Establishment of Rat Dermal Papilla Cell Lines that Sustain the Potency to Induce Hair Follicles from Afollicular Skin

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Dermal papilla cells in culture show a lower proliferative capacity compared with dermal fibroblasts, and lose their *in situ* potency to induce hair follicles in the epidermis at more than 10 passage numbers. This study overcomes these limitations of cultured papilla cells and for the first time demonstrates that papilla cells can be serially cultured for a long period without losing their hair-inductive potency. Outgrowth and the ensuing proliferation of papilla cells were markedly stimulated when explants of rat vibrissa papillae were cultured with rat sole-derived keratinocytes. Such feeder effects of the keratinocytes could be replaced to some extent with conditioned medium of the cells. Serial cultivation of papilla cells was established by maintaining them in the conditioned medium in which they were subcultured for more than 90 passages with an approximate population doubling time of 30 h, a value similar to that of rat dermal fibroblasts. During the subculture, they showed morphologic characteristics and phenotypic expressions of original papilla cells. Even after at least 70 passages, papilla cells sustained the innate hair follicle inductive ability at a level comparable with that of intact dermal papillae. The established cell lines did not show tumorigenicity when they were subcutaneously implanted into nude mice. The culture method developed in this study should facillitate the search for a biochemical entity of dermal papilla cells. ***Key words: epidermal–dermal interaction/hair induction/keratinocyte conditioned medium. J Invest Dermatol 111:767–775, 1998***

Hair follicles are developmentally distinguished as hair germs formed by a series of interactions between the embryonic ectoderm and the mesoderm (Hardy, 1992). These interactions continue during the development, growth, and differentiation of hair follicles. Although not experimentally proven, progenies of the interacting mesodermal cells are believed to be incorporated into a specialized portion of the follicle (hair bulb) as dermal papilla cells. Using the rodent vibrissa follicle as a model and the transplantation technique, Oliver (1967) demonstrated that dermal papilla is key in supporting the growth of hair. Reynolds and Jahoda (1991, 1992) reported that dermal papilla cells can also induce new hair follicles from afollicular skin epidermis.

To better understand the interactions between keratinocytes and papilla cells, researchers have cultured papilla cells (Jahoda and Oliver, 1981, 1984; Messenger, 1984) and showed that cultured papilla cells sustain their original ability to elicit hair growth (Jahoda *et al*, 1984); however, papilla cells grow slowly *in vitro* as compared with dermal fibroblasts and lose their inductive ability at more than 10 passages (Jahoda *et al*, 1984; Horne *et al*, 1986; Lichti *et al*, 1993; Weinberg *et al*, 1993).

In this study, we therefore developed a method to establish cell lines of dermal papilla cells that sustain their hair-inductive ability. Cocultivation of papilla cells with keratinocytes was found to support the rapid growth of the papilla cells. This feeder effect was replaceable to some extent by a conditioned medium (CM) of keratinocytes. By adding CM into culture medium, we succeeded in serial cultivation of papilla cells for more than 90 passages. During subcultivations these cells proliferated at a rate comparable with that of rat dermal fibroblasts and sustained their hair-inductive potency. To our knowledge, our study is the first demonstration that papilla cells can be serially cultured for a long period without losing their hair-inductive ability.

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Abbreviations: Ab, antibody; CM, conditioned medium; PDT, population doubling time.

MATERIALS AND METHODS

Animals, chemicals, reagents, and culture materials Materials were obtained as follows: male and female Fisher 344 (F344) rats from Japan SLC (Shizuoka, Japan); BALB/C nu/nu mice from Charles River (Yokohama, Japan); dispase from Sanko Jun-yaku (Tokyo, Japan); 0.25% trypsin-1 mM ethylenediaminetetraacetic acid solution, Dulbecco's modified Eagle's medium (DMEM), and colcemid from Life Technologies (Gaithersburg, MD); fetal bovine serum (FBS) from Hyclone Laboratories (Logan, UT); bovine serum albumin and PKH26-GL from Sigma (St. Louis, MO); bromodeoxyuridine (BrdU) from Amersham International (Bucks, U.K.); anti-BrdU antibody (Ab) from Dako A/S (Glostrup, Denmark); anti-vimentin Ab and anti-cytokeratin 14 (CK14) Ab from YLEM S.r.I. (Rome, Italy); horseradish peroxdase-labeled anti-mouse IgM Ab from Oreganon Teknika (Durham, NC); Vectastain ABC kits from Vector Laboratories (Burlingame, CA); and 13.5 mm Sumilon Celldesks from Sumitomo Bakelite (Tokyo, Japan). All other chemicals and reagents not specified above were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Primary culture of keratinocytes and preparation of CM Pieces of skin (0.5–2 cm^2 in area) were excised from the sole of 5–8 wk old F344 rats that had been lethally anesthetized. The pieces were then treated with 1000 units

per ml dispase dissolved in DMEM containing 10% FBS (DMEM/FBS) at 4°C overnight. The epidermis was removed from the dermis, incubated in phosphate-buffered saline containing 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid at 37°C for 10 min, then placed in DMEM/FBS.

For primary culture, keratinocytes were removed from the epidermis by scraping with the back portion of the tips of clawed forceps, filtered through a 50 μm nylon mesh. The CM of keratinocytes was obtained from the primary culture in which 2.2×10^6 cells were incubated in 10 cm diameter tissue culture dishes containing 10 ml of DMEM/FBS. Under these conditions the cells grew rapidly and reached confluency around day 5. We prepared two CM (CM5 and CM8). For CM5, culture medium was removed at day 5 when the cells were preconfluent, and passed through 0.22 μm membrane filters. For CM8, the cells were cultured with fresh medium for an additional 3 d, during which time the cells became confluent, and the CM was prepared in a similar manner as CM5.

We prepared two other CM to check if active CM could be prepared from the keratinocyte culture in which the keratinocytes are cultured in a serum-free medium: CM7(–) to obtain serum-free CM and CM7(+) to examine the effects of the serum in the conditioning process. For both CM, the cells were cultured in DMEM/FBS for 5 d in a similar manner as CM5 and CM8, and then cultured with fresh serum-free DMEM for 24 h to wash serum-derived ingredients out of the culture. The cells were then cultured for an additional 24 h in either serum-free DMEM or DMEM/FBS and the CM7(–) and CM7(+) removed, respectively.

All CM were stored in the dark at 4°C and used within 1 mo of preparation.

Cultivation of papilla cells Vibrissa follicles were excised from the upper lip of 5–8 wk old F344 rats. Dermal papillae were removed from the anagen follicles using a fine needle and a pair of forceps. Eight explants per 35 mm dish were cultured with 2 ml medium for four different cultures: (i) control culture, in DMEM/FBS alone; (ii) coculture, in DMEM/FBS in the presence of 4×10^5 keratinocytes; (iii) FBS/CM culture, in a 1:1 mixture of DMEM/FBS and one of the CM, i.e., CM5, CM7(+), CM7(–) supplemented with 10% FBS [CM7(–)/FBS], or CM8; and (iv) FBS-free CM culture, in a 1:1 mixture of DMEM and CM7(–).

