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# Calcium Regulation of Growth, Differentiation and Keratinization of Cultured Cells from Murine Hair and Hair Follicles: An Ultrastructural Study

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*Summary.* **Calcium regulation of murine hair and hair follicle cells grown in culture was electron-microscopically studied and the quantitative analysis of both growth and differentiation was performed. Some (47%) of the cultured cells were immature and others (53%) were already differentiated on Day 1 of the culture. In low calcium (0.03 mM) media, all of the cultured cells were immature on Day 3, and 90% of the cells were differentiated on Day 6. Twenty-five % of the latter cells were identified as inner root sheath cells, 55% as cortical or outer root sheath cells and 10% as medulla cells. The features of the cultured cells in high calcium (1.0 mM) media were different; although all of the cells were immature on Day 3, keratinization occurred in 27% of the cultured cells on Day 6. These keratinized cells were analogous in ultrastructure to those of *in vivo* keratinized inner root sheath or cortical cells. Twenty-two % of the cells showed degeneration. Most of the cells cultured in media with calcium levels lower than 0.03 mM showed ultrastructural features of degeneration on Day 6. These findings suggest that calcium plays an important role in the regulation of growth, differentiation and keratinization of cultured hair and hair follicle cells.**

## INTRODUCTION

Recently we have reported a method for culturing cells from the murine hair and hair follicle,[1)] and observed that immature cells proliferate *in vitro* and differentiate towards several subpopulations corresponding to the *in vivo* cell layers of the hair and hair follicle.[2)]

The concentration of calcium ions in the extracellular environment has been found to regulate the growth and differentiation of epidermal cells.[3-5)] In our previous study, the hair and hair follicle cells were cultured in medium containing 0.03 mM calcium, and several ultrastructural markers for hair and hair follicle differentiation such as the appearance of medulla granules, trichohyalin granules and aggregation of filaments were observed after Day 6 of the culture.[2)] In the present study, we examined the ultrastructural features of hair and hair follicle cells cultured in media with varying levels of calcium. In order to elucidate the role of calcium in the regulation of growth and differentiation, the cultured cells were classified into several subpopulations according to their ultrastructures, and the growth and differentiation of the cultured cells was quantitatively analyzed.

## METHODS

### Cell culture

Hair and hair follicle cells were isolated from 4-day-old C3H mice as previously described[1,2)] and $1.5 \times 10^6$ cells were plated in 35mm collagen coated dishes (Corning, NY, USA) in Dulbecco's modified Eagle minimum essential medium (DMEM, Nissui, Tokyo, Japan) with 10% fetal bovine serum (FBS, Biocell, LA, USA). One day after cultivation, the media were discarded and the cells were maintained in MCDB 153 with various calcium concentrations. MCDB 153 without calcium was obtained from Kojin Bio. Co. (Saitama, Japan) and calcium chloride was then added to a final concentration of 0.015, 0.03 or 1.0 mM. Plates were routinely incubated at 37℃ in a humidified atmosphere of 5% $CO_2$ in air. For growth kinetic studies, cells were harvested from duplicate dishes on culture Days 1, 3 and 6, and counted in a hemocytometer chamber.

### Electron microscopy

The cells cultured for 1, 3 or 6 days were resuspended by the treatment of a mixture of 0.1% EDTA and 0.25% trypsin at 37℃ for 10 min. These cells were then precipitated by centrifugation at 1,000 rpm and fixed with 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at 4℃ for 2 h. They were postfixed with 1% osmium tetroxide in the same buffer at 4℃ for 2 h, dehydrated in graded concentrations of ethanol and propylene oxide, and embedded in Epon. Ultrathin sections were cut with diamond knives in Ultracut microtomes (JEOL, Tokyo), mounted on grids, double stained with 1% uranyl acetate and Reynolds' lead citrate, and observed in a JEM-100SX electron microscope (JEOL). For a quantitative analysis of cell growth and differentiation, more than 40 cells in each material were observed and classified into immature, differentiated, keratinized and degenerated cells.

## RESULTS

### Cell growth curves

Fig. 1 shows the growth curves of cultured cells in media with various calcium concentrations. The number of cells in media with 0.03 mM and 1.0 mM calcium increased on Day 3 to more than twice of that on Day 1, keeping this same level until Day 6. However, the proliferation of the cells was inhibited on Day 3 with calcium levels lower than 0.03 mM; in sach cases, the number of the cells increased on Day 6 (Fig. 1).

