaced into 96-well plates in 100  $\mu\text{l}$  of complete medium and incubated in a 37°C, 5%  $\text{CO}_2$  incubator. After culturing for 2 hr (0 h for cell proliferation assay), 20  $\mu\text{l}$  of 5%  $\text{CO}_2$  incubator and then CellTiter 96<sup>®</sup> Aqueous One Solution (Promega, Madison, WI, USA) were added to each well and cells were then incubated for 1 h at 37°C and 5%  $\text{CO}_2$ . To stop the reaction, 25  $\mu\text{l}$  of a 10% SDS solution were added and absorbance was measured at 492 and 690 nm. The cell proliferation was measured every 24 h interval with spectrophotometric plate reader (Labsystems Multiskan MS).

### Cell Cycle Analysis

A431 cells that were exposure conditioned 3D matrix for 4 days were harvested described as an above and cells were washing with PBS, the cells were resuspended in 0.4 ml of PBS and 1 ml of ice-cold absolute ethanol was added and mixed immediately. Cells were

fixed at  $-20^{\circ}\text{C}$  for a minimum of two hours and then washed with PBS. Cells were incubated with propidium iodide,  $20\ \mu\text{g}/\text{ml}$  and RNase,  $200\ \mu\text{g}/\text{ml}$ , for 30 minutes at room temperature in the dark. Cells were analyzed on a Becton Dickinson FACS Calibur flow cytometer (BD Biosciences, San Jose, CA). Intact cells were gated in the FSC/SSC plot to exclude small debris. Cell cycle was determined using Mod Fit LT software (Verity Software House, Inc., Topsham, ME).

### Telomerase Activity Assay

High molecular weight genomic DNA from A431 cells exposed conditioned 3D matrix for 4 days was isolated by phenol-chloroform-isoamylalcohol extraction or by using the All Prep DNA/RNA/Protein kit (Qiagen, GmbH, D-40724 Hilden). Telomerase activity was analyzed using the TRAPeze XL Telomerase Detection Kit (Chemicon, Temecula, CA) according to manufacturer's suggested protocol. Twenty-five microliters of the PCR product was mixed with  $5\ \mu\text{l}$  loading dye containing bromophenol blue and xylene cyanol ( $0.25\%$  each in  $50\%$  glycerol/ $50\ \text{mmol/l}$  ethylenediaminetetraacetic acid [EDTA]), then electrophoresed on  $10\%$  non-denaturing polyacrylamide gel (no urea) at 150 volts until the bromophenol blue just ran off the gel. After electrophoresis, the gel was stained with ethidium bromide. The TRAP products were visualized as a ladder with six base increments starting at 50 nucleotides. Telomerase activity was expressed as a signal intensity of the ladder measured by Image J computer program (v1.41), compared with that of TSR8.

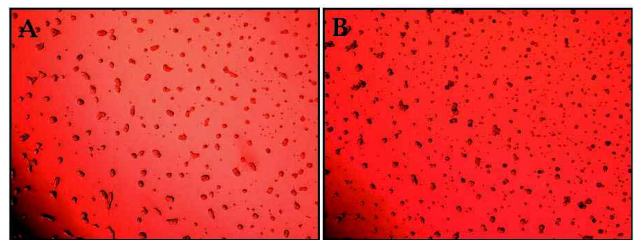
### Cellular Aging Assay

Cellular senescence of the cells from A431 cells exposed with conditioned 3D matrix for 4 days was analyzed by pH 6.0-dependent  $\beta$ -galactosidase expression a marker for senescence along with senescent related morphology (5). After the cells were exposed with conditioned 3D matrix for 4 days, cells were washed twice with PBS, fixed with  $0.2\%$  glutaraldehyde for 15 min, washed with PBS and then incubated with staining solution ( $1\ \text{mg}/\text{ml}$  5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactosidase in dimethylformamide,  $5\ \text{mM}$  potassium ferrocyanide,  $5\ \text{mM}$  potassium ferricyanide,  $150\ \text{mM}$  NaCl,  $40\ \text{mM}$  citric acid/sodium phosphate, pH 6.0, and  $2\ \text{mM}$   $\text{MgCl}_2$ ). Following overnight incubation at  $37^{\circ}\text{C}$ , the cells were washed twice with PBS, and photographed under a light microscope.