In the coculture, keratinocytes were labeled using PKH26-GL red fluorescent dyes according to the protocol provided by the manufacturer and plated in dishes simultaneously with papillae. For all cultures, the cells were refed twice a week during the period of culture. When the papilla cells grew to full confluency, they were detached from dishes by treating with 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid, and subjected to the first subcultivation at a density of 5.5×10^3 cells per cm^2. Subsequently, they were serially subcultured by passaging every week in a similar manner. The growth of papilla cells in control cultures was slow and limited.

The chromosome complement was analyzed for growing papilla cells at the eighty-sixth passage. The cells were subjected to the metaphase preparation by incubating them for 2 h in the medium containing 0.1 μg colcemid per ml, followed by staining with Giemsa. Chromosomes were counted in 62 preparations in which chromosomes had been spread well.

Measurement of growth of papilla cells Dermal papillae were explant-cultured as described in the preceding section and photographed using a phase contrast microscope. The growth of papilla cells was determined by measuring the area of clusters formed by papilla cells outgrown from the explants seen in these photographs using NIH Image 1.61 software. The growth of papilla cells was also determined by measuring the BrdU-labeling index as follows. Papilla cells at day 7 of explant cultures were treated with BrdU for 6 or 12 h, fixed with cold ethanol, and subjected to immunocytochemistry with anti-BrdU antibody as described previously (Tateno and Yoshizato, 1996). The number of all papilla cells and the number of labeled papilla cells were determined using a phase contrast microscope and the labeling indexes were calculated. The coculture results were statistically analyzed using Student's unpaired t test and the CM culture results by Fisher's protected least significant difference.

Immunocytochemistry We previously reported a series of monoclonal antibodies (MoAb) against hair follicles (Matsuzaki *et al*, 1996). To characterize the cultured papilla cells we used two of these MoAb, ORS-4 (K1310) and D-2 (K1311), in this study; ORS-4 specifically reacts with cells of both the hair matrix and the outer root sheath, and D-2 binds to cells of both the dermal papilla and the dermal sheath with a reactivity similar to Ab against vimentin (Matsuzaki *et al*, 1996).

Ten thousand papilla cells were cultured on Celldesks placed in 24 well plates, allowed to grow to confluence, fixed with cold acetone, and blocked with 5% bovine serum albumin. The cells were treated with ORS-4, D-2, anti-vimentin Ab, and anti-cytokeratin 14 Ab at 4°C overnight. Bound MoAb were visualized with peroxidase-labeled second Ab or with a Vectastain ABC kit using diaminobenzidine as a substrate. Cells were counterstained with hematoxylin or methyl green. Twenty thousand keratinocytes were cultured on Celldesks and were similarly subjected to immunocytochemistry.

Assay for hair follicle-inductive activity of cultured papilla cells Square pieces (3 × 3 mm) of sole skin were removed from 8 wk old female F344 rats and mildly digested with 500 U dispase per ml at 37°C for 3 min to make a pocket-like space between the epidermis and the dermis. Papilla cells under serial cultivations were grown to confluence in 10 mm tissue culture dishes containing 10 ml of 50% CM as above, scraped off gently by using a cell scraper, and transferred to 6 mm bacteriologic dishes. The cells were then cultured while being gently shaken by a rotary shaker in an incubator for 1 d, during which time they aggregated into spheroids containing $\approx 10^4$ cells. A total of seven such spheroids were prepared from papilla cells serially cultured for 20–70 passages in CM5 (CM5 spheroids) and a total of 14 in CM8 (CM8 spheroids). The spheroids were inserted into the spaces of the sole skin pieces. As positive controls, seven implants (three and four skin pieces implanted in two independent experiments) were made by inserting freshly isolated papillae into the sole skin. The experimentals consisted of seven implants containing CM5 spheroids (two, two, and three skin pieces implanted in three independent experiments) and 14 implants containing CM8 spheroids (five, three, and six skin pieces implanted in three independent experiments). Two types of skin pieces were prepared as negative controls: 10 pieces of intact skin that had received no treatment (three, three, and four pieces, respectively, implanted in three independent experiments) and seven pieces of skin that had been inserted with spheroids of 10^4 Swiss 3T3 cells (three and four pieces implanted in two independent experiments). Swiss 3T3 cells have been reported not to show any hair follicle-inductive ability (Lichti *et al*, 1993; Weinberg *et al*, 1993). Each skin piece was implanted under a kidney capsule of 8 wk old female F344 rats according to the method described by Kobayashi and Nishimura (1989). Eight weeks later, the animals were sacrificed and the implants were removed from them. The implants were subjected to histologic examinations, or immunohistochemically analyzed in which 6 to 8 μm thick serial frozen sections were treated with ORS-4 or D-2, and processed as described in the section *Immunocytochemistry*. The assay results were statistically analyzed using Fisher's exact probability test.

Tumorigenicity assay of papilla cells Papilla cells at 90 passages in CM5 or CM8 were checked for the tumorigenicity. A suspension of these papilla cells in phosphate-buffered saline was subcutaneously inoculated into two 5 wk old female BALB/C nu/nu mice; one mouse received an injection of 5 million dermal papilla cells cultured in CM5 (CM5 cells) in 100 μl of phosphate-buffered saline at two different sites, and the other mouse received an injection of 2.5 million CM8 cells in 100 μl of phosphate-buffered saline at two different sites. For 2 mo, we observed the mice for development of tumors from the inocula. The experiment was repeated twice to confirm the reproducibility of the results.

RESULTS

Growth characteristics of cultured papilla cells Isolated vibrissal dermal papillae were cultured for 5 d with or without sole keratinocytes, which had been labeled with fluorescent dyes to distinguish them from the dermal papilla cells. Keratinocytes markedly enhanced the outgrowth of papilla cells from the explants (compare **Fig 1*A*** with **Fig 1*B***). Papilla cells appeared to grow while excluding keratinocytes around the growing edges of clusters of papilla cells (compare **Fig 1*B*** with **Fig 1*C***). This notion was also supported when cocultures (**Fig 1*B*, *C***) were compared with cultures of keratinocytes alone (**Fig 1*D*, *E***). Keratinocytes enhanced the number of papilla cells per cluster grown during 7 d by a factor of 2.7 (**Fig 2*A***). Similarly, the keratinocytes enhanced the BrdU-labeling indexes of papilla cells by a factor of 2.6 (**Fig 2*B***), indicating that keratinocytes actually stimulated the growth of papilla cells.

