# Development of Isolation and Cultivation Method for Outer Root Sheath Cells from Human Hair Follicle and Construction of Bioartificial Skin

**Young-Kwon Seo[1], Doo-Hoon Lee[1], Youn-Ho Shin[1], Bo-Young You[1], Kyung-Mi Lee [1,5], Key-Yong Song[2], Seong-Jun Seo[3], Sung-Joo Whang[4], Young-Jin Kim[5], Eun-Kyung Yang[6], Chang-Seo Park[7], Ih-Seop Chang[8], and Jung-Keug Park[1]***

[1] Department of Chemical and Biochemical Engineering, Dongguk University, Seoul 100-715, Korea
[2] Department of Pathology, College of Medicine Chung-Ang University, Seoul 156-756, Korea
[3] Department of Dermatology, College of Medicine Chung-Ang University, Seoul 156-756, Korea
[4] Hair Clinic Center, CNP Cha and Park Hospital, Seoul 135-893, Korea
[5] Biomedical Research Center, Lifecord. Co., Suwon 442-749, Korea
[6] R&D Center, Bioland. Ltd., Chun-An 330-863, Korea
[7] Biotech BU, Doosan.Corporation, Kyonggi-Do 449-844, Korea
[8] R&D Center, Amore Pacific. Ltd., Kyonggi-Do 449-729, Korea

**Abstract** Obtaining a sufficient amount of healthy keratinocytes from a small tissue is difficult. However, ORS cells can be a good source of epithelium since they are easily obtainable and patients do not have to suffer from scar formation at donor sites. Accordingly, the current study modified the conventional primary culture technique to overcome the low propagation and easy aging of epithelial cells during culturing. In a conventional primary culture, the average yield of human ORS cells is $2.1 \times 10^3$ cells/follicle based on direct incubation in a trypsin (0.1%)/EDTA *(0.02%) solution for 15 min at 37°C, however, our modified method was able to obtain about* $6.9 \times 10^3$ cells/follicle using a two-step enzyme digestion method involving dispase (1.2 U/mL) and a trypsin (0.1%)/EDTA (0.02%) solution. Thus, the yield of primary cultured ORS cells could be increasd three times higher. Furthermore, a total of $2.0 \times 10^7$ cells was obtained in a serum-free medium, while a modified E-medium with mitomycin C-treated feeder cells produced a total of $6.3 \times 10^7$ cells over 17 days when starting with $7.5 \times 10^4$ cells. Finally, we confirmed the effectiveness of our ORS cell isolation method by presenting their ability for reconstructing the bioartificial skin epithelium *in vitro*

*Keywords*: hair follicle, outer root sheath cells, primary culture, bioartificial skin, tissue engineering

## INTRODUCTION

The applications of tissue engineering include the formation of living tissue for cell biology research, wound repair, test systems for therapeutics, and drug delivery [1]. Intensive investigations have already resulted in developing technologies for a variety of organs and applications.

The most advanced developments have been achieved in skin replacement [2-6]. Bioartificial skin is composed of two tissues, a connective tissue or dermis and covering epidermis. Fibroblasts migrate from adjacent tissues into the wound region, then proliferate and synthesize a collagen-rich extracellular matrix that effectively fills the wound, while the extracellular matrix of the dermis provides the structural and biological support for the epidermis [7]. Meanwhile, keratinocytes synthesize and secrete the basic fibroblast growth factor (bFGF) and endothelin-1, stimulate mitogens and the proliferation of melanocytes *in vitro,* and control hypertrophic scars and fibrosis formation in wound healing [8]. As such, one or more cell types are used in manufacturing bioartificial skin and clinical applications [9-11].

Various methods have already been developed for cultivating keratinocytes, usually involving disaggregation though a combined mechanical and enzymatic action. Many approaches include the culture of human epidermal keratinocytes on fibronectin or collagen-coated dishes or at reduced $Ca^{2+}$ levels in media containing chemically defined supplements and a bovine pituitary extract. Using growth-arrested murine 3T3 cells, Rheinwald and Green reported on the rapid growth of human neonatal and adult keratinocytes in primary cultures seeded at a low density [12].

***Corresponding author**
Tel: +82-2-2260-3365 Fax: +82-2-2271-3489
e-mail: jkpark@dongguk.edu

In the case of an allograft, since epithelial cells from adult skin have a stronger immunity than fibroblasts, the use of autologuous epithelial cells is better than allogenic cells. Yet, epithelial cells have a low propagation rate and easily age during culture periods *in vitro*, thus many attempts have been made to discover stem cells for skin epithelial cells.