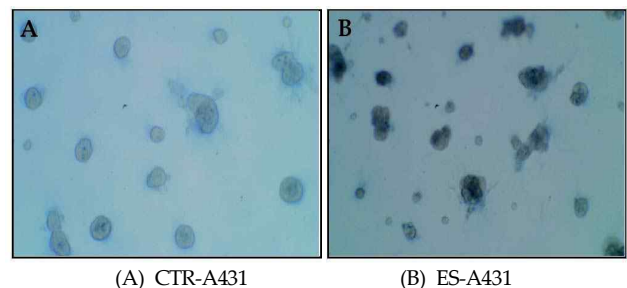
## RESULTS

### The ES-Preconditioned Microenvironment Induces Morphological Changes

In previous study, ES-preconditioned microenvironment altered the cancer cell properties in human melanoma cells (11). We modified the 3-D-matrigel matrix culture model using ES cell (TT2 mouse ES cell)-preconditioned matrigel matrix (18) and used same method as before we published (11). An approximate  $0.5\text{-cm}$  thickness of matrigel was prepared and a single suspension of ES cells was seeded onto the matrigel. The cells were maintained with ES culture medium for 4 days and then the medium was removed. This is designated as ES-preconditioned matrigel (ES-matrigel). Single cell suspensions of A431 cells were seeded onto the ES-matrigel with complete growth medium and cul-

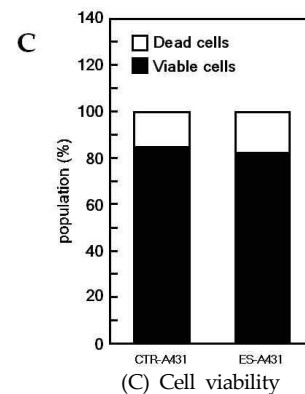


**Fig. 1. The microenvironment of mouse embryonic stem cells induces shrunk colony formation (A-B).** A431 cells were showed the normal colony formation on the Matrigel Matrix (A), small and shrunk colony formation on the ES conditioned Matrigel Matrix (B).



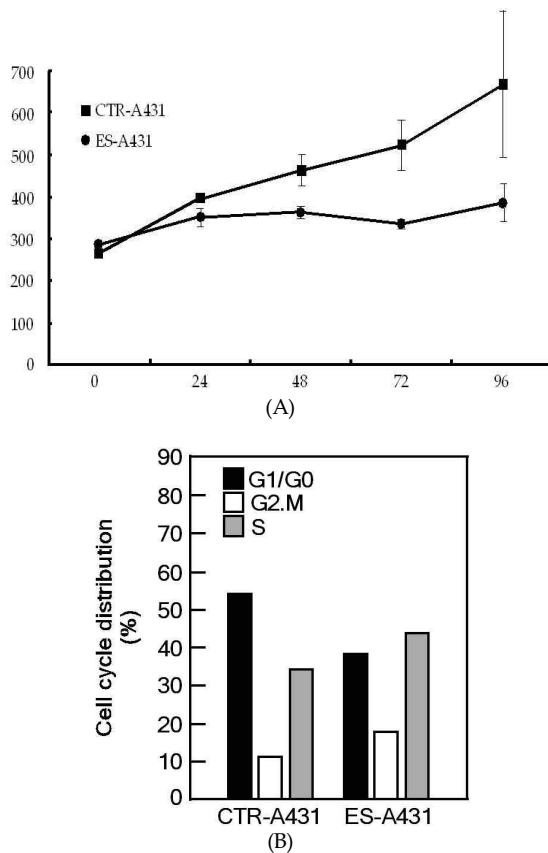
(A) CTR-A431

(B) ES-A431



(C) Cell viability

**Fig. 2. Change the cell viability of cancer cells on the control and ES conditioned matrix.** A431 cells were cultured for 3day with or without ES cell preconditioning. The viabilities were compared to that of control and ES cell preconditioning. Data are shown as means  $\pm$ S.E.M.