Both CM5 and CM8 obtained from cultures of keratinocytes were also effective in stimulating the outgrowth of papilla cells from explant-cultured papillae (**Fig 3**). The area of clusters formed by papilla cells (**Fig 4**), the cell number per cluster (**Fig 5*A***), and the BrdU-labeling index (**Fig 5*B***) were used as measures of this outgrowth. All CM increased this cluster area: at day 7, compared with that for the control medium (**Fig 4**), the cluster area increased by a factor of 2.5 for CM5, 5 for CM7(+), and 3.5 for CM7(–)/FBS; and at day 6, by ≈3.3 for CM8 (data not shown). Both the total number of cells in the clusters and the labeling index were higher in culture with CM5 and CM7(+) than in culture with control medium (**Fig 5**). Although the CM7(–) showed no growth-stimulatory effects when combined with serum-

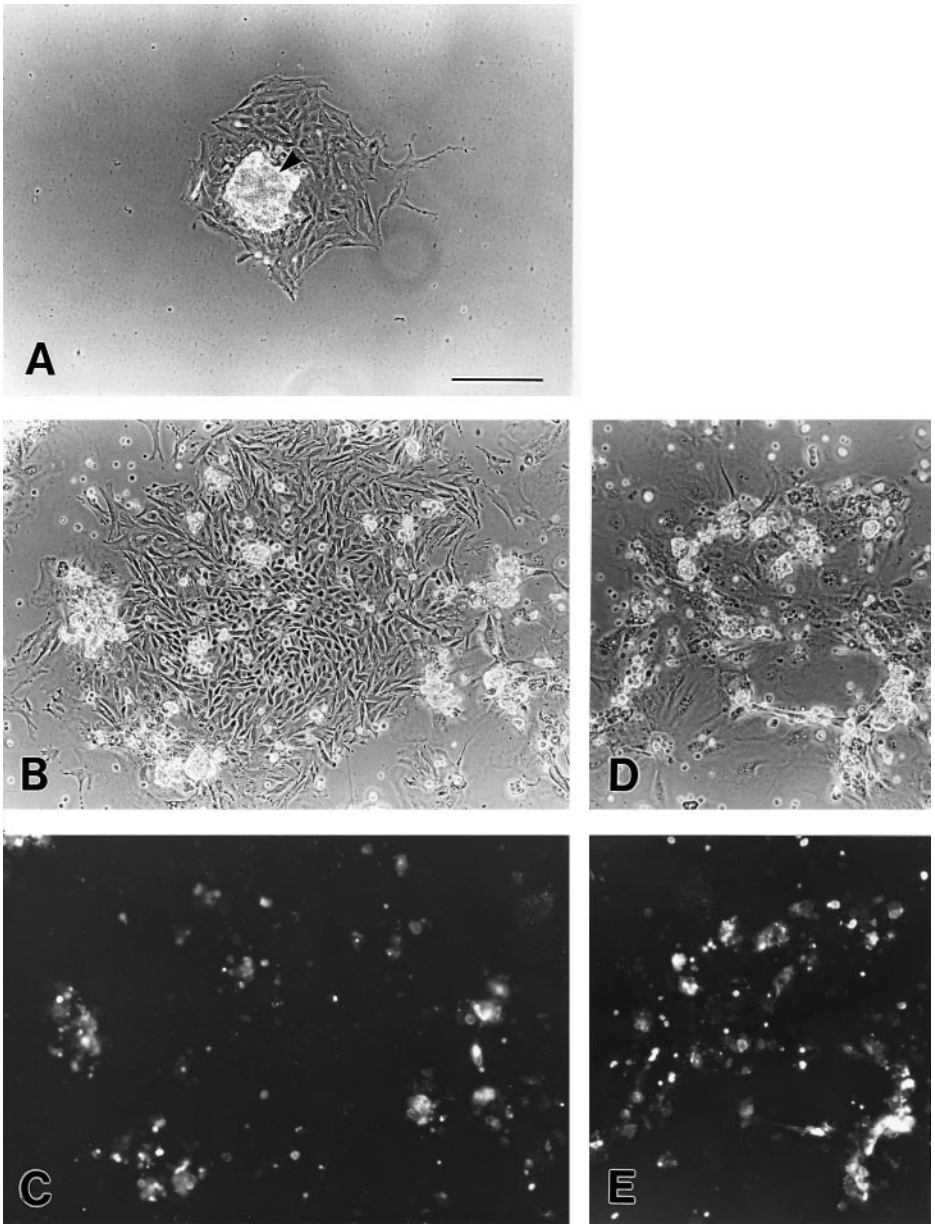

Figure 1. Enhanced outgrowth of papilla cells from explants in the presence of keratinocytes. Eight vibrissa papillae were explanted in 35 mm dishes containing 2 ml of DMEM/FBS in the absence (*A*) or presence (*B*, *C*) of 4×10^5 sole keratinocytes labeled with PKH26 red fluorescent dyes. Photographs were taken at 5 d in culture through a phase contrast microscope (*A*, *B*). The field of (*B*) was photographed through a fluorescent microscope (*C*). An *arrowhead* in (*A*) shows the site to which the papillae originally attached. Sole keratinocytes (4×10^5 cells) labeled with PKH26 dyes were cultured alone for 5 d and were photographed through a phase contrast microscope (*D*) or a fluoroscent microscope (E). *Scale bar*: 100 μm.

free DMEM, CM7(–) stimulated growth of papilla cells comparable with CM5 when the medium mixture was supplemented with 10% FBS. The extent of stimulation of CM7(–)/FBS was less than CM7(+). This might be related to the difference in physiologic activity of keratinocytes between cultures with and without serum.

Papilla cells cultured in CM could be serially cultured without affecting their growth potential (**Fig 6**). For CM5 and CM8, the population doubling time (PDT) of cells showed wide variability (standard deviations in **Fig 6**; actual data ranged from 25 to 200 h) at an early phase of passage, then approached a constant value (between 25 and 35 h) after 20 passages. The dermal papilla cells could be serially cultured in CM over 90 passages. A small but significant difference was observed in PDT between the cells in CM5 (25 h) and those in CM8 (30 h). In contrast, PDT in control medium was around 150 h at the first passage and then increased to 550 h at the fourth passage, after which the cells grew so slowly that it took more than 2 wk for the next subcultivation; this made the passage frequency of once a week impractical.

Characterization of serially cultured dermal papilla cells Papilla cells in the outgrowth from explants were spindle-like in control medium and became flat in their appearance during the primary culture. The cells under subcultures in CM (CM cells) also started to change their morphology from passage number 2 to flat irregular forms with fibrous structures in the cytoplasm. This morphology continued to about passage number 10 (**Fig 7*A*, *D***). Then around passage number 20, the cells shrank into uniform, spindle-like, densely packed structures (**Fig 7*B*, *E***), and from passage number 40 tended to aggregate into clumps, which is a characteristic of cultured papilla cells (**Fig 7*C*, *F***). The cells exhibited the contact inhibition of growth at confluence. Two noticeable differences in characteristics between CM5 and CM8 cells were that the formation of clumps took place earlier in CM8 than in CM5 (compare **Fig 7*E*** with **Fig 7*B***), and that the adhesiveness of CM5 cells to a culture dish was weaker than that of CM8 cells because the former were much more easily detached from the dish by trypsin treatment upon subcultivations.