Recently, stem cells for various epithelial cell populations of the skin have been located in the ORS tissues of hair follicles. In addition, since ORS cells can be regarded as undifferentiated epidermal keratinocytes, they represent a source of easily and repeatedly available keratinocytes [13]

Hair follicle epithelial cells have also been found to play a role in skin stem cell biology, while the follicular stem cell population has a dual function in making hair and contributing to the construction of the skin epidermis [14].

To further investigate the biology of ORS cells, various cultivation methods have been developed, mainly by explanting plucked human hair follicles on various growth-substrates, such as bovine eye lens capsules, collagen-coated dishes, and tissue-culture plastic. Recently, it was reported that primary cultures of dispersed ORS cells require feeder cells because of the small number of ORS cells available per hair follicle, thereby greatly reducing the plating efficiency of this cell type when cultured without feeder cells [15-20].

Normally, the primary cultivation of ORS cells from the follicles is carried out by incubation in trypsin (0.3%)/EDTA (0.02%) for 4 min at 37°C or in trypsin (0.1%)/EDTA (0.02%) for 15-20 min at 37°C, or via an explantation method [21,22], yet the yield is very low.

Accordingly, the current study developed a 2-step enzyme digestion method for cultivating ORS cells that yields twice the number of cells within a shorter time compared to explant culture methods. In addition, the use of a 3T3 feeder cell culture system was found to produce a much higher number of ORS cells than a serum-free culture method [23,24].

Finally, bioartificial skin was reconstructed using three major components, collagen, dermal fibroblasts, and ORS cells, based on a tissue engineering technique. Since the ORS cell layer plays an important role as the epidermis, it was confirmed that the cultivated ORS cells were progenitor cells of skin epithelial cells.

## MATERIALS AND METHODS

### Isolation and Cultivation of Dermal Fibroblasts

Normal human fibroblasts were obtained from surgically obtained scalp skin. A human scalp skin biopsy was aseptically carried out at the CNP Cha and Park Hospital (Myongdong Clinic, Seoul, Korea). Whole follicles dissected from the scalp skin were incubated for the ORS cell culture, while the rest of the tissue was washed three times in sterile PBS containing Antimycotic-antibicotic (Gibco BRL, Grand Islands, NY., USA, 20 mL/L). Thereafter, the tissue was digested in 1.2 units/mL dispase (Gibco BRL, Grand Islands, NY., USA.) in PBS for 16 h at 4°C and incubated with a trypsin (0.05%)/EDTA (0.02%) solution for 15 min at 37°C. A suspension consisting of single cells was obtained by vigorous pipetting in DMEM supplemented with 10% FBS (fetal bovine serum, Gibco BRL). After centrifugation for 10 min at 800 rpm, the fibroblasts were resuspended in a fresh culture medium and seeded at a density of $1 \times 10^4$ cells/cm$^2$. The medium was changed three times a week.

### Preparation of Feeder Cells

The culture of the NIH J2 3T3 feeder cells was propagated in 3 parts DMEM and 1 part Ham's F12 supplemented with 10% FBS. Confluent cultures were subcultivated in a 1:3 ratio every 3 days. To prepare the feeder cells, $1 \times 10^4$ cells/cm$^2$ were incubated in DMEM/Ham's F12 containing 10% FBS and 8 μg/mL mitomycin C (Sigma) for 1.5 h at 37°C. The post-mitotic feeder cells obtained by this procedure were then rinsed 4 times with PBS, detached with trypsin (0.05%)/EDTA (0.02%), and replated at a density of $1.0 \times 10^4$ cells/cm$^2$ in DMEM/Ham's F12 containing 10% FBS.

### Isolation and Cultivation of ORS Cells from Hair Follicles

Normal human hair follicles were obtained from surgically obtained scalp skin. A human scalp skin biopsy was aseptically carried out at the CNP Cha and Park Hospital (Myongdong, Seoul, Korea). Whole follicles were dissected from the scalp skin and washed three times in sterile PBS containing Antimycotic-antibicotic (Gibco BRL, 20 mL /L).