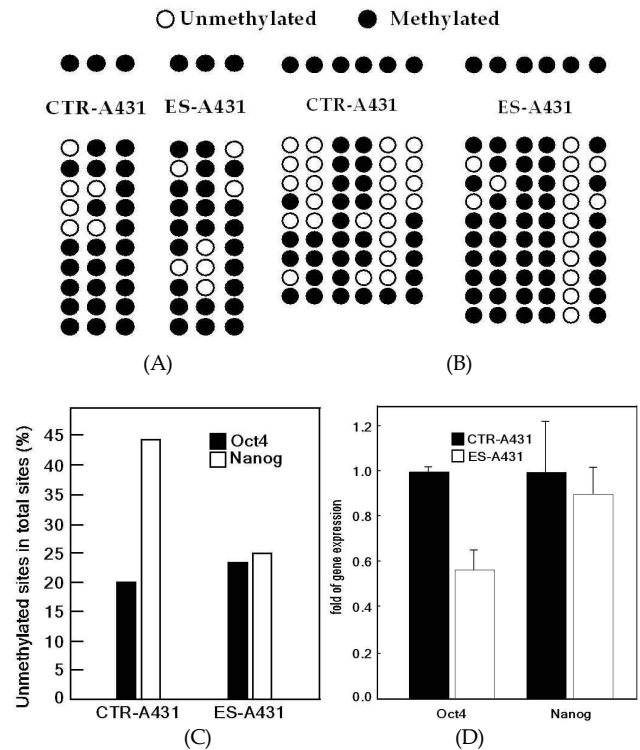


**Fig. 3. ES conditioned matrix matrigel suppresses cancer cell proliferation.** The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium assay was used to examine proliferation of cancer cells. A431 that exposed with ES cells showed a marked decrease in the rate of proliferation compared with control group (A). A431 cells also exhibited an decreased rate of cell proliferation but not causing cell accumulation of G1 phase (B).

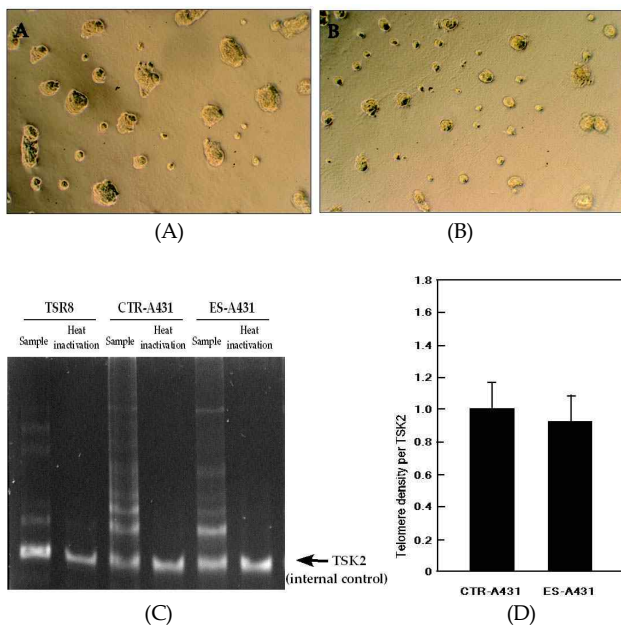
tured for 4 days. We observed a significant inhibition of A431 cell growth when cells were cultured on ES-matrigel compared with control (Ctr)-matrigel (Fig. 1). To determine whether this morphological change is related to cell viability, we performed trypan blue staining. The results showed no significant difference in viability between cells cultured on Ctr- or ES-matrigel, suggesting that the ES-matrigel matrix doesn't affect cancer cell viability (Fig. 2). Also we count total cell number of A431 cells cultured in Ctr- or ES-matrigel. The results showed ES-matrigel reduced cell proliferation (Fig. 3(A)). On the basis of these data, we investigated cell cycle progression at this time point. The ES microenvironment consistently increased the number of cells in the G1/G0 cell cycle phase and suppressed the S and G2/M cell cycle phases compared with control (Fig. 3(B)). Therefore, the ES-preconditioned microenvironment seems to alter A431 cell morphology and reduced cell proliferation without affecting viability.