Phenotype specificities were determined (**Fig 8**) using MoAb D-2, which specifically recognize cells of the dermal papilla and the dermal sheath, and using ORS-4, which specifically recognizes cells of the hair matrix and the outer root sheath (Matsuzaki *et al*, 1996). Papilla cells in either CM5 or CM8 after the 30th passage were positive in D-2 staining and negative in ORS-4-antigen expression (**Fig 8*A–D***). In addition, the cells were reactive with anti-vimentin Ab but not with antibodies against CK14, which is a lineage specific marker of keratinocytes (**Fig 8*E*, *F***). These results supported that serially cultured dermal papilla cells sustained their original characteristics and that no

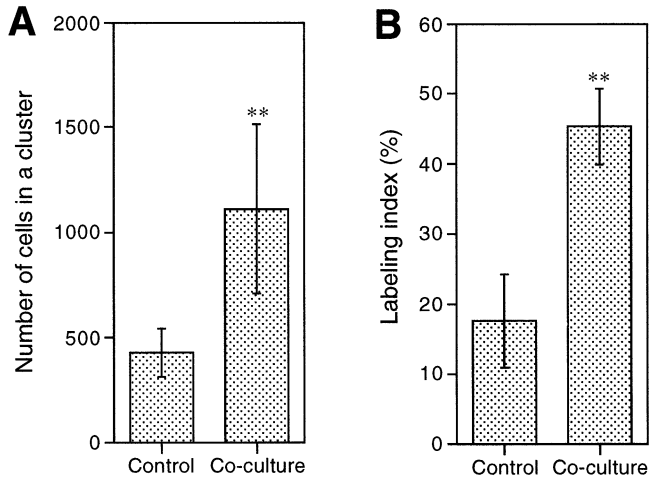

Figure 2. Effect of keratinocytes on the growth of papilla cells. Vibrissa papillae used in the experiment shown in **Fig 1** were cultured for 7 d and were incubated with BrdU for 12 h. The number of total papilla cells and BrdU-labeled papilla cells was determined in each of eight clusters on photographic prints. (*A*) Total number of cells in each cluster. (*B*) Labeling index of papilla cells. Each column represents the mean ± SD of eight independent papillae. Significant difference from the control; $^{**}p < 0.01$.

epidermis-derived cells contaminated the culture. Control stainings showed that sole keratinocytes were negative to vimentin and positive to CK14 (**Fig 8*G*, *H***).

The chromosome composition was determined for papilla cells at 86 passages. Fifty-three per cent of CM5 cells examined had the diploid number of chromosomes (n = 42). Other CM5 cells showed aneuploidy but their deviation from the diploid number was small, ranging from –3 to +2, with the cells with 43 chromosomes being the most frequent. The CM8 cells differed significantly from the CM5 cells in their chromosome compositions; CM8 cells showed a wide range of aneuploidy, from n = 36–84 chromosomes, with the majority being between 78 and 81.

CM5 papilla cells at 90 passages showed no tumorigenicity for more than 2 mo after they were implanted into nude mice; similarly, neither did CM8 cells, in spite of their severe aneuploidy. The tumorigenicity check was repeated twice for each of CM5 and CM8 cells, and identical results were obtained, thus confirming reproducibility.

Hair follicle induction by serially cultured papilla cells The hair follicle-inductive ability is a unique and prominent characteristic of dermal papilla cells. We determined if the serially cultured papilla cells studied here sustained this activity. Cell aggregates (spheroids) were prepared from papilla cells that had been collected from culture dishes at different passages indicated in **Table I**. The spheroids were then assayed for their hair-inductive ability by inserting them into sole skin pieces, which were then implanted

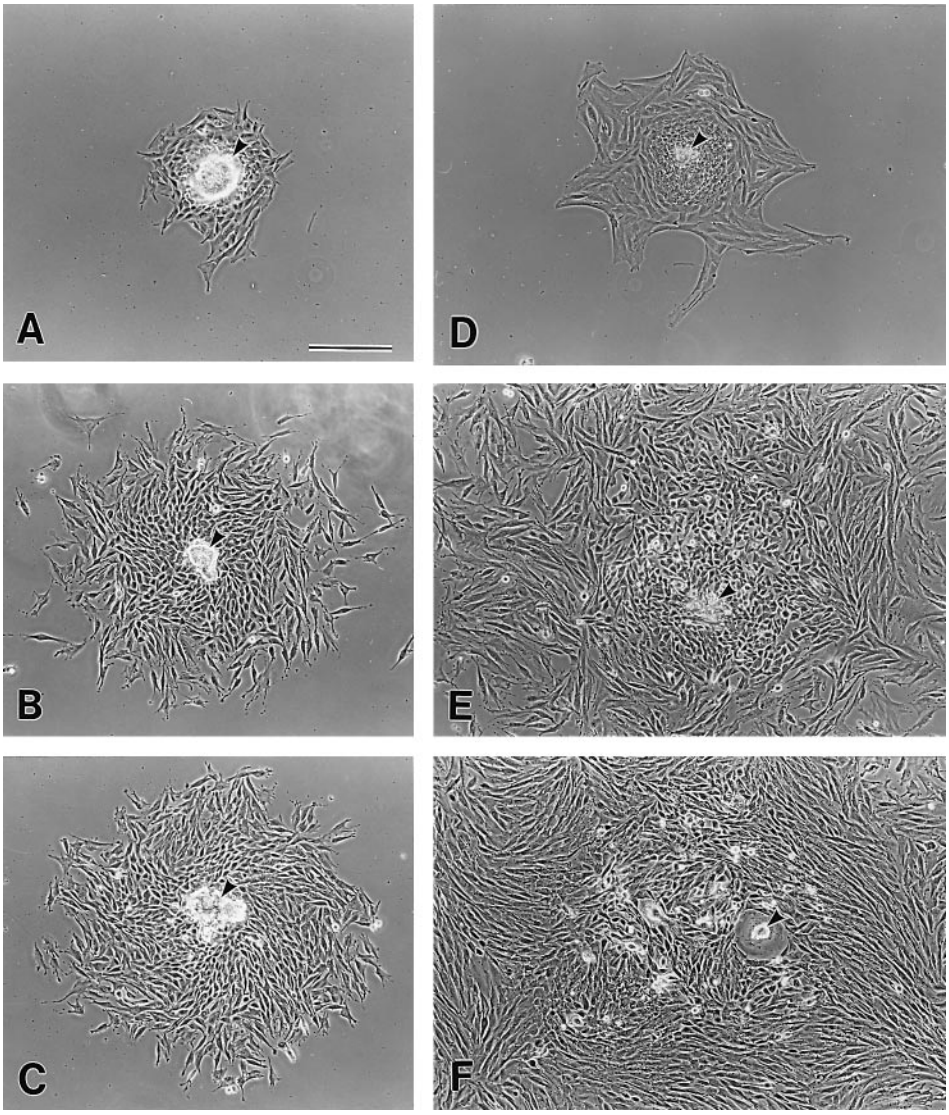

Figure 3. Keratinocyte-CM-induced stimulation of outgrowth of papilla cells from explants. Vibrissa papillae were explant-cultured in unconditioned medium (*A*, *D*), 50% CM5 (*B*, *E*), and 50% CM8 (*C*, *F*), and then photographed on day 3 (*A*, *B*, *C*) and day 6 (*D*, *E*, *F*). *Arrowheads* show the original papillae. *Scale bar*: 100 μm.