The isolation of the ORS cells from the hair follicles was carried out using three different methods. First, the hair follicles were loosened by incubation in 1.2 units/mL dispase (Gibco BRL, Grand Islands, NY., USA.) in PBS for 16 h at 4°C followed by incubation in a trypsin (0.05%)/EDTA (0.02%) solution for 15 min at 37°C. Second, direct incubation in a trypsin (0.1%)/EDTA (0.02%) solution for 15 min at 37°C was performed. Then, a suspension consisting of single cells was obtained by vigorous pipetting of the follicles at least 30 times in DMEM supplemented with 10% FBS. Finally, after centrifugation for 10 min at 1000 rpm, the ORS cells were resuspended in a culture medium and seeded at a density of $3.0 \times 10^3$ cells/cm$^2$. Third, an explantation cultivation method was performed after incubation in trypsin (0.05%)/EDTA (0.02%) for 15 min on a type I collagen coated tissue culture dish in a serum-free medium.

The primary cultures were performed in a modified E-medium or Keratinocyte-Serum Free Medium (K-SFM, Gibco BRL). The modified E-medium was composed of 3 parts DMEM and 1 part Ham's F12 supplemented with 10% FBS, transferrin (5 g/mL), insulin (5 mg/mL), EGF (5 ng/mL), hydrocortisone (0.4 mg/mL), cholera toxin ($10^{-10}$ M), triiodothyronine (T3, $2 \times 10^{-11}$ M), and Antimycotic-antibicotic (Gibco, 10 mL/L). In the case of the primary culture performed in the modified E-media, the

ORS cells were co-cultured with mitomycin C-treated NIH J2 3T3 feeder cells. The cells were grown in different media at 37°C in a 5% $CO^2$ incubator. The first medium change was performed after 5 days of culture.

The K-SFM consisted of an MCDB 153 medium supplemented with insulin (0.005 g/L), hydrocortisone (0.074 mg/L), T3 (0.0067 mg/L), a bovine pituitary extract (50 mg/L), and epidermal growth factor (EGF, 5 mg/L). Plus, since the calcium ion concentration of K-SFM was very low, calcium ions were added up to 0.15 mM

### Cell Growth Analysis of Cultured ORS Cells

For cell counting, single cell preparations were obtained by incubating the samples for an additional 10 min at 37°C with a 0.05% trypsin solution. Aliquots of the samples were mixed with trypan blue and the viable cells counted using a hemocytometer.

The population doubling level (PDL) was calculated using the following equation,

$PDL = \log (X_1/X_0) / \log 2$,

where $X_0$ is the initial cell number and X1 is the final cell number[25].

Plus, the rate of cell growth was characterized by the average growth rate, which was calculated as Growth rate $= (1/X)(dX/dt)$, where $X$ is the first subculture cell number and t is time.

### Statistical Analysis

Data for trypsin treatment and dispase / trypsin treatment were statistically evaluated using Student's *t*-test. Data are given as means±SD. The difference between the means was considered significant when $P \leq 0.05$.

### Construction of Artificial Skin

The *in vitro* construction of bioartificial skin was initiated by casting cell/collagen mixtures as a dermal equivalent onto a 3 mm porous polycarbonate membrane of the culture insert (MILLICELL, Millipore, USA). The dermal equivalent cultures were prepared according to the modified method of Bell *et. al.* [4]. The fibroblasts were suspended at a concentration of $5.0 \times 10^5$ cells/mL DMEM containing 10% FBS and harvested at 800 rpm by centrifugation. The cell pellet was gently stirred with a solution containing seven volumes of a 5 mg/mL collagen solution (Cell Gen, Koken, Japan), two volumes of 5 × DMEM, and one volume of 0.05 N NaOH containing 2.2% sodium bicarbonate and a 200 mM HEPES buffer solution. Aliquots of the cell/collagen mixtures were poured into the culture insert. The temperature was then raised to 37°C and the collagen solution gelled by incubation. After culturing the dermal equivalent for 2 days, the culture medium was aspirated to leave the gel surface exposed, then the ORS cells were applied to the surface at an initial density of $5.0 \times 10^5$ cells/cm$^2$. The construct was then submerged for two days in the culture medium both inside and outside the culture insert to allow the ORS cells to spread and cover the surface of the dermal equivalent. To develop a multilayered artificial skin the whole system was lifted at the air-liquid interface by removing the inside medium of the insert. After 10 days of incubation at the air-liquid interface, the bioartificial skin was fixed in 10% neutral buffered formalin for 2 h at 4°C. The fixed samples were then embedded in paraffin and 5 mm-sectioned paraffin ribbons stained with hematoxylin and eosin.