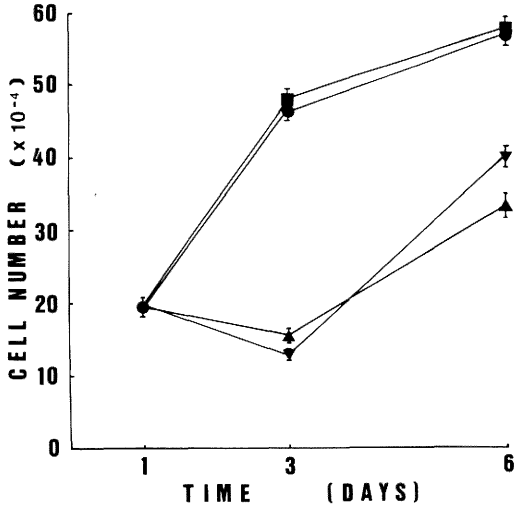



**Fig. 1.** Growth curves of cultured hair and hair follicle cells in MCDB 153 with various calcium concentrations. $1.5\times10^6$ of cells were inoculated into 35 mm collagen coated dishes in 10% FBS-DMEM. One day after plating, cells were allowed to grow in MCDB 153. Each point on the curves represents the mean ±SE of numbers of cells in three separate experiments performed in duplicate. ▲0 mM, ▼0.015 mM, ●0.03 mM, ■1.0 mM.

### Electron micrographic findings

Ultrastructurally, the cultured cells were divided into immature cells and differentiated cells. The former cells contained free ribosomes, but few filaments or few specific granules in the cytoplasm. The latter cells were further classified into four groups: (1) cells having many medulla granules; (2) cells having many or huge trichohyalin granules; (3) cells having a moderate or large amount of filaments not associated with specific granules; and (4) keratinized cell's. The cells in groups (1) and (2) always contained a small number of filaments and were considered to be differentiated medulla cells and inner root sheath (IRS) cells respectively, because of the presence of the specific granules. The cells group (3) should be cortical cells or outer root sheath (ORS) cells because of the abundant cytoplasmic filaments.[6,8,9] It was ultrastructurally impossible to distinguish between cortical differetiation and ORS differentiation of the cultured cells.

The cells cultured for 1 day showed either immature or differentiated features (Fig. 2). Immature

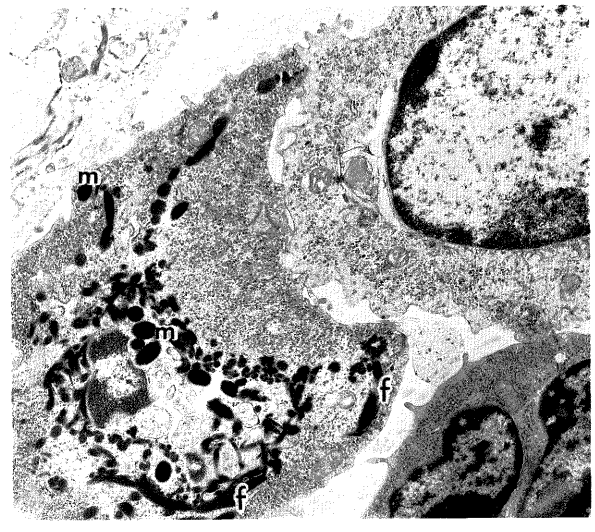
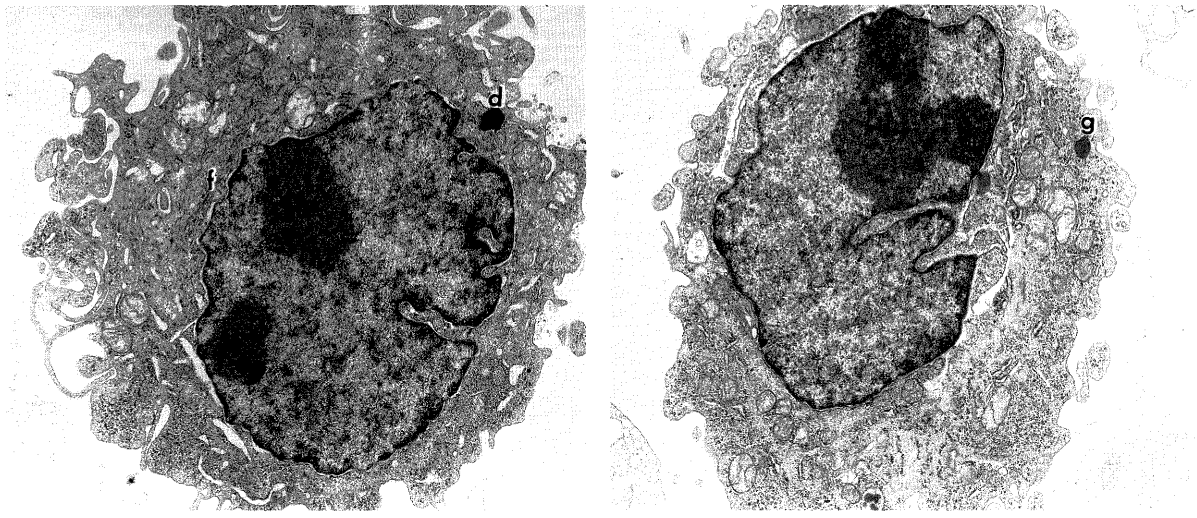