### The ES-Preconditioned Microenvironment Induces Loss of Cancer Cell Properties

Epigenetic alterations is one of the criteria for reprogramming processes. Previous study indicated that the 3-D matrix preconditioned by human ES cells induces melanoma spheroid or shrink formation and epigenetic changes in melanoma cells (11, 18). Also, another study revealed that the methylation status of *Oct3/4* and *Nanog* is altered by reprogramming of somatic cells to induced pluripotent stem (iPS) cells (17). On the basis of these data, we examined whether the ES microenvironment affects the methylation status of *Oct3/4* or *Nanog* in A431 cells. Our data generated by bisulfite DNA sequencing showed a slight increase in *Oct3/4* and decrease in demethylation of the *Nanog* promoter in A431 cells cultured on ES-matrigel compared with Ctr-matrigel (Fig. 4(A) and 4(B)). *Nanog* methylation was increased methylation and shown the percentage of un-



**Fig. 4. Bisulfite sequencing analysis of DNA methylation changes in ES cell conditioned matrigel matrix and quantitative gene expression of Oct4 and Nanog.** A431 cells cultured on the ES cells conditioned matrigel matrix or without ES cells conditioned. These cells were examined for cytosine methylation in CpG dinucleotides within shown promoter regions of the human Oct 4 (A) and Nanog (B). A431 cells were little bit increased the demethylation sites in the Oct4 region (A). In A431 cells, Nanog methylation in the promoter region was increased. (B). Diagrams show the percentage of unmethylated sites (C). Quantitative RT-PCR analysis of Oct4 and Nanog. ES-A431 cells expressed less than 0.7-fold lower levels of Oct4 compared with CTR-A431 (D first), whereas Nanog expression was little bit decreased (D second).



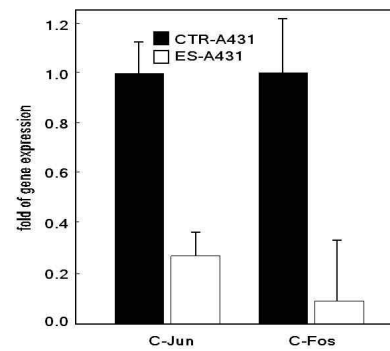
**Fig. 5. Senescence B-galactosidase staining and telomerase activity of cancer cells by ES conditioned matrigel matrix.** ES cells were seeded at a density of 50,000 cells per twelve well plate and 3 days later the ES cells were replaced with cancer cells (B), or without ES cell preconditioned matrix and seeded cancer cells (A). Three days later the cells were washed, fixed and stained for SA-b-gal (Dimri *et al.*, 1995). Effects of the ES cells preconditioned matrigel matrix on telomerase activity in cancer cells. The intracellular activity of telomerase by ES preconditioned matrigel matrix (C) and graphic representation of the experiment (D). TSK8 (positive control); Lanes 1~2, CTR-A431; Lanes 3~4, ES-A431; Lanes 5~6. All samples had negative controls that used heat inactivated samples. Both cancer cells have no different telomerase activity and negative control didn't get any telomere band (C).

methylated sites (Fig. 4(C)). These results imply that the ES microenvironment might not dramatically induce epigenetic changes and suggests instead that another mechanism might be involved in reprogramming.