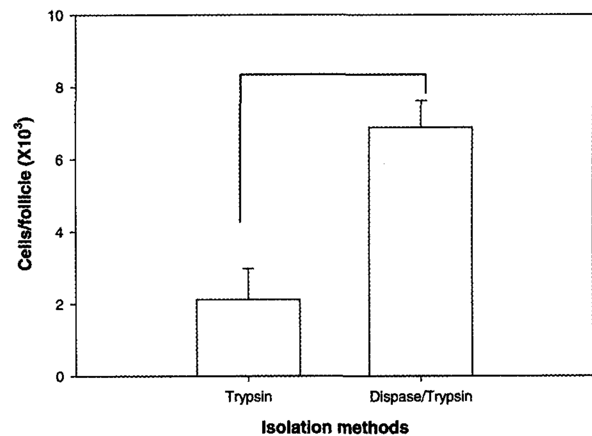


**Fig. 1.** Isolation yield of ORS cells according to enzyme digestion method. Data are shown as the mean±standard derivation for five culture from two independent isolations. $P \leq 0.01$.

## RESULTS AND DISCUSSION

### Enhancement of Primary and Cell Culture Method

The conventional method of cultivating ORS cells from hair follicles is either incubation in trypsin (0.1~0.3%)/EDTA (0.02%) for 4~20 min at 37°C or an explant cultivation method. When the concentration of trypsin or enzyme treatment time is increased, the number of primary cells recovered increases, yet the number of adhesion cells decreases and the cell morphology is transformed (data not shown).

However, the current study modified the conventional primary culture method into two-step enzyme digestion, thereby producing a highly viable primary cell yield. Normally, the primary yield of human ORS cells is about $2.1 \times 10^3$ cells/follicle after direct incubation in a trypsin (0.1%)/EDTA (0.02%) solution for 15 min at 37°C. Yet, $6.9 \times 10^3$ cells/follicle was achieved with the proposed two-step enzyme digestion method using dispase and a trypsin (0.05%)/EDTA (0.02%) solution. These results are shown in Fig. 1.

Figure 2 shows the cell growth curve during five weeks of a primary culture of human ORS cells from hair follicles in the K-SFM medium, where the inoculum cell density was $3.0 \times 10^3$ cells/cm$^2$ in a 35 mm culture dish. Based on the initiation of primary cells from five hair follicles, a yield of around $3.0 \times 10^6$ ORS cells was obtained

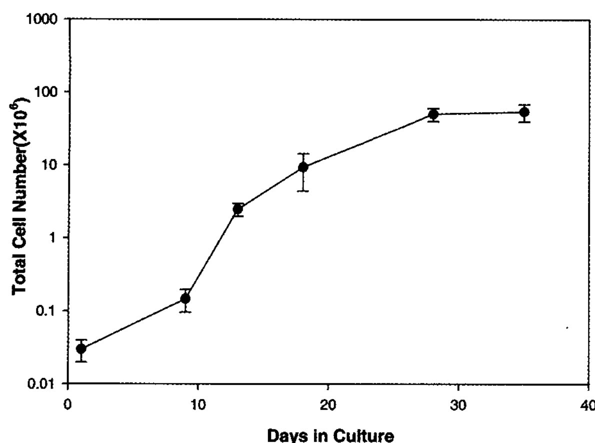


**Fig. 2.** Cell growth curve of human primary keratinocytes in K-SFM medium. A total of $3.0 \times 10^4$ ORS cells were inoculated onto a 35 mm culture dish, and the inoculum cell density was $3.0 \times 10^3$ ORS cells/cm$^2$.

