

# 배양조건에 의한 HPV16 E6/E7 불멸화 구강상피세포의 특성 변경

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〈Abstract〉

## Alteration of Epithelial Properties by Culture Condition in HPV16 E6/E7-immortalized Human Oral Keratinocytes

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Cellular microenvironment is an essential issue for regulating epithelial characteristics through the alteration of intricate signaling pathways and intercellular communications in different cell types. Thus, microenvironment influences tumor initiation, progression, and metastasis. This study aimed to investigate the relationship between microenvironment and epithelial property in HPV16 E6/E7-immortalized human oral keratinocytes (IHOKs). To investigate characteristics of IHOK cultured in different media, two media were used, which included keratinocyte growth media (KGM), F-medium composed of 3:1 ratio of DMEM and F-12 (P media) supplemented with 10% FBS and 1% penicillin/streptomycin. Proliferative property and invasive and migratory activity were observed. As results, proliferating activities of IHOK in different culture condition were changed. Likewise, migratory and invasive activities were also different depending on media types. These results suggest that cellular microenvironment can affect modification of biological properties of epithelial cells.

*Key words* : Microenvironment, Invasion, Media, Immortalized Oral Keratinocytes

### I . Introduction

Microenvironmental signals impact the ability of cells to adapt to the local environment<sup>1)</sup>. Homeostasis of normal tissue is preserved by vigorous interactions between epithelial cells and their microenvironment. Disrupting this homeostasis can induce aberrant cell proliferation, adhesion, and migration that might promote malignant behavior<sup>2)</sup>. In

cancer, the tumor microenvironment is characterized by communication such as cell to cell interactions and cell to extracellular matrix (ECM) contacts, and by the chemical environment such as soluble factors secreted by the cells and the diverse nutrient, and by the mechanical aspects<sup>3)</sup>. Indeed, microenvironment in tumor influences tumor progression, metastasis, and generation of tumor initiating cells<sup>4)</sup>. Therefore, study for cellular microenvironment is important for understanding of epithelial physiology and therapeutic approaches of cancer treatment<sup>5)</sup>.

Human keratinocytes have been cultured by different growth media to investigate the relation between keratinocytes and microenvironment. In the beginning of keratinocytes culture, 3T3 feeder layer was used to improve

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proliferation of keratinocytes<sup>6)</sup>. On the other hand, there was a culture system used both 3T3 feeder layer and fibroblast conditioned medium. In addition, serum-free medium or low calcium serum-free medium except feeder fibroblasts was feasibly used in keratinocyte culture<sup>7,8)</sup>. These different culture conditions affected to proliferation and differentiation of keratinocytes.

HPV E6/E7-immortalized human keratinocytes (IHOKs) have been used in keratinocyte differentiation and cancer research and are considered to be a very good as model cell line for studying epithelial cancers<sup>9)</sup>. Culture conditions can have a significant influence on motility and cell growth.

In this study, IHOKs that were cultured in different media showed various modifications in growth, migration and invasion. Because simple biological research without comprehensive explorations about culture conditions can lead to wrong direction of study, this study aimed at investigating the modulation of epithelial properties by microenvironment in different culture conditions.

## II. Materials & Methods

### 1. Reagents and Antibodies

Antibodies against p21 (mouse monoclonal antibody, 1:2000), CDK4 (mouse monoclonal antibody, 1:2000), vimentin (rabbit polyclonal antibody, 1:1000), and cyclin D1 (rabbit polyclonal antibody, 1:1000), were obtained from Cell Signaling Technology, Beverly, MA, USA. Antibodies against p27 (mouse monoclonal antibody, 1:500) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma, respectively. Type I collagen was purchased from Cellmatrix (Japan).