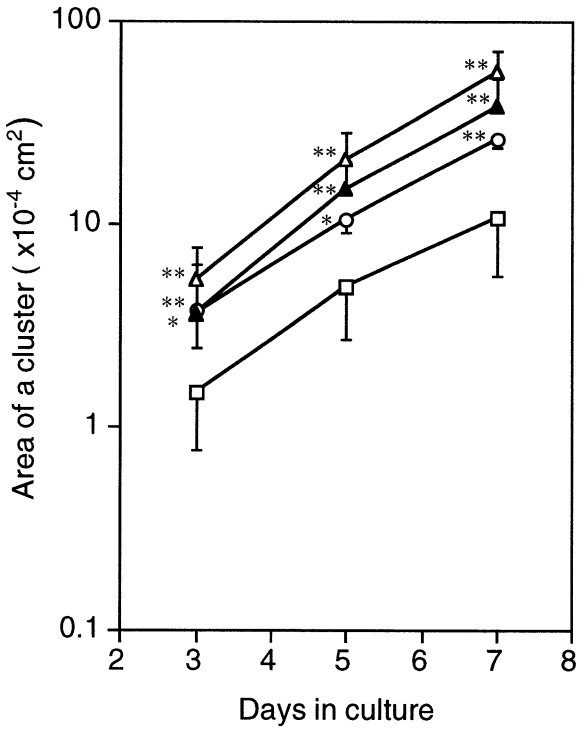

Figure 4. Quantitation of growth of papilla cells in keratinocyte-CM. Vibrissa papillae used in the experiment of **Fig 3** were cultured up to 7 d. Photographs were taken using a phase contrast microscope at day 3, 5, and 7, and then used to determine the area of clusters formed by papilla cells outgrown from the explants. Each point represents the mean ± SD of eight independent experiments. □, control medium; ○, CM5; △, CM7(+); ▲, CM7(–)/FBS. Significant difference from the control; $^{**}p < 0.01$, $^{*}p < 0.05$.

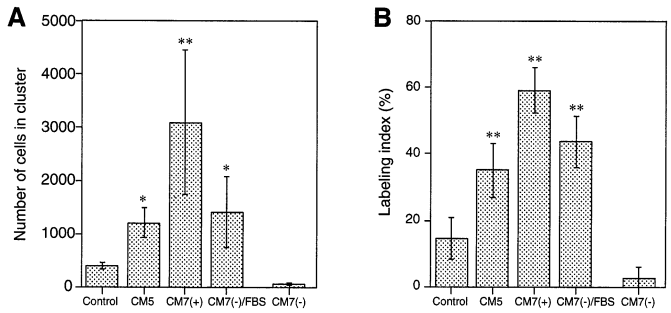

Figure 5. Effect of CM on the growth of papilla cells. Vibrissa papillae cultured for 7 d in the experiment of **Fig 4** were incubated with BrdU for 6 h. (*A*) The number of cells in the cluster derived from each isolated papilla was counted on photographic prints. (*B*) BrdU-labeling index was determined for the cells in each cluster described in (*A*). Each column represents the mean ± SD of eight independent papillae. Significant difference from the control; $^{**}p < 0.01$, $^{*}p < 0.05$.

under a kidney capsule of rats. The inductive ability was apparently high and macroscopically visible in three cases of CM8 cells (**Fig 9**); hair shafts grew out of the implant. Because other implants did not show such macroscopic alterations, the induction of hair follicles was histologically examined on serial sections of each implant. The tissues of hair matrix and dermal papilla were also immunohistochemically identified in the CM cell-induced tissues by specific MoAb of ORS-4 and D-2 (**Fig 10**). Intact papillae induced hair follicles in six of seven implants (**Table I**). Most implants with CM5 cells (86%) and with CM8 cells (93%) developed tissues that were histologically typical of hair follicles. Hair bulbs and hair shafts were induced even in the control explants, although the rate of incidence was low (14%–20%). Incidence of the induction by intact papilla cells or CM cells was statistically significant at a level of $p < 0.05$ compared with negative control cells (3T3 cells) or the implants without inserts.

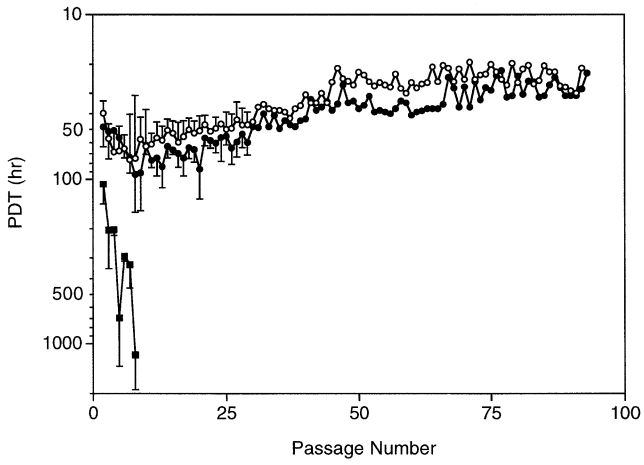

Figure 6. Serial cultivations of papilla cells in CM. Papilla cells were serially cultured in unconditioned medium (■), 50% CM5 (○), and 50% CM8 (●). PDT were determined at each passage of subcultures. Each data point up to the 29th passage represents the mean ± SD of PDT. Four independent subculture experiments were performed for CM5 and CM8, one culture was terminated at passage 11, one at passage 29, one at passage 31, and the final one at passage 92.

DISCUSSION

Cultivation of dermal papilla cells has been a useful method for researchers seeking to reveal the mechanism behind the epitheliomesenchymal interaction that leads to the formation of a hair follicle. The first such trial was reported by Jahoda and Oliver (1981) for rat vibrissal papillae and by Messenger (1984) for human dermal papillae, in which singular morphologic characteristics of the papillae were described, but growth potential and hair follicle-inductive potency were not investigated. Since then, several attempts have been made to characterize the growth capacity of cultured papilla cells of rat (Jahoda and Oliver, 1984) and human origin (Katsuoka *et al*, 1986, 1987; Messenger *et al*, 1986; Warren *et al*, 1992; Warren and Wong, 1994). Jahoda *et al* (1984) first showed that cultured rat vibrissal dermal papilla cells sustain their hair-inductive activity; however, papilla cells in culture lose this activity in later passage number (Horne *et al*, 1986; Lichti *et al*, 1993; Weinberg *et al*, 1993).