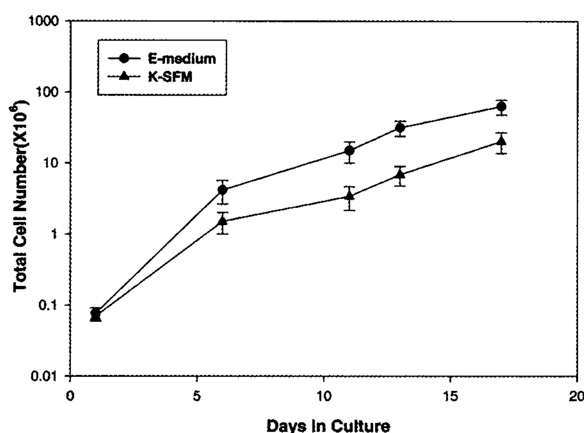


**Fig. 3.** Effect of different media on growth of human ORS cells. K-SFM indicates keratinocyte serum-free medium, while E-medium indicates DMEM/Ham's F12 with added supplements and 10% fetal bovine serum. The inoculum cell density was $7.5 \times 10^3$ ORS cells/cm$^2$. The error bars show the mean standard deviations for more than five determinations.

within 2 weeks, while a total of $4.0 \times 10^7$ ORS cells was obtained after 4 weeks.

The primary ORS cells exhibited donor-to-donor differences in their growth characteristics and the dish of primary cells reached a 70~80 % confluence within 7 to 10 days followed by sequential transfers. The doubling time during the lag phase in the primary culture was 36~48 h, while the doubling time during the exponential phase of the ORS cells was 16~20 h.

Fig. 3 shows the effect of different media on the growth of the human ORS cells. The modified E-medium was used with mitomycin C-treated NIH J2 3T3 feeder cells. The modified E-medium was prepared by DMEM and Ham's F12 media supplemented with 10% FBS, transferrin, insulin, EGF, hydrocortisone, cholera toxin, and T3. In the current experiment, the calcium ion concentrations in the K-SFM and modified E-medium were 0.15 mM and 1.5 mM, respectively.

When considering the growth curve and cell morphology, serum supplementation and co-culturing with feeder cells in the modified E-medium system promoted cell proliferation and maintained a normal morphology. Calcium is necessary for desmosome formation, stratification, and terminal differentiation in human epithelial cells [26]. Usually serum and a high concentration of calcium ion stimulate cell differentiation rather than proliferation, yet the soluble factors and cytokines secreted by the NIH J2 3T3 feeder cells inhibited differentiation of the ORS cells and stimulated proliferation of the cells.

Using the cell growth curve in Fig. 3, the average growth rate of the human ORS cell was calculated for 10 days (from 7 to 17days). The averge growth rate of the serum-free media culture, K-SFM, was 1.04 day$^{-1}$, while, that of the 10% serum-containing culture, modified E-medium, was 1.42 day$^{-1}$. We can see that the growth of ORS cells in the modified E-medium was higher than that in K-SFM. The population doubling level of the ORS cells in the E-medium was 9.7, while that in K-SFM was 8.1 after 17days of culture.

Fig. 4A clearly shows polygonal ORS cells growing out from the ORS of a plated hair follicle 7 days after an explant culture. The ORS cells were densely packed, exhibited a polygonal morphology, and spread with orientation. However, this method is unsuitable as a primary cultivation technique, because the probability of out growth is low, the chance of different cell contamination is high, and the lag phase is too long.

The ORS cells exhibited a colony formation, grew in a cobblestone pattern, and processed conspicuous nuclei within 6 days of primary cultivation in K-SFM, as seen in Fig 4B. Fig. 4C shows the ORS cells co-cultured with mitomycin C-treated NIH J2 3T3 feeder cells. After 5-6 days of culture, single ORS cells or groups of ORS cells surrounded by post-mitotic feeder cells became visible.

Under a microscope, differences were observed in the cell morphology after 28 days (passage 4 times) of culture, as shown in Fig. 5. The ORS cell colonies continuously grew, pushing aside the surrounding feeder cells, as seen in Fig. 5A. The proliferating ORS cells were densely packed and displayed a typical epitheloid morphology with a high nuclear-cytoplasmic ratio, which was essentially identical to healthy cells [24,27]. While the cells in the modified E-medium had a normal ORS cell morphology, the cells cultured in the K-SFM and modified E-medium without feeder cells exhibited a more squamous shape, as shown in Figs. 5B and 5C. It was found that subcultivation was optimal when the ORS cells were in a preconfluent stage.