### 2. Cell culture

We used IHOK, which was reported before<sup>10)</sup>. For

analyzing changes of properties according to different media, two of keratinocyte culture media were used. IHOK was cultured in keratinocyte growth media (KGM, Lonza, MD, USA) with supplementary bullet kit (Lonza, MD, USA). IHOK cultured in KGM was named as IHOK-KGM. F-medium was consist of Dulbecco's Modified Eagles Medium (DMEM; Gibco BRL, USA) and Ham's Nutrient Mixture-F12 (Gibco BRL, USA) culture medium at a ratio of 3:1(P media), supplemented with 10% Fetal bovine serum (FBS) and 1% penicillin/streptomycin. IHOK cultured in F medium was named as IHOK-F. Normal oral gingival fibroblasts were derived from patients who underwent wisdom tooth extraction without mucosal disease. Informed consent was given by the patients for this study (IRB-2-2009-0002). Gingival fibroblasts were selected by a verseyne solution (0.2% EDTA in PBS) supplemented with 0.04% glucose from explanted tissues; the cells were maintained in F media.

### 3. Immunoblotting

Cells grown to 70% confluency were lysed in Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA). After lysis, protein complexes were boiled for 5 min in sodium dodecyl sulfate (SDS) sample buffer and separated on 10% SDS-PAGE. Then, proteins were transferred and then blocked in 5% milk in TBST (tris buffered saline contained tween 20). Blots were then incubated with appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and detection was done by chemiluminescence (Santa Cruz Biotechnology).

### 4. Cell proliferation assay

The proliferation of IHOKs was measured by MTT [3-(4,5-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. After seeding cells, the absorbance was measured for

up to 72h. The experiments were performed in triplicate.

## 5. Transwell invasion assay

IHOKs were cultured to 70% confluence. Harvested cells were resuspended in P media for IHOK-F and in KGM without supplementary bullet kit for IHOK-KGM. Then cells were seeded in upper wells of 24-well transwell chambers (Coster) coated with Type I collagen for 48h. And each culture media were put in the lower wells and upper wells seeded cells were combined with lower wells. After incubation for 48h cells were fixed in 10% formalin solution and stained in 0.025% crystal violet (Junsei, Japan). The invaded cells were counted and the mean for each chamber was determined. Each invasion assay was performed in triplicate.

## 6. Transwell migration assay

The transwell migration assay was carried out without coating Type I collagen and other procedures were same to above transwell invasion assay.

## 7. Three dimensional cell culture

For 3-dimensional culture, 300  $\mu$ l of a type I-A collagen mixture was made by mixing 8 volumes of an ice-cold collagen solution with 1 volume of 10 $\times$  reconstitution solution and 1 volume of 10 $\times$  DMEM. Normal gingival fibroblasts ( $1.5 \times 10^5$  cells, IRB-2-2009-0002) were then added to the mixture. The mixtures of collagen and normal gingival fibroblasts were transferred to a Millicell (3.0  $\mu$ m pore size, 12 mm diameter; Millipore, MA, USA). After 24h, two of IHOKs ( $3 \times 10^5$  cells) were put on the dermal equivalents and submerged in the culture medium (P media supplemented with 10% FBS, 1% penicillin/streptomycin, 0.1  $\mu$ g/ml cholera toxin, 0.4  $\mu$ g/ml hydrocortisone, 5  $\mu$ g/ml insulin, 5  $\mu$ g/ml apo-transferrin, and 2  $\mu$ g/ml 3'-5-triiodo-L-thyronine)

for 4 days. The cells were then removed the medium from the surface and cultured for 2 weeks. The tissues were fixed in 10% formalin and embedded in paraffin. The tissues were analyzed histologically through staining with hematoxylin and eosin.

## 8. Gelatin zymography

MMP-2 and MMP-9 activities were analyzed by gelatin zymography. IHOKs were incubated in each culture media for 48h. After electrophoresis using 8% SDS polyacrylamide gel copolymerized with 0.2% gelatin, the gel was renaturated in zymogram renaturation buffer (Bio-Red Laboratories) at room temperature for 1 h, and then incubated in zymogram development buffer (Bio-Red Laboratories) for 16h at 37 $^{\circ}$ C. The gels were stained with 0.25% Coomassie blue and then destained.

## 9. Statistical analysis

Data were analyzed for comparing difference between control and experimental groups in invasion assay, transwell migration assay, three dimensional cell culture and Zymography. were used. The significant difference determined through Mann-whitney test (SPSS Inc, IBM, Chicago, US) in results was reported as  $P < 0.05$ .

