An embryonic pineal body as a multipotent system in cell differentiation

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Summary

The differentiating potency of pineal cells from 8-day quail embryos was studied with cell culture. It was found that the differentiation of striated muscle fibres occurred abundantly in the pineal cells cultured in hypertonic culture conditions. Muscle nature of these fibres was confirmed by utilizing the antiserum against the striated muscle type creatine kinase (MM-CK). When CO₂, NAHCO₃, NaCl, KCl and MgCl₂ were added in hypertonic concentrations, extensive myogenesis occurred in cultured pineal cells. Myogenesis in pineal cultures began as early as 2 days and, after 3 days in the medium with 75 mM additional NaCl, reached 100-fold when compared with that in the isotonic medium. Muscle fibres from pineal cells in culture were similar in morphology to the skeletal muscle fibres of mesodermal origin in situ. Myogenesis of pineal cells under hypertonic conditions was accompanied by the synthesis of a unique 56×10⁶ Mr protein, which was not found in the intrinsic muscle cells. Clonal cell culture revealed that about 80% of clonable pineal cells were myogenic precursors. Pineal cells of 8-day quail embryos were not only myogenic but oculopotent (melanogenic and lentoidogenic) in cultures. This study examined whether multipotential progenitor cells with both potentials are present in the pineal or not. The results showed that at least 16% of all clonable pineal cells were multipotent precursors. The embryonic pineal is considered to be a typical multipotent system in parallel with the pigmented and neural retina, the neural crest and the teratocarcinoma.

Key words: pineal body, ectopic myogenesis, melanogenesis, multipotency in differentiation, cell culture, transdifferentiation.

Introduction

The pineal body of aves and mammal is located on the roof of the third ventricle and has an endocrine function. Major constituents of the pineal are endocrine pinealocytes and supportive glial cells (Collin, 1971). In addition to these, ectopic striated muscle fibres also occur at a low frequency in the in situ pineal of some mammalian species (see Watanabe et al. 1981). When the pineal cells of neonatal rats were cultured in vitro, some muscle fibres were always observed (Watanabe et al. 1981). Thus, it is certain that the pineal contains cells that have the potential for muscle differentiation. There have been several reports of muscle differentiation of cells derived from nervous tissues. Lennon & Peterson (1979) found the spontaneous differentiation of muscle fibres from rat glial cell lines. Wier & Lennon (1981) showed that cells of the rat optic nerve, probably glial cells, differentiated into striated muscle fibres. One of the main aims of the present paper is to investigate which type of cells in the pineal is myogenic. Cell culture studies have already shown, besides myogenesis, the differentiation of pigmented epithelial cells and lens cells in pineal cultures (Watanabe et al. 1985). Such expression of ‘oculopotency’ of pineal cells may be relevant to the fact that the pineal body is an eye-like organ in lower vertebrates before its conversion to an endocrine organ in avian species (Eakin, 1970). In the present experiments, we have tried to discover whether there are two separate types of progenitor cells with ‘oculopotency’ and myogenic potency, respectively, or common bipotent progenitors are present in the embryonic pineal.
Materials and methods

Materials
Fertilized quail eggs were obtained from a local quail yard and 8-day embryos were used for culture experiments.

Primary culture
Pineals were removed from the brain with forceps and were treated with 0.1% collagenase (Sigma) and 100 IU ml⁻¹ dispase (Sanko, Japan) each for 30 min at 37°C. After these processes, pineals were almost free from mesenchymal cells, while the pineal epithelium (parenchyme) remained intact (Fig. 1). The cleaned pineals were dissociated into single cells with 1 mM-EDTA for 15 min at 37°C.

Basal culture medium (medium E) was Eagles' MEM (Nissui, Japan) supplemented with 250 µg ml⁻¹ sodium pyruvate, 50 µg ml⁻¹ ascorbic acid, 0.1 IU ml⁻¹ insulin (Sigma), 32 IU ml⁻¹ penicillin, 40 µg ml⁻¹ streptomycin (Meiji, Japan), 2.5 µg ml⁻¹ fungizon (Gibco), 1% chicken serum (Flow) and 10% heat-inactivated horse serum (Gibco). For the high ionic culture condition, stock solution of 2.5 mM-NaCl, KCl, NAHCO₃ and 1 mM-MgCl₂ was added to medium E. Cells were inoculated in plastic dishes (Corning) coated with collagen (1.4 x 10⁶ cells cm⁻²). Cultures in hypertonic conditions started 24 h after inoculating the cells, since hypertonicity might inhibit the cell-substrate adhesion.