To our knowledge, our study is the first demonstration that when appropriately cultured, papilla cells can be serially cultured for a long period without losing their hair-inductive ability. This study was initiated by the finding that the outgrowth of papilla cells is greatly stimulated by the presence of keratinocytes in the culture. The feeder effect of keratinocytes could be partly replaced with CM of keratinocytes. We succeeded in repeatedly multiplying the hair-inductive papilla cells *in vitro*.

Our study clearly demonstrates that the CM of keratinocytes contains a potent mitogenic factor(s) for rat dermal papilla cells. This factor did not decrease its activity even in the CM that had been stored at 4°C for a month, indicating that the factor(s) is stable for a relatively long period (data not shown). The molecular weight of the factor(s) should be greater than 5 K, because the mitogenic activity was found in the retained fraction of ultrafiltration with a 5 K-cut membrane filter (data not shown). Warren and Wong (1994) also demonstrated that human keratinocytes stimulate the mitogenic activity of dermal papilla cells derived from human scalp hair. This stimulation is partly mediated by certain diffusible factor(s) with a molecular weight of more than 3 K. Furthermore, Kratz *et al* (1991) reported that CM of human keratinocytes has a mitogenic activity toward various types of cells, including endothelial cells, keratinocytes, smooth muscle cells, and mouse 3T3 cells. Moore *et al* (1991) showed that keratinocytes express several growth

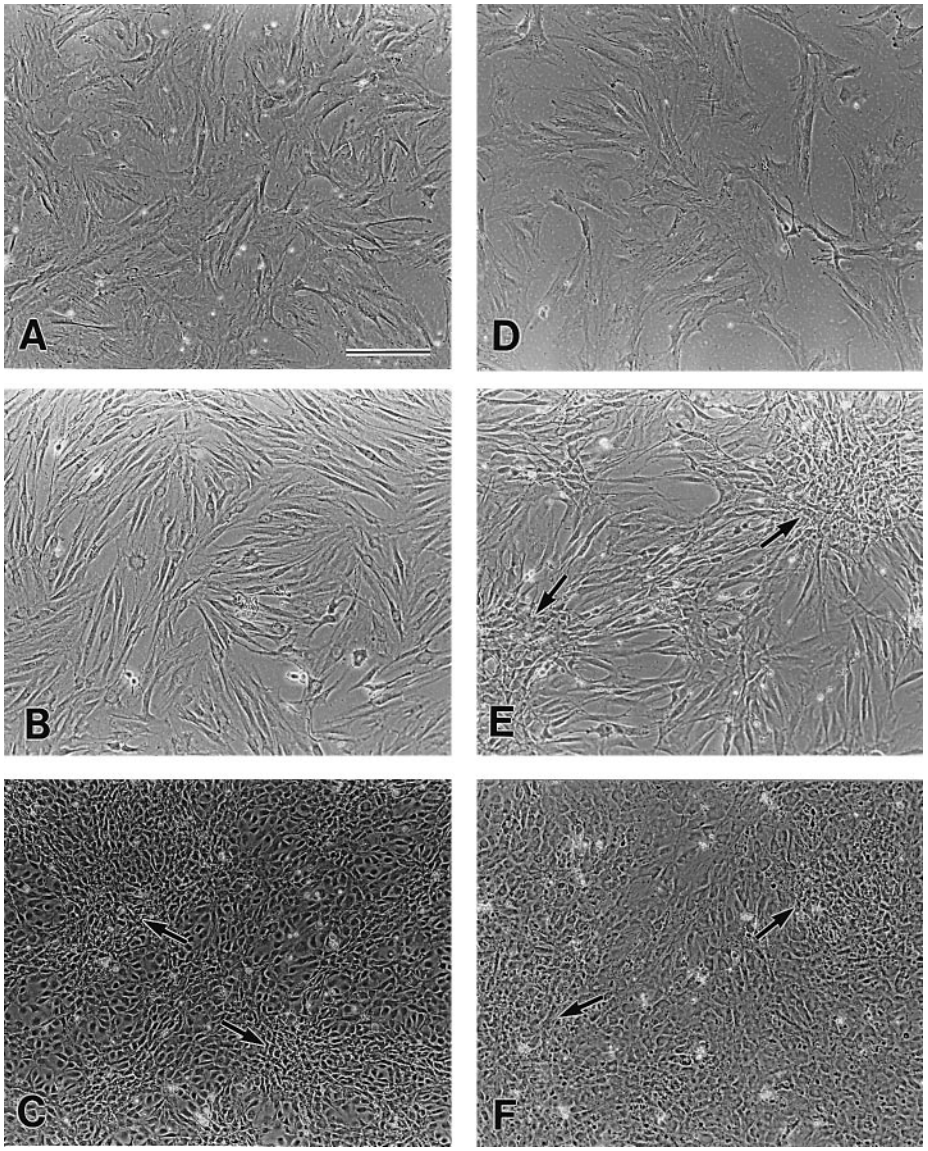

Figure 7. Phase-contrast microscopic views of dermal papilla cells during subcultivations in CM. Papilla cells were serially cultured in CM5 (*A*, *B*, *C*) and CM8 (*D*, *E*, *F*), and were photographed at the fifth (*A*, *D*), twentieth (*B*, *E*), and fifty-ninth passage (*C*, *F*). *Arrows* show multilayered aggregates (clumps). *Scale bar*: 100 μm.

factors *in vivo* and also *in vitro*, such as FGF1, FGF2, TGFα, TGFβ, and epidermal growth factor. We have not biochemically identified the responsible mitogen in the CM that we studied here.

Several studies have previously revealed several notable features of cultured papilla cells: (i) the cells are flat and polygonal (Messenger *et al*, 1986); (ii) they tend to form multilayered aggregates due to their highly adhesive property (Jahoda and Oliver, 1984); (iii) they grow slowly (Jahoda and Oliver, 1984); and (iv) they have the inductive ability to elicit hair growth from follicular and afollicular keratinocytes (Jahoda *et al*, 1984; Reynolds and Jahoda, 1992). These features contrast with the features of dermal fibroblasts. Our results confirmed these notable features of cultured dermal papilla cells. Papilla cell strains cultured with CM formed multilayered aggregates, which were more conspicuous after the cells had reached confluence. The cells were spindle-like, and expressed papilla-specific antigens but not keratinocyte-specific antigens. These notable appearances could be sustained during serial cultivations up to at least 90 passages. Importantly, papilla cells cultured in CM of keratinocytes sustained their inductive ability even at the 70th passage. These results indicate that CM is effective in activating the growth of papilla cells and maintaining their hair-inductive potential; however, the chromosome composition of the strains showed a certain degree of divergence from the normal diploidy. Irrespective of their aneuploidy, the strains showed no signs of tumorigenicity when implanted into nude mice.