# III . Results

## 1. Modulation of cell morphology by culture conditions

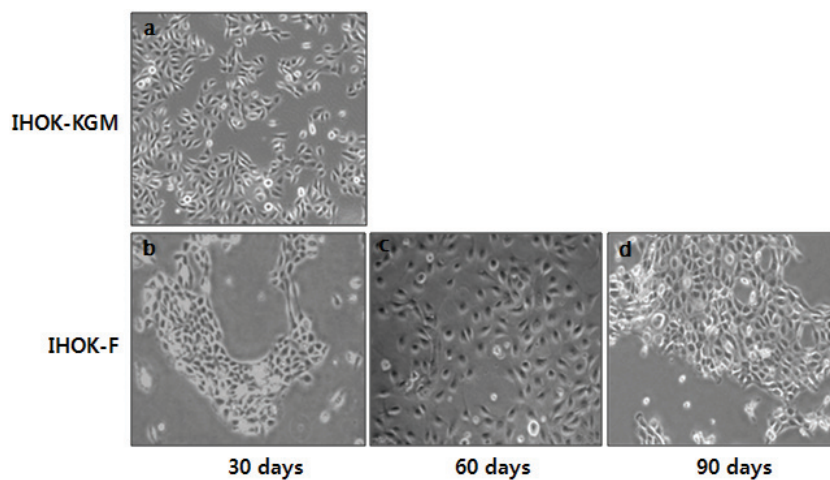
HPV E6/E7 IHOKs, which exhibit epithelial cobblestone-like morphology, were maintained in KGM media (IHOK-KGM). To investigate the changes of keratinocytes property by different culture conditions, IHOK-KGM was

cultured in F media (IHOK-F). When IHOKs were cultured in F media for 30 days, the cells were made clusters, and in 60 days, the cells were lost cell to cell contacts and scattered. However, when these cells were cultured for 90 days, IHOK-F showed increased size of cytoplasm and clustered morphology similar to early stage of culture(Fig. 1).

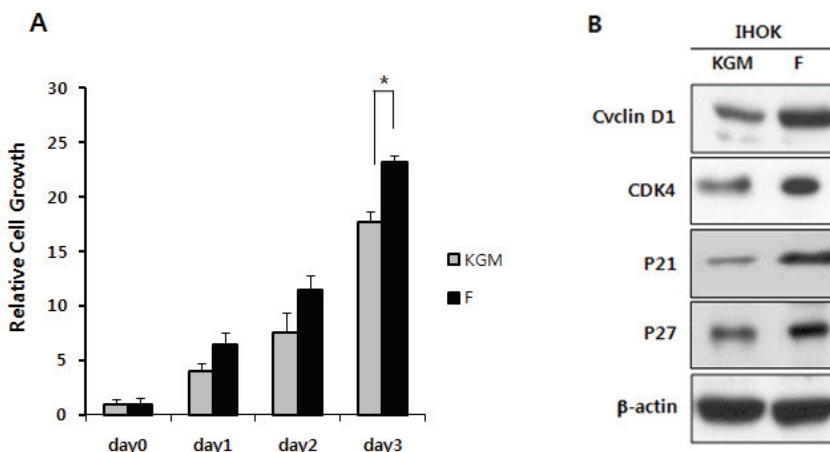
## 2. Growth activity and expression of cell cycle regulating factors by different culture conditions

To compare the proliferation rate between IHOK-KGM and IHOK altered by culturing in different media for 90 days, MTT assay was performed. Relative growth rate of

IHOK-F was higher than that of IHOK-KGM for 3days (Fig. 2A). Because some difference was shown in growth rate between IHOK-KGM and IHOK-F, we investigated cell cycle regulating proteins using Western blotting. Cyclin D1 and CDK4, cell cycle turning protein, were upregulated in IHOK-F compared to IHOK-KGM. Likewise, p21 and p27, cell cycle inhibitors, were up-regulated in IHOK-F, exhibiting complex microenvironmental change by different culture conditions (Fig. 2B). These results indicate that different culture conditions can alter proliferative capability and the expression of cell cycle regulating proteins.