Clonal analysis with limiting dilution
Cells were precultured for 2 days, harvested and dissociated with 0.1% collagenase, and then with a buffered saline containing 0.25% trypsin and 0.5 mM-EDTA each for 30 min at 37°C. By this procedure, a cell suspension with almost single cells (over 95%) was obtained. However, complete elimination of 2- to 3-celled aggregates was not possible. Into each well of a 96-well microplate (Corning), 10 singly dissociated cells were inoculated. This cell density, which gave statistically monoclonal growth in each well, was determined by the limiting dilution (cf. Lefkovitz & Waldman, 1979). Thus, this type of culture will be designated as 'clonal' in this paper. A mixture of fresh medium (2 vol) and a conditioned medium (1 vol) was used for clonal culture. To prepare the conditioned medium, pineal cells (2 x 10⁶ per 6cm dish) were cultured for 7 days and throughout the following 7 days the medium was changed every day and the harvested medium was frozen to be used as the conditioned medium.

To determine the frequency of myogenic precursors in the pineal, cells were precultured in medium E for 2 days and then transferred into clonal cultures using medium E with 75 mM additional NaCl for another 3 weeks. Finally, they were processed for immunostaining of creatine kinase specific for skeletal muscle fibres (MM-CK).

To detect any possible multipotency of pineal cells, cells precultured for 2 days were transferred to clonal culture for another 2 weeks. The medium used in the preculture and in the clonal culture was Ham's F12 (Nissui, Japan) supplemented with 10% fetal bovine serum (Gibco), which is known to have a tendency to suppress cell differentiation (Pinset & Whalen, 1984). Then, colonies were individually dissociated into single cells with enzyme treatment. Cells obtained from individual colony were divided into a pair of daughter cultures and cultured for 3 weeks, using 24-well multiplates (Corning). One daughter culture of each colony was nourished with medium E to detect the differentiation of pigmented epithelial cells and processed for DOPA histochemistry (see below), whereas the other was cultured in the hypertonic medium E to detect the differentiation of skeletal muscle fibres and processed for MM-CK immunostaining.

Immunological methods
MM-CK and brain-specific creatine kinase (BB-CK) (Eppenberger et al. 1964; Perriard et al. 1978) were purified from the breast muscle of adult quails and from the brain of cockerels, respectively, according to Keutel et al. (1972). MM-CK was used for immunizing rabbits. The fact that the antiserum is highly MM-CK-specific was confirmed by several independent techniques. In double-diffusion test, anti-MM-CK serum formed a precipitin line against purified MM-CK from the breast muscle, but not against BB-CK from the brain. During immunostaining, the antiserum stained cultured muscle cells, whereas cultured fibroblasts, brain cells and neural retinal cells were negative. ELISA (sandwich method) detected as little as 1 pg MM-CK and gave a linear reaction from 1 pg to 1 ng MM-CK. BB-CK up to 1 µg was under the detection limit of our system.

Immunostain for MM-CK was done with the PAP method (Sternberger et al. 1970). Cells were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, overnight at room temperature and washed with phosphate-buffered saline containing 0.1% Triton X-100 (TPBS). The specimens were successively treated with anti-MM-CK (1:400 dilution), goat anti-rabbit-IgG serum (1:100 dilution, MBL, Japan), and peroxidase–anti-peroxidase complex (1:100 dilution, DAKO) for 30 min at room temperature, respectively, and then were washed with TPBS for 15 min after the antibody treatment. After another wash with PBS, colouration of HRP was done on ice for 30 min with AEC substrates (3-amino-9-ethyl-carbazole, Aldrich).

Fig. 1. Thin section of an isolated 8-day-old embryonic pineal cleaned by enzyme treatments. Note the almost complete removal of mesenchymal cells, whereas the epithelial elements remain intact. Bar, 50µm.
The activity of MM-CK was determined with ELISA. To do this, cultured pineal cells were frozen and thawed several times, and the supernatants were used to quantify MM-CK. From anti-MM-CK serum, HRP-Fab' was prepared following Hashida et al. (1984). IgG fraction of anti-MM-CK (0.04 mg ml\(^{-1}\) in 0.1 M-bicarbonate), 0.1 ml well\(^{-1}\), was adsorbed to 96-well microtitre plate (Nunk) overnight at 4°C. Plates were stored stably for months in PBS containing 0.1% bovine serum albumin and 0.1% NaN\(_3\). Before assay, plates were washed with TPBS. Standards, specimen MM-CK and BB-CK were diluted serially with TPBS and 0.1 ml of each was incubated in IgG-coated plates for 2 h at room temperature. After washing with TPBS for 15 min, 0.1 ml of a solution of HRP-Fab' (400 ng ml\(^{-1}\)) was added to the well and incubated overnight at room temperature. After thorough washing, HRP was coloured with 0.05 ml ABTS substrates (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (Kirkegaard & Perry Laboratories Inc. MD)) by a 2 h incubation. After adding 0.25 ml water, absorbance at 405 nm was read with a spectrophotometer.