As cited in the previous section, papilla cells lose their hair-inductive activity during repeated subcultivations. In contrast, our results show that papilla cells that have been serially cultured for a long time can induce hair follicles from the afollicular epidermis. We used papilla cells from the same species and tissues as Jahoda *et al* (1984), who could not demonstrate the hair-inductive ability of papilla cells at passage numbers higher than 10. A major difference between that study and ours is our use of CM (we use CM, whereas Jahoda *et al* did not). This means that the CM we prepared significantly improves the culture condition of papilla cells for maintaining inherent characteristics of the cells.

The hair-inductive activity of cultured papilla cells was determined by stuffing the cells into a small piece of sole skin and implanting it under a kidney capsule. The skin was obtained from the central part of the sole to avoid contamination of follicular tissues from the surrounding hairy skin. In addition, foot pad areas were removed from the implants because we observed a relatively high incidence of spontaneous hair formation in these areas (unpublished observations). In spite of following such a careful experimental protocol, we found hair follicles that unexpectedly formed in negative control implants at a rate of incidence of 14%–20%. This result was

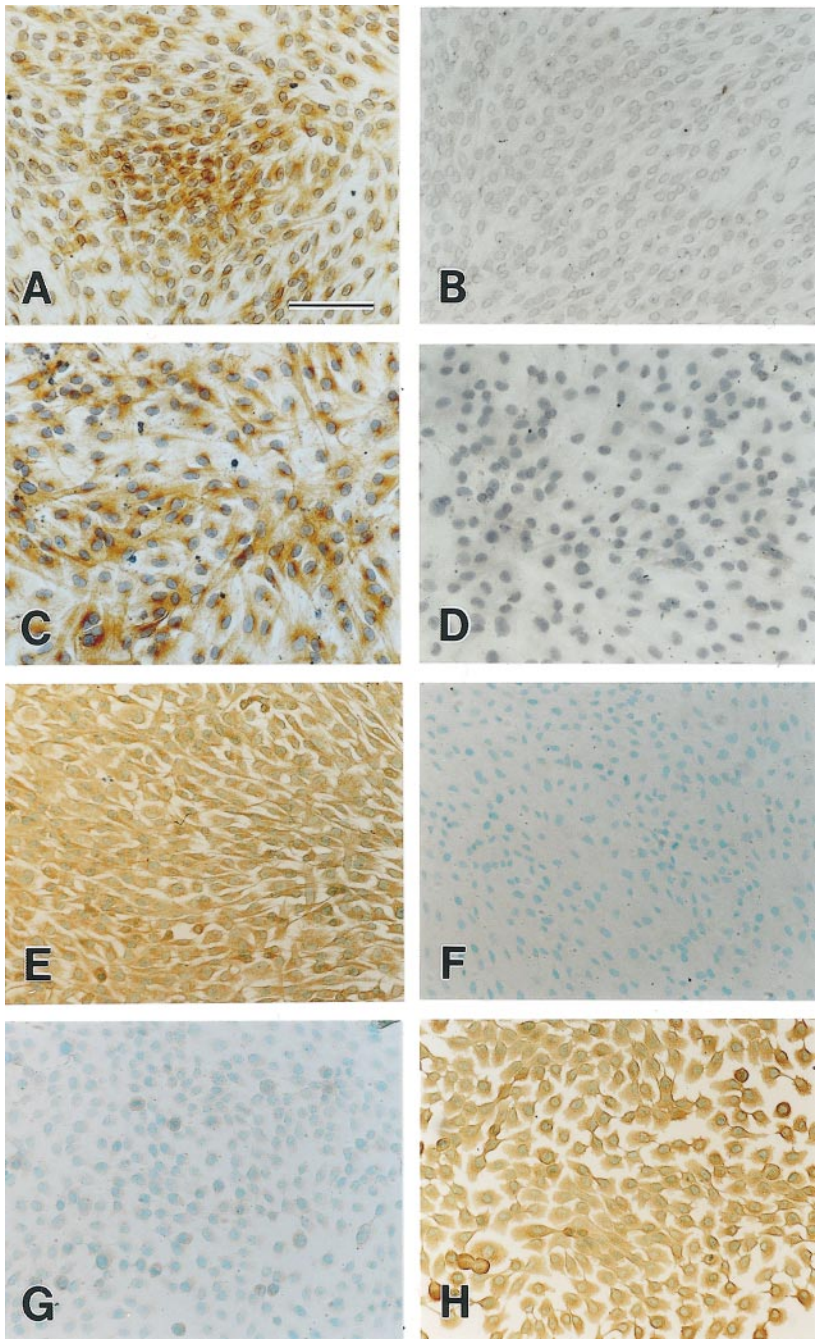

Figure 8. Immunohistochemical characterization of serially cultured papilla cells. Papilla cells were cultured in CM5 (*A*, *B*, *E*, *F*) and CM8 (*C*, *D*), and were immunohistochemically characterized at passage 45 (*A–D*) and 37 (*E*, *F*), respectively, using MoAb D-2 (*A*, *C*), MoAb ORS-4 (*B*, *D*), anti-vimentin Ab (*E*), and anti-CK 14 Ab (*F*). Bound MoAb were visualized by peroxidase activity (brown color). Photos G and H are immunostainings of keratinocytes using anti-vimentin Ab and anti-CK 14 Ab, respectively. *Scale bar*: 100 μm.

Table I. Hair induction assay

Inserts	Passage number	No. of implants		No. of hair bulbs induced (%)[a]		No. of shaft-producing bulbs (%)[a]	
No inserts	–		10		2 (20)		2 (20)
Swiss 3T3 cells	–		7		1 (14)		1 (14)
Intact papillae	–		7		6[b] (86)		6[b] (86)
CM5 papilla cells	33	2		2		1	
	36	2	7	2	6[b] (86)	2	4[b] (57)
	70	3		2		1	
CM8 papilla cells	20	5		5		5	
	37	3	14	2	13[c] (93)	2	13[c] (93)
	56	6		6		6	

[a]Numbers in parentheses show the rate (%) of incidence of induction of hair bulbs or shaft-producing bulbs.
[b]Significant difference from the implant without inserts; $p < 0.05$.
[c]Significant difference from the implant without inserts; $p < 0.01$.