**Fig 1. Culture of IHOK using two different media**  
Control IHOK-KGM cells were cultured in two different media, a, Control IHOK-KGM cells; b, c, and d, IHOK-KGM cells were cultured in F media for 30day, 60days, and 90days, respectively.



**Fig. 2. Effect of culture microenvironment in cell proliferation and cell cycle regulating protein expression of IHOKs.**

A, Cells were cultured in each culture media. Cell viability was measured by MTT assay for 3 days (\* $p < 0.05$  by  $t$ -test). B, IHOKs were cultured for 90 days by two media, Western blotting was carried out for expression of cell cycle turning proteins (Cyclin D1, CDK4) and cell cycle inhibitors (p21, p27) in each IHOK.

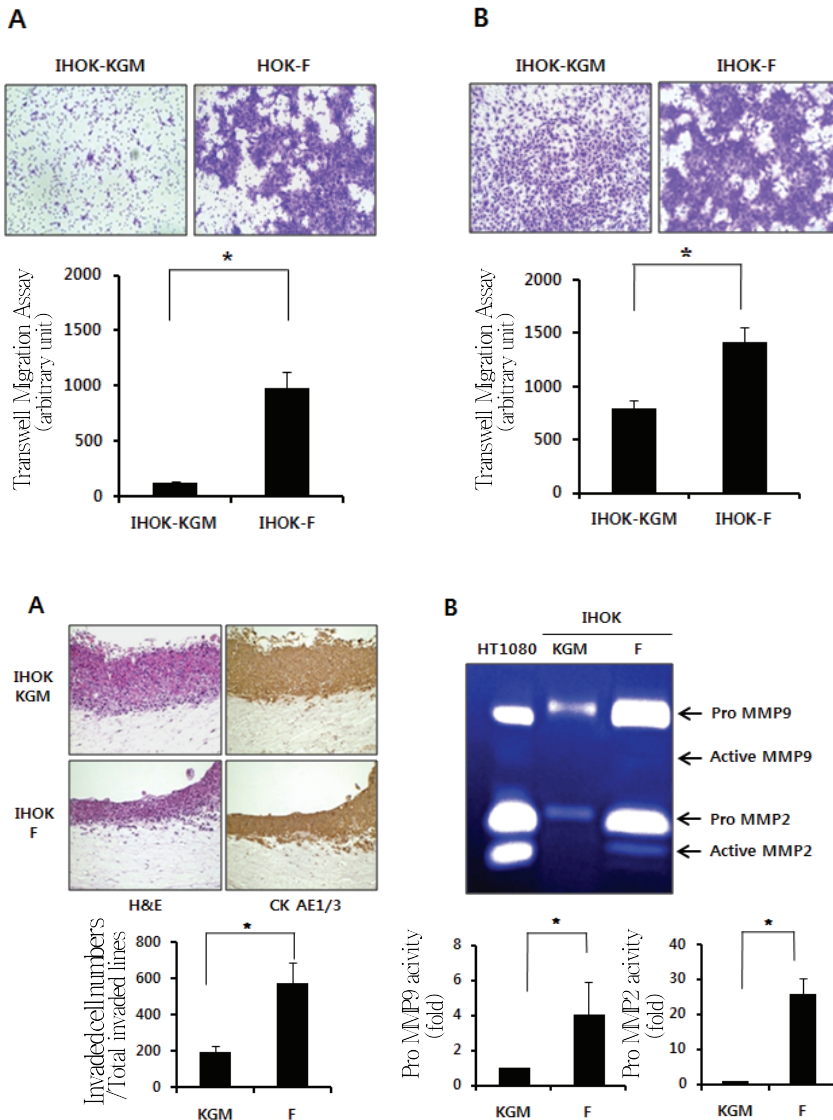
### 3. Transwell migratory and invasive activity of cells cultured by different media

To determine whether there are some difference in the cell migratory and invasive activity according to media conditions, transwell migration assay and invasion assay were carried out. After 48h, migrated and invaded cells were measured. In case of applying each culture media to lower chamber of transwell, migratory activity of IHOK-F was higher than IHOK-KGM by 7.66 fold ( $p < 0,05$ ) (Fig. 3A). In addition, invasive activity of IHOK-F in type I

collagen-coated transwell was higher than IHOK-KGM by 1.79 fold ( $p < 0,05$ ) (Fig. 3B). Altogether, these results indicate that IHOK cells have acquired prominent migratory and invasive capability in F media.