**DOPA histochemistry**

DOPA histochemistry of Rodriguez & McGavran (1969) was adapted to culture cells.

**Electron microscopy**

Cells were fixed in situ with the culture substrate with 2.5% glutaraldehyde in 0.1 m-phosphate buffer pH 7.4 for 30 min and postfixed with 1% OsO\(_4\) in the buffer for 2 h at 4°C, dehydrated with ethanol–butanol series and embedded with Epon 812. After polymerization, the plastic substrate was taken away from the Epon block mechanically. Thin sections were cut tangentially to the surface of dishes. Sections were contrasted with uranyl acetate and lead citrate, then examined using an electron microscope (Hitachi H-600).

**Analysis of a protein composition in the cells**

The pineal cells or breast muscle cells were cultured in 24-well plates in medium E supplemented with various concentrations of NaCl (0–125 mM). After an incubation of 2 h in a medium containing 10 \(\mu\)Mml\(^{-1}\) \([\text{U}^\text{35S}]\)methionine, cells were washed with 150 mM-NaCl containing 1 mM-CaCl\(_2\), solubilized with 200 \(\mu\)l of Laemmli’s sample buffer (Laemmli, 1970) and incubated in boiling water for 3 min. The homogenate of the muscles of 13-day-old quail embryos were treated similarly for electrophoresis. 10 \(\mu\)l of samples and molecular markers (Bio Rad) were loaded on to a 12% separation gel following Laemmli (1970), using a microslab electrophoresis apparatus (Marysol, Japan). After electrophoresis, gels were stained with Coomassie blue and dried onto filter papers (Chromatography paper 3MM, Whatman) for autoradiography.

**Results**

**Effects of various culture conditions on pineal cell cultures**

In the early stage of culturing (up to about 7 days), most of the pineal cells were of epithelial shape in medium E under 5% CO\(_2\) atmosphere. There were some fibroblastic cells, but no muscle fibres were found. In addition, some small round cells with short processes attached on the top of epithelial sheets were observed, but they disappeared around one week in culture. However, when pineal cells were cultured in medium E under 12% CO\(_2\) atmosphere, elongated multinucleate cells with morphology similar to that of skeletal muscle fibres were sometimes observed around 2 weeks. In the same atmosphere, the differentiation of muscle fibres was enhanced when additional NaHCO\(_3\), NaCl, KCl (25 mM) or MgCl\(_2\) (10 mM) was added into medium E. Quantification of MM-CK in 3 weeks cultures showed that all these additives increased the amount of MM-CK in pineal cultures (Table 1). The original in situ pineals did not contain any detectable amount of MM-CK.

**Effect of NaCl concentration on the amount of MM-CK in pineal cells**

Pineal cells were cultured under 5% atmosphere in medium E containing various concentrations of additional NaCl. After 2 weeks in culture, the amounts of MM-CK and of total soluble protein in the cells were assayed. Three separate culture experiments were conducted for this purpose. MM-CK was about 1 \(\mu\)g mg\(^{-1}\) protein in isotonic culture conditions and it increased strikingly with the increase of additional NaCl and reached plateau at more than 100 ng mg\(^{-1}\) under additional 75 mM-NaCl (Fig. 2A). The level of MM-CK was decreased under additional 125 mM-NaCl. Since a substantial number of aberrant cells appeared under additional 100 mM- and 125 mM-NaCl, in the latter series of experiments, medium E with additional 75 mM-NaCl was chosen as the hypertonic medium E to enhance myogenesis in cultured pineal cells. MM-CK content in pineal cells cultured in the hypertonic medium E increased from 6 days to 14 days (Fig. 2B). Application of the hypertonic medium E was effective at any stages during 14 days' culture to increase the amount of MM-CK. The longer the cells stayed in the hypertonic medium E, the more MM-CK was produced (Fig. 3).

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>MM-CK/plate (ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% CO(_2)</td>
<td>1</td>
</tr>
<tr>
<td>12% CO(_2)</td>
<td>5</td>
</tr>
<tr>
<td>+25 mm-NaHCO(_3)</td>
<td>50</td>
</tr>
<tr>
<td>+25 mm-NaCl</td>
<td>400</td>
</tr>
<tr>
<td>+25 mm-KCl</td>
<td>150</td>
</tr>
<tr>
<td>+10 mm-MgCl(_2)</td>
<td>33</td>
</tr>
</tbody>
</table>

Table 1. **MM-CK synthesis of pineal cells cultured in various conditions for 2 weeks.** Values are an average obtained from two separate cultures.
Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Culture period (days)

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

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MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)

Addition of NaCl (ITIM)

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MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

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MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

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Creatine kinase/total water soluble proteins (ng mg⁻¹)

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Creatine kinase/total water soluble proteins (mg)

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Creatine kinase/total water soluble proteins (ng mg⁻¹)

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Creatine kinase/total water soluble proteins (ng mg⁻¹)

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Addition of NaCl (ITIM)

MEM + 75 mM-NaCl

MEM + 0 mM-NaCl

Creatine kinase/total water soluble proteins (ng mg⁻¹)

Creatine kinase/total water soluble proteins (mg)
It was found that the number of MM-CK-positive cells increased with an increase in hypertonic medium (Fig. 5). The number of cells in the hypertonic medium was significantly higher than those in the isotonic medium. These results suggest that the pineal cells have the potential for muscle differentiation.