**Fig. 2.** An electron micrograph of hair and hair follicle cells cultured for 1 day. The immature cell (right) contains a large nucleus, many ribosomes and few filaments in the cytoplasm. The differentiated cell (left) contains many melanosomes (*m*) and filaments (*f*) in the cytoplasm. ×8,000

**Table. 1.** Morphological classification and quantitative study of the cultured hair and hair follicle cells

| Calcium cells | Culture days | Differentiated cells | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Cells with filaments | | | | | |
| | | Immature cells | Medulla cells | Moderate | Rich | IRS cells | Keratinized cells | Degenerated cells | Totals |
| | 1 day | 22 (46.8) | 2 (4.3) | 19 (40.4) | 4 (8.5) | 0 (0) | 0 (0) | 0 (0) | 47 (100) |
| 0.03mM | 3 days | 44 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 44 (100) |
| | 6 days | 3 (7.5) | 4 (10) | 7 (17.5) | 15 (37.5) | 10 (25) | 0 (0) | 1 (2.5) | 40 (100) |
| 0.1mM | 3 days | 63 (100) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 63 (100) |
| | 6 days | 7 (11.9) | 0 (0) | 10 (16.9) | 7 (11.9) | 6 (10.2) | 16 (27.1) | 13 (22.0) | 59 (100) |

Each number expresses the number of the subject cells.
Each bracketed number expresses the percentage (%) of the subject in the total number of the cells counted in each cell culture.
IRS, inner root sheath