### 4. Invasiveness of IHOKs in 3-dimension cell growth model and Zymography

The invasive activity of two IHOKs was assessed by a 3-dimensional cell growth model which is alike to *in vivo* conditions. Two IHOKs showed infiltrative growth into the



**Fig. 3. Transwell migration and invasion activities of IHOKs by two different media**

A, Each cell line was seeded on a transwell chamber, and the cells migrated to the other side of the chamber were counted at 48hr. In lower chamber, each culture media were applied. B, For invasion assay, each cell line was seeded on a transwell chamber precoated with collagen type I. After 48h, cells invaded the collagen and moved to the other side of the chamber were counted. Each assay was performed in triplicate ( $*p < 0,05$  by *t*-test).

**Fig. 4. Assessment for invasive activity of two IHOKs using three-dimensional culture and Zymography.**

A, IHOK-KGM and IHOK-F cell lines were seeded on top of mixture of normal gingival fibroblasts and collagen type I. IHOKs were cultured under air-liquid condition for 14 days. Left side: hematoxylin-eosin staining, Right side: immunohistochemical staining for cytokeratin AE1/3. B, Each cell was incubated in each culture media for 48h. The conditioned media were collected and subjected to gelatin zymography. Gelatin zymography was performed using electrophoresis gels contain 0,2% gelatin ( $*p < 0,05$  by *t*-test).

dermal equivalent, however, invaded cell numbers were higher in IHOK-F than in IHOK-KGM by 3 fold ( $p < 0,05$ ) just like in transwell assay. For discrimination of IHOKs and fibroblasts of dermis, immunohistochemical staining of cytokeratin AE1/3 was carried out (Fig. 4A). This invasive activity was established by gelatin zymography. When conditioned media from each cell was used, gelatinolytic activity of pro MMP-9 and pro MMP-2 were remarkably up-regulated in IHOK-F compared with IHOK-KGM as 4 fold and 25,9 fold, respectively (Fig. 4B).

#### IV. Discussion

The components of media used to culture have been widely investigated for growth and proliferation of cells, without consideration as to modification of diverse properties. In this study, we showed properties alteration of IHOK by two different media. KGM used for establishing immortalization of oral keratinocytes is specially designed to serum-free and low-calcium (0,15 mM) medium to support the growth of human and animal primary derived cells. Serum-containing culture media significantly increase the amount of undesired cells such as fibroblasts and melanocytes in primary culture of oral or skin epithelial cells. The characteristics of keratinocytes stem cell are better preserved in serum-free media<sup>11</sup>. Keratinocytes usually proliferate in low-calcium medium (0,15mM CaCl<sub>2</sub>) and differentiate in high-calcium medium<sup>12</sup>. Therefore, low-calcium (less than 0,15 mM) medium was developed to permit long-term proliferation of cells<sup>13</sup>, and to maintain a monolayer culture and prevent differentiation<sup>14</sup>.

In this study, IHOK-F cultured in serum-containing F media showed higher growth rate, migratory activities, and invasive activities than IHOK-KGM, suggesting that different growth media induce different properties of culture cells.

In current study, characteristics change of IHOKs in different media was considered to be due to change of media condition. Besides IHOK, phenotype changes for cancer cell and fibroblast in different culture conditions were reported. For example, biochemical changes of human small cell lung cancer associated to culture conditions were reported<sup>15</sup>. In addition, alterations of drug sensitivity were examined under various culture conditions in colon cancer cell lines<sup>16</sup>, and a comparison of metabolic activity was reported in prostate cancer cell culture<sup>17</sup>. Currently, protein profile of human dermal fibroblasts was investigated according to culture condition<sup>18</sup>.

Taken together, this study suggests that extensive and multifaceted investigations for culture conditions are required to in the way of selection for cell lines, which are used in research determining biological characteristics of some cells. In addition, it is required for cancer therapy inhibiting invasion or metastasis to develop target therapy of modifying microenvironment.

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