#### The ES-Preconditioned Microenvironment Not Induces Cellular Senescence

In this context, we reasoned that the ES-preconditioned microenvironment might induce cellular senescence. To examine this possibility, we analyzed cellular senescence-associated  $\beta$ -galactosidase activity by staining with X-gal in A431 cells cultured on ES-matrigel. A431 cells cultured on ES-matrigel (Fig. 5(B)), but not Ctr-matrigel (Fig. 5(A)), were not stained by X-gal, suggesting that the ES-preconditioned microenvironment can't induce cellular senescence in A431 cells.

Consistent with this observation, the telomerase activity was no change in A431 cells cultured on ES-matrigel (Fig. 5(C) and (D)). Therefore, these results showed that the ES cell-preconditioned microenvironment was not related to inducing cellular senescence.



**Fig. 6. Quantitative RT-PCR analysis of CTR-A431, ES-A431. quantitative gene expression of c-Jun and c-Fos (A).** Cancer cells exposed with ES conditioned matrix matrigel were loss of oncogenic gene expression, ES-A431 cells expressed less than 0.3-fold lower levels of c-Jun compared with CTR-A431 (first), whereas c-Fos expression had 0.7-fold decreased (second).

#### ES-Preconditioned Microenvironment Suppress Oncogenic Gene Expression

Interestingly, our real-time PCR data revealed that mRNA level of *c-jun* was decreased about 70% in cells cultured on ES-matrigel compared with control (Fig. 6). Moreover, *c-fos* expression robustly suppressed about 90% compared with control (Fig. 6). These results indicate that ES-preconditioned microenvironment suppresses oncogenic gene expression such as *c-jun* and *c-fos*, results in inhibition of A431 cell proliferation. These results strongly support ES microenvironment that suppresses the tumorigenic phenotype of A431.

## DISCUSSION

The 3-D microenvironment culture system using ES cells was originally developed by the Hendrix group (18). This system consists of complex experimental steps, such as seeding the ES cells onto a 3-D matrigel matrix for 3~4 days, remove out the ES cells, and washing the matrix. We change this system slightly and follow the method before we published (11). Instead of using melanoma cells (SK-MEL-28), we observed epidermoid carcinoma cells (A431). Before, we observed a dramatic change in the morphology of human SK-MEK-28 and SK-MEL-5 and mouse B16 melanoma cells with culturing in the mouse ES-preconditioned microenvironment. Furthermore, independent cell growth and anchorage-dependent colony cell growth were markedly decreased by the ES-preconditioned microenvironment. We speculated that A431 cells have a same effect as a melanoma cells by the ES-preconditioned microenvironment. We show that same dramatic change in the morphology (Fig. 1), cell proliferation (Fig. 3), oncogenic gene (*c-Jun* and *c-Fos*) by the ES-preconditioned microenvironment in A431 cells. But we also show a di-

fferent phenomenon for methylation (Fig. 4(A) and 4(B)), stem cell marker gene expression (Fig. 4(D)), telomerase activity (Fig. 5(C) and 5(D)), and senescence b-gal staining (Fig. 5(A) and 5(B)). This phenomenon might be regulated by another cellular mechanism not a lefty1(18) and Gremlin(11).

Interestingly, we found that the ES microenvironment induces oncogenic gene (*c-Jun* and *c-Fos*) expression of A431 cells. Activating protein-1 (AP-1) is a dimeric transcription factor composed primarily of members of the Jun family of proteins (*c-Jun*, Jun B, Jun D) that dimerize to form homodimers or heterodimers with Fos family of proteins (*c-Fos*, Fos B, Fra-1, Fra-2) (7, 22). AP-1 has linked to cellular transformation as oncogenic H-Ras and v-Src and other tumor promoters can induce AP-1 activity (20, 22, 24) 24). AP-1 component proteins are overexpressed in various cancers and *c-Jun* has also been associated with a more aggressive phenotype in cancer cells (1, 4, 13, 16, 19, 21, 25, 26). The altered gene expression of AP-1 in A431 cells is implicated in tumorigenic process, including cell proliferation (Fig. 3A). Ap-1 also involved to regulation of cancer cell progression and proliferation (20). Therefore, inhibition of AP-1 component results in decreased proliferation in A431 cells.

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