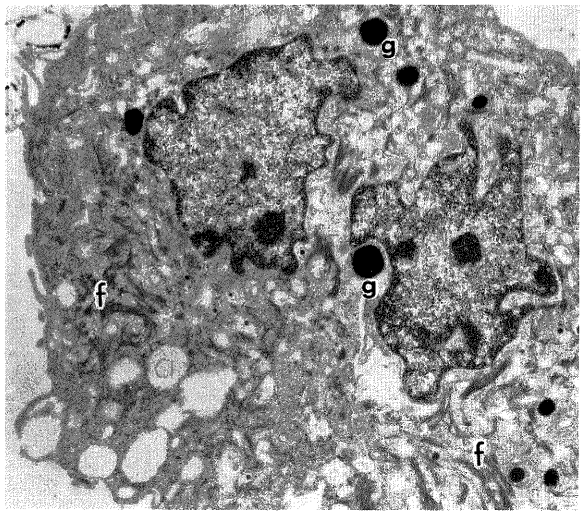



**a** **b**

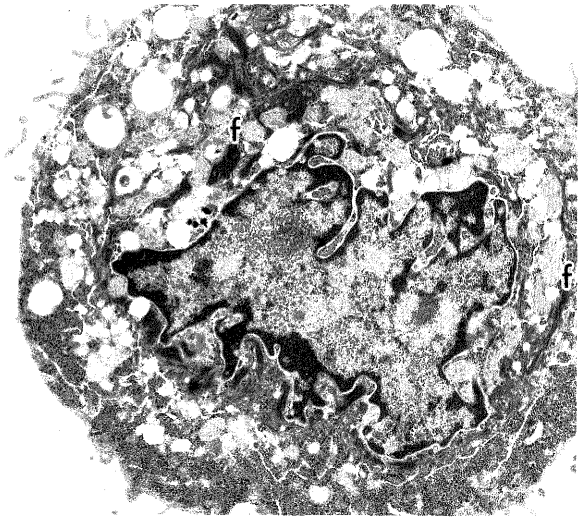
**Fig. 3.** Electron micrographs of hair and hair follicle cells cultured in a 0.03 mM calcium medium for 3 days. **a.** an immature cell. The cell contains many ribosomes and a small number of filaments (*f*). d, lysosomal dense body. **b.** an immature cell having a few round electron dense deposits (*g*) like medulla granules. a, b ×8,000

cells comprised 46.8% of all the cells, counted the others (53.2%) being differentiated (Table 1). In the differentiated cells, 4.3% were medulla cells and 48.9% were cortical or ORS cells; however, IRS cells and keratinized cells were not observed.

After the medium was changed to MCDB 153 with 0.03 mM calcium on Day 1, all the counted cells were electron microscopically immature on Day 3 (Table 1); they were small in size, possessed a large nucleus and contained many free ribosomes and a few filaments in the cytoplasm (Fig. 3a). Some cells already revealed a few medulla granules, although they were believed to be immature (Fig. 3b). On Day 6 of the culture, most (90%) of the cells were differentiated and only 7.5% of the counted cells were immature (Table 1). They were identified as differentiated medulla cells (Fig. 4a), cortical or ORS cells (Fig. 4b), or IRS cells (Fig. 4c) based on their ultrastructural




4a

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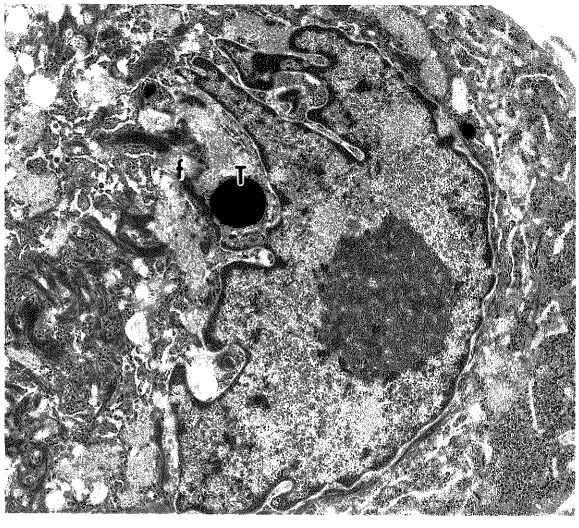


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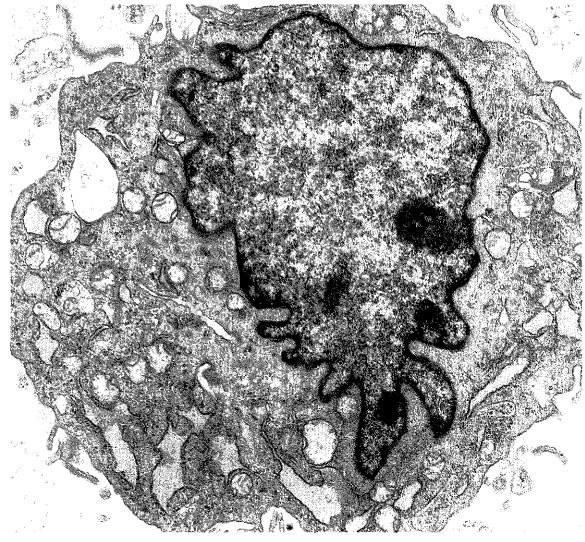

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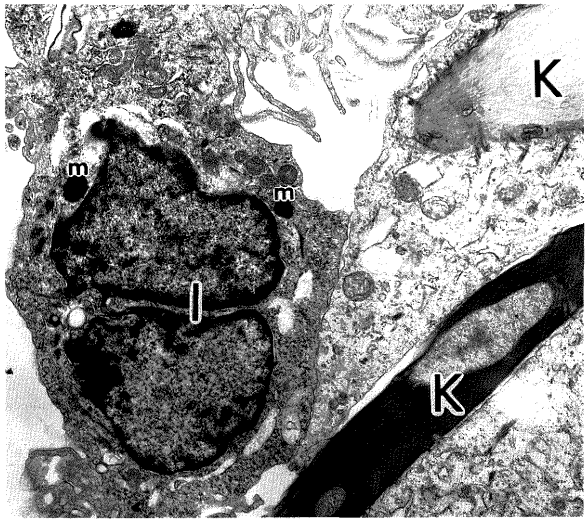
**Fig. 5** An electron micrograph of hair and hair follicle cells in a 1.0 mM calcium medium for 3 days. The cell contains a large nucleus, many ribosomes and a few filaments. ×8,000