As regards the preparation of the feeder cells, density is a crucial point. If the density is high, the ORS cell colonies are hampered in their laternal expansion by the opposite tendency of the feeder cells, which increase in cell size in a postmitotic state. Conversely, the growth stimu-

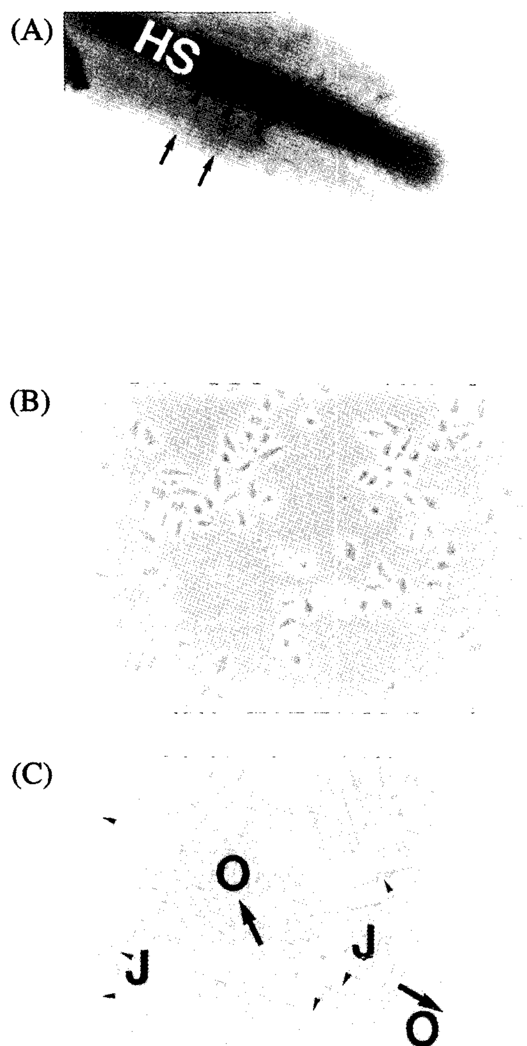


**Fig. 4.** (A) Phase-contrast photograph of human hair follicle attached to dish surface after treatment in trypsin (0.05%) for 20 min after 7 days of explant culture. The arrowheads indicate the inner root sheath, while the arrows mark the outer root sheath (ORS). Polygonal cells were growing out from the ORS of the placed hair follicles. HS indicate a hair shaft. (B) ORS cells formed colonies and processed conspicuous nuclei after 6 days of culture in K-SFM. (C) ORS cells co-cultured with NIH J2 3T3 cells (J) after 6 days of culture in modified E-medium. Small islands (arrow) of compactly arranged ORS cells (O) were easily recognizable by their epithelial morphology. The ORS cells were surrounded by feeder cells (arrow head; J), which were easily recognizable by their much larger size and pleiomorphic shape (× 100).