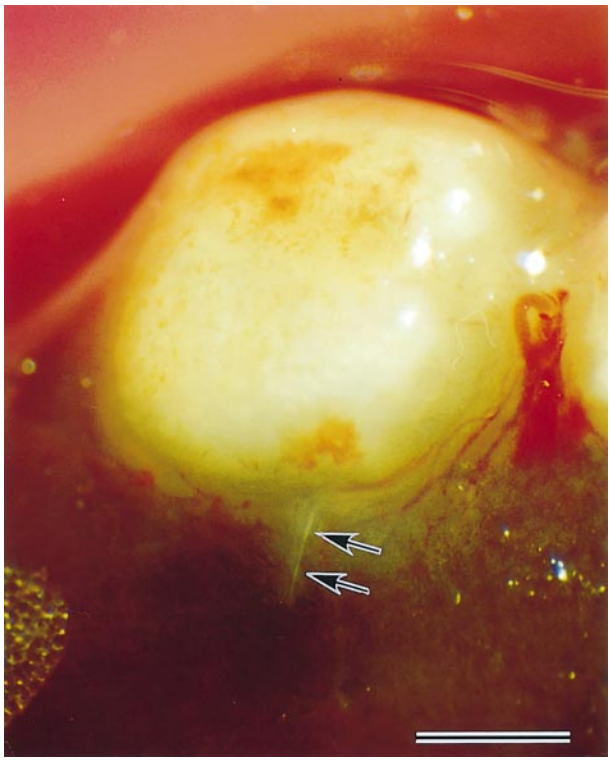

Figure 9. Hair-inductive ability of cultured papilla cells. CM8 cells at passage 56 were inserted between the epidermis and the dermis of sole skin, and implanted under a kidney capsule for 8 wk. *Arrows* show a hair shaft protruding from the implant. *Scale bar*: 1 mm.

reproducible in three independent experiments. Previously, spontaneous hair formation was also unexpectedly found when upper halves of rat vibrissal follicles were implanted under a kidney capsule, which had not been thought to induce the follicles unless hair-inductive cells or dermal papilla cells were inserted (Matsuzaki *et al*, 1996). The environment under a rat kidney capsule might facilitate such a spontaneous hair formation from the negative control explants.

Papilla cells cultured with CM8 started to aggregate at a lower cell density than those with CM5, and the hair-inductive ability of CM8 cells was slightly higher than that of the CM5 cells, suggesting that CM8 cells sustained more of the native characteristics of papilla cells. Furthermore, the CM5 cells were smaller and less adhesive than CM8 cells. Interestingly, these two characteristics of CM5 cells changed to those of CM8 cells when the culture medium was replaced with the medium containing CM8, whereas such changes did not occur when CM5 cells were continued to be cultured in CM5 medium, and *vice versa*. Such differences between the two CM may be attributable to the state in which keratinocytes were cultured during the preparation of each CM; CM5 was prepared when keratinocytes were in a preconfluent state, whereas CM8 was in a postconfluent state. Cells grow actively in a preconfluent state and cease to grow in the confluent state. Keratinocytes in the confluent state may secrete proteins different from proteins secreted by cells in the preconfluent state. At present the molecule(s) that is responsible for the difference between CM5 and CM8 is not known.

CM7(–) prepared in serum-free DMEM sustained the mitogenic activity toward papilla cells at about 74% of that of CM7(+) when the CM7(–) was assayed by adding fresh FBS at 10%. This retention indicates that keratinocytes can secrete an active principle(s) in the absence of FBS, thereby making it advantageous to biochemically isolate it from the CM. When the assay of CM7(–) was done without adding 10% fresh FBS, no growth stimulation was observed, demonstrating the necessity of some factor(s) in FBS for the active principle to work on papilla cells.

Attempts to maintain functional dermal papilla cells during repeated

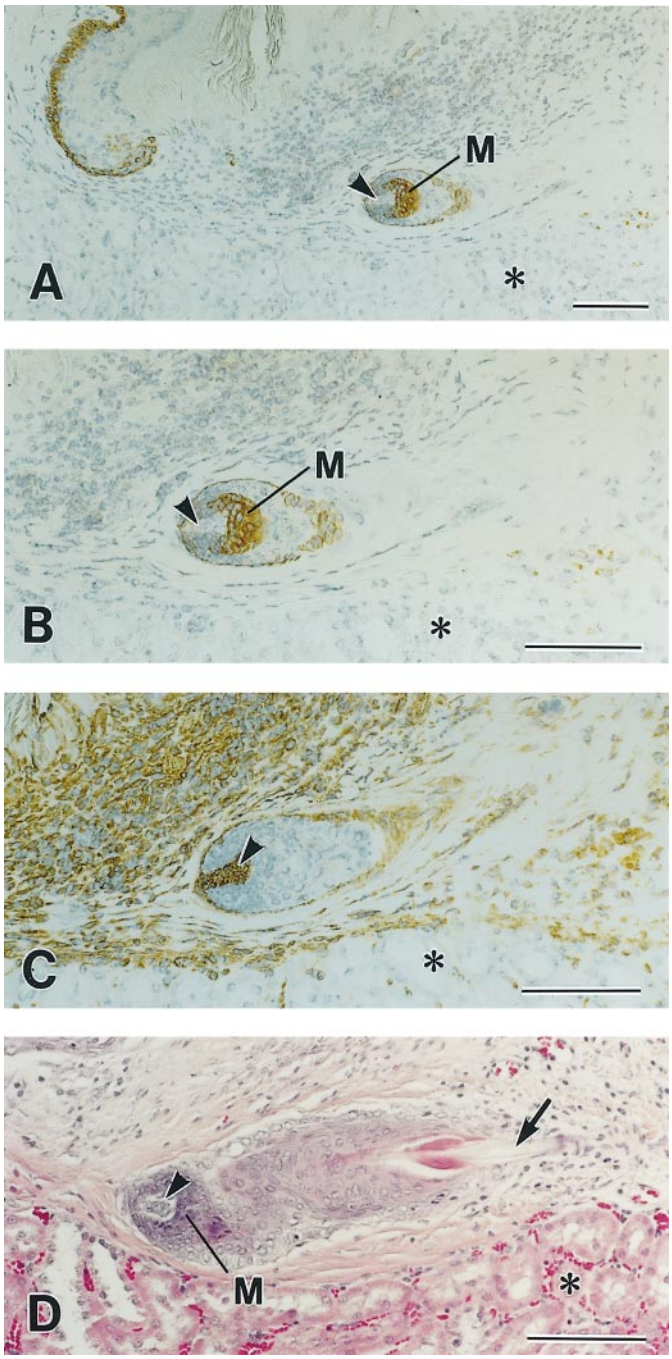

Figure 10. Immunohistochemistry of hair follicles induced by cultured papilla cells. Structures of hair follicles induced by CM8 cells at passage 56 were immunohistochemically characterized using MoAb of ORS-4 and D-2. (*A*) Section stained with ORS-4. (*B*) Enlarged view of reproduced dermal papilla (*arrowhead*) in (*A*), clearly showing the hair bulb. Positive stains are visible in the hair matrix (M). (*C*) Section adjacent to (*B*), showing that both a dermal papilla (*arrowhead*) and the surrounding tissues of the dermal sheath were intensely stained by D-2. (*D*) Haematoxylin and eosin staining on a section of an induced hair follicle. Cultured papilla cells induced typical structures of the matrix (M) and hair shaft (*arrow*). *Arrowheads* show the reproduced dermal papilla, and asterisks show the kidney tissue. *Scale bar*: 100 μm.

passages in culture have not been successful. At least two research groups tried to overcome this problem by immortalizing early passage dermal papilla cells with viral immortalizing genes (Bayley *et al*, 1991; Lichti *et al*, 1993); however, depending on the strains, these cells showed no or a low hair-inductive ability. The culture method of dermal papilla cells that we developed should aid in the discovery of

a biochemical entity that can stimulate hair growth and induce hair follicles from afollicular skin.

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