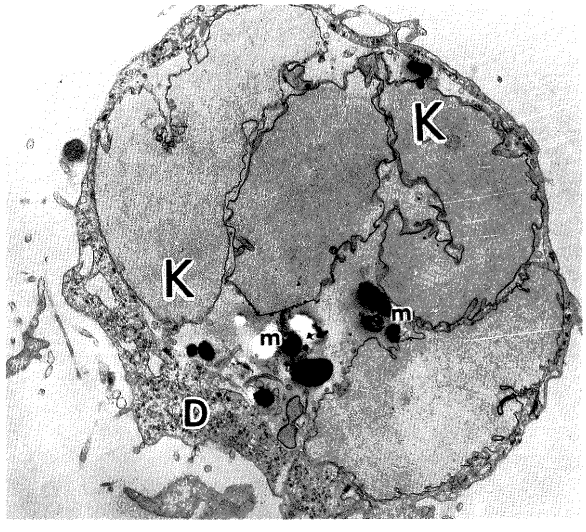
findings. The numbers of medulla cells, cortical or ORS cells and IRS cells were 10%, 55% and 25%, respectively, of the counted cells (Table 1).

In several cultures, the medium was changed to MCDB 153 with 1.0 mM calcium 1 day after seeding. On Day 3, all the cells were immature (Fig. 5), similar to those in the low calcium medium on Day 3 as described above. On Day 6 of the culture, keratinization occurred in 27.1% of the cultured cells. As shown in Fig. 6a, keratinized cells were often present intermingled with immature cells. Some keratinized cells were encircled by a degenerated cell (Fig. 6b). The keratinized cells had none of the nuclei or other organelles, and were filled with keratin filaments. Most of the keratinized cells were flattened and spindle-shaped. At higher magnification, the filaments were arranged in a so-called fingerprint "keratin pattern"[6] (Fig. 6c). Furthermore, some keratinized cells formed a marginal band[7] along the inner sur-

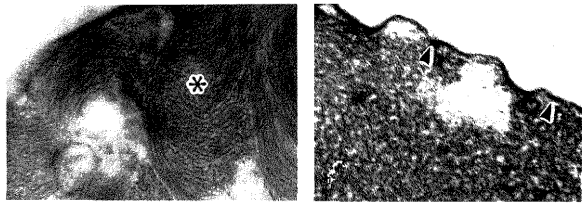
**Fig. 4.** Electron micrographs of hair and hair follicle cells cultured in a 0.03 mM calcium medium for 6 days. **a.** a cell showing differentiation of the medulla. Note the many round electron dense deposits (*g*) like medulla granules. *f*: filaments. **b.** a cell showing hair cortical or ORS differentiation. The cell contains thick bundles of filaments (*f*), but still many ribosomes in the cytoplasm. **c.** a cell showing IRS differentiation. Note several variously sized electron dense deposits (*T*) associated with some filaments (*f*). a-c ×8,000




6a




6b



6cd

**Fig. 6** Electron micrographs of hair and hair follicle cells in a 1.0 mM calcium medium for 6 days. **a.** karatinized cells are filled with a dense or loose filamentous material, and nuclei and other organelles are not seen in the cytoplasm. m: melanosome. **b.** Four keratinized cells (*k*) infolded by a degenerated cell (*D*). Nuclei and other organelles are not seen and the cytoplasm is filled with many filaments. *m*: melanosome. **c.** The keratinized cell showing fingerprint "keratin pattern (*)" in the cytoplasm. **d.** The keratinized cell. The filaments with high density are seen in the interfilamentous substance of the density. A marginal band (arrow heads) is produced. a, b ×8,000 c ×65,000 d ×75,000

face of the cell membrane (Fig. 6d). The prominent difference between the high calcium culture and the low calcium one was the presence of keratinized cells and a larger number (22%) of degenerated cells; these had some lipid droplets and vacuoles in their cytoplasm.