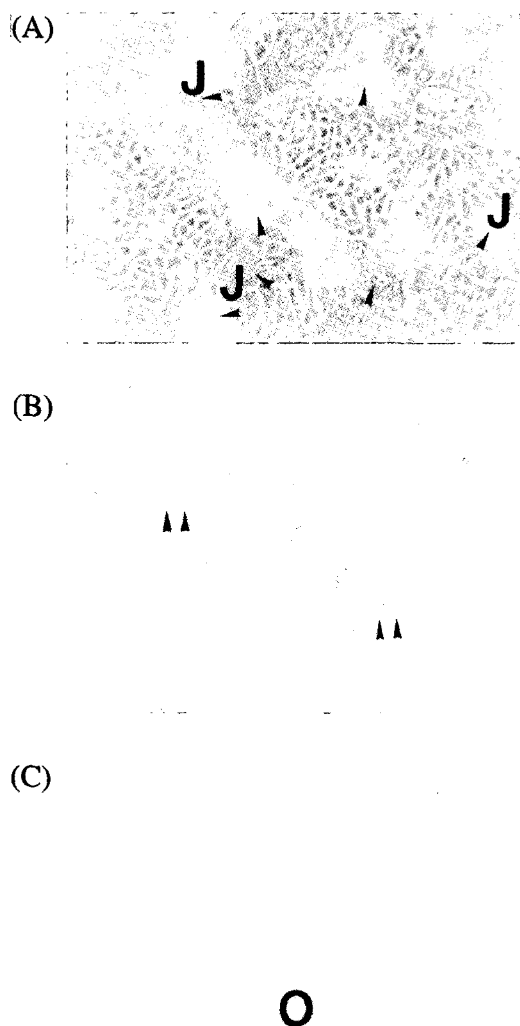


**Fig. 5.** Phase-contrast photograph of human primary ORS cells in various media (× 100). Differences were observed in the cell morphology after 28 days of culture (passage 4 times). (A) Expanded ORS cell islands detached from feeder cells. The ORS cells reached an 80% confluence. (J=feeder cells) (B) ORS cells cultured in K-SFM. (C) ORS cells cultured in modified E-medium without NIH J2 3T3 feeder cells (O = ORS cells). The ORS cells cultured in the modified E-medium co-cultured with NIH J2 3T3 feeder cells exhibited a normal morphology (A), yet the ORS cells cultured in K-SFM (B) and the modified E-medium without NIH J2 3T3 feeder cells (C) exhibited a more squamous shape (arrow head).

latory capacity of the feeder cells is reduced if the feeder cells are present at low a density [24].

As a result, feeder cells are very important, as the soluble factors and cytokines they produce stimulate cell proliferation and inhibit differentiation. The serum-free medium was easily used for primary cultivation, yet for the long-term culture of ORS cells, this medium was unsuitable.

Consequently, the best long-term culture method for ORS cells was found to be a two-step enzyme digestion treatment and culturing in a modified E-medium with feeder cells.

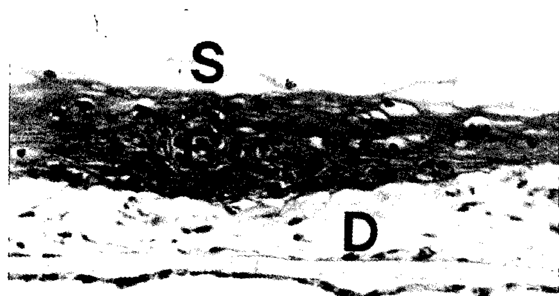


**Fig. 6.** Phase-contrast photograph of bioartificial skin cultured *in vitro* composed of human ORS cells and collagen gel matrix containing human fibroblasts. Morphology of human artificial skin stained with hematoxylin and eosin. Histological examination revealed an epidermal layer (E) with stratification (S) and dermal substrate (D) containing collagen with fibroblasts. The stratified epidermal layer was composed of approximately 5~6 ORS cell layers. The epidermis consisted of stratified cell layers formed by the continuous proliferation and differentiation of cells from basal ORS cells to a stratum corneum layer (S = stratum corneum, E = epidermis, D = dermis, × 200).

### Histological Analysis of Artificial Skin

The morphology of the resulting bioartificial skin is shown in Fig. 6. The construct was fixed for light microscopy after 10 days of culture at the air-liquid interface. An histological examination revealed an epidermal layer (E) with stratification (S) and dermal substrate (D) containing collagen with cells. The stratified epidermal layer was composed of approximately 5~6 ORS cell layers. The epidermis consisted of stratified cell layers formed by the continuous proliferation and differentiation of cells from basal ORS cells to a stratum corneum layer.

As such, it was confirmed that the structure of the bioartificial skin made from the cultured ORS cells or keratinocytes from adult skin was very similar to the structure of real skin. Therefore, it was concluded that the cultured ORS cells were progenitor cells of skin epithelial cells, thereby representing a source of easily and repeatedly available keratinocytes.

## CONCLUSION

Viable epithelial cells can easily be obtained by plucking hair follicles in the anagen stage or from whole follicles dissected from surgically obtained scalp skin. Thus, cultured ORS cells serve as an even better material to cover skin defects because they are obtained from plucked follicles with minimal distress to the donor.

Accordingly, the goal of the current study was to develop an efficient *in vitro* cultivation method for ORS cells obtained from human hair follicles and the formation of a skin substrate that can be grafted on a freshly excised or chronic skin wound.

As such, a two-step enzyme digestion method was found to be very useful, as many viable ORS cells could be recovered without damage. Normally, the primary cell yield of human ORS cells is about $2.1 \times 10^3$ cells/follicle based on conventional direct incubation in trypsin, however, two-step enzyme digestion with dispase and trypsin produced about $6.9 \times 10^3$ cells/follicle. Furthermore, a total of $2.0 \times 10^7$ cells were obtained in a serum-free medium and $6.3 \times 10^7$ cells in a modified E-medium with mitomycin C-treated feeder cells during 17 days of culture, when starting with only $7.5 \times 10^4$ cells. Finally, it was found that the primary culture method and type of medium had an influence on both the cell growth rate and the cell morphology.

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