Most of the cells showed cell degeneration in both 0 and 0.015 mM calcium levels on Day 6. Their nuclei became pycnotic and many vacuoles appeared in their cytoplasm.

## DISCUSSION

Although many electron microscopic studies of hair structures *in vivo* have been carried out,[6,8-10] the morphologic details of the cultured cells deriving from hairs and hair follicles have not been well examined. The present quantitative classification of the cultured cells by the electron microscope gave new information concerning their growth and differentiation. The cells attached to dishes on Day 1 included both already differentiated cells and im mature cells in a proportion of about 1:1. The differentiated cells at this time might be cells already differentiated *in vivo* in hairs and hair and hair follicles, because the cortex-deriving cells always contained an adequate and constant number of melanosomes in the cytoplasm (see Fig. 2). Since no cells with trichohyalin granules were observed, the already differentiated IRS cells could not probably attach to the dishes. All the differentiated cells seemed to detach from the dishes and disappear by Day 3. Therefore, only the immature cells divided, increased in number and differentiated after Day 3. The growth curve of the cultured cells showed that the number of the cells on Day 3 was twice of that on Day 1. As far as the cell growth curve showed, the cells appeared to divide only once. However, this classification could give an accurate estimate of the cell division: since immature cells, in other words, the attachable and proliferative cells, occupied half the number of the cultured cells on Day 1, at least two cell divisions occurred in 2 days. On Day 6, differentiated cells rich in medulla granules, trichohyalin granules or filaments appeared; these cells could be regarded as cells differentiated *in vitro* from the immature cells on Day 3. Trichohyalin granules were often found in the cells grown in low calcium (0.03 mM) media. Culrured epidermal cells grown in low calcium media have been reported to be similar to *in vivo* epidermal basal cells, and to contain no keratohyalin granules in their cytoplasm.[11] This

difference in cell differentiation between hair and hair follicle cells and epidermal cells seems to be important in examining the biological features of the former cells. The medulla granules appeared in the early stages of cultivation. This is consistent with the *in vivo* observation that medulla granules in association with free ribosomes are already seen in the immature medulla cells in the hair matrix.[6] The medulla *in vivo* comprises the majority of hair volume in mice.[9] The percentage occupied by the medulla cells in the present culture cells was 10%.

Marked morphological changes induced by elevating the extracellular calcium concentration were found on Day 6 of the culture; keratinized cells, which were never seen at a 0.03mM calcium concentration, were often observed and the degenerated cells increased in number. As the cytoplasm of the medulla cells becomes vacuolaed *in vivo*,[6] some of the degenerated cells in culture might be differentiated medulla cells, although it seems impossible to decide whether those vacuoles were produced in the normal process of differentiation or by artifacts caused by the culture. Some keratinized cells seemed to be cortical cells; in their cytoplasm, bundles of filaments (keratin filaments) formed a fingerprint-like "keratin pattern".[6] Some keratinized cells formed a marginal band along the inner side of the cell membrane. Such a marginal band is often seen in the keratinized cuticle, IRS or ORS *in vivo*.[6,9] Among these keratinized cells, homogeneously less electron-dense cells filled with fine cytoplasmic filaments as shown in fig. 6b seen to be keratinized IRS cells. However, it is difficult to determine precisely the cell types of all the keratinized cells only from their morphological findings.

The possibility that the cultured hair and hair follicle cells could continue to keep the characteristics of the immature cells in media with calcium levels lower than 0.03mM for a long time was examined, since the majority of the cultured cells had already shown differentiated cell features on Day 6 at a 0.03 mM concentration of calcium. However, such very low calcium levels inhibited the normal growth of the cells. Although the calcium concentration optimum for cell growth may be varied by additional components,[12] it is supposed that the cultured hair and hair follicle cells require extracellular calcium levels higher than at least 0.03 mM for their growth. Similarly, other epithelial cells require extracellular calcium levels higher than 0.01 mM.[13]

The present study suggests that concentrations of extracellular calcium can regulate the growth, differentiation and keratinization of the cultured cells deriving from hairs and hair follicles. The analyses of quantitative and/or qualitative characteristics of cell growth and differentiation seem to be very important for further biochemical or pharmacological studies using the present culture system.

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