**Multipotency of pineal cells**

Four independent experiments were conducted to examine the possible multipotency of pineal cells to differentiate into both muscle and ocular pathways, and the results are summarized in Table 2. The average clonal efficiency of experiments was about 4%. Cells in clonal growth were mostly epithelial in shape. Cells coming into close contact with neighbours grew particularly well and those cells were transferred to daughter cultures. When multiple colonies were grown in a single well, no transfer was made. Continued growth of cells after the transfer was observed in 51 pairs out of 82 colonies transferred. We found 8 (16%) multipotent pairs which contained both DOPA-positive cells and MM-CK-positive cells. Unipotent pairs positive only to DOPA reaction were 14 (27%) and those only positive to MM-CK were 13 (26%). The remaining 16 pairs (31%) consisted of unidentified cells only. Most of DOPA-positive cells were polygonal epithelial cells similar to the pigmented epithelial cells of the retina (Fig. 9A). MM-CK-positive cells were mostly elongated cells (Fig. 9B), although some of them were epithelial.
Polypeptide of pineal cells cultured in the hypertonic medium
When pineal cells were cultured in medium supplemented with higher concentration of NaCl than the physiological concentration, they expressed a protein with an apparent relative molecular mass of $56 \times 10^3$ (P56; Fig. 10A), which was absent in the cells cultured in the isotonic medium. The amount of P56

Fig. 6. MM–CK immunostaining of pineal cells cultured for 2 weeks in the hypertonic medium (A) and in the isotonic medium (B). Note that MM–CK positive cells in the hypertonic medium are mostly elongated multinucleated cells seen all over the dish. MM–CK-positive cells in the isotonic medium are very scarce and primitive in shape. Bar, 500 μm.

Fig. 7. Fine structure of muscle fibres formed in a 4-week pineal culture in the hypertonic medium. Arranged myofibrils (my), numerous mitochondria (mi) and eccentric nuclei (n), similar to those found in normal muscle fibres, are observed. Bar, 2 μm.
in pineal cells increased with the culturing time in the hypertonic medium. The autoradiograms showed that the accumulation of P56 was due to novel synthesis (Fig. 10B). P56 was not detected in the muscle tissue of 13-day-old embryos and in 6-day cultures of 8-day-old quail embryonic muscle (Fig. 11). It seems that P56 was detected specifically in the pineal cells cultured in the medium permissive to muscle differentiation.

**Discussion**

The pineal of 8-day-old quail embryos consists of the epithelium of follicles and mesenchymal cells. As to the origin of muscle cells observed in the pineal cultures in the present experiments (see also Watanabe et al. 1981 as to the neonatal rat pineal cells in vitro), the possibility of contamination of precursors of head musculatures can be eliminated. This can be stated not only by a careful elimination of adherent

<table>
<thead>
<tr>
<th>Cloning efficiency*</th>
<th>Daughter cultures</th>
<th>Growing colonies</th>
<th>DOPA &amp; MM-CK</th>
<th>Only DOPA</th>
<th>Only MM-CK</th>
<th>Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>18/72</td>
<td>12 pairs</td>
<td>7 pairs</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>35/72</td>
<td>22 pairs</td>
<td>15 pairs</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>64/192</td>
<td>24 pairs</td>
<td>12 pairs</td>
<td>1</td>
<td>6</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>47/96</td>
<td>24 pairs</td>
<td>17 pairs</td>
<td>4</td>
<td>2</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>164/432</td>
<td>82 pairs</td>
<td>51 pairs</td>
<td>8</td>
<td>14</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(100 %)</td>
<td>(16 %)</td>
<td>(27 %)</td>
<td>(26 %)</td>
<td>(31 %)</td>
</tr>
</tbody>
</table>

* The number of wells with growing colonies per the number of wells.

Fig. 8. MM–CK immunostaining of pineal cells cultured at the clonal cell density in each well for 3 weeks in the hypertonic medium. Large muscle colonies (arrows) are visible with naked eyes.

![Fig. 8](image_url)

Table 2. Examination of possible multipotency of pineal cells by daughter cultures of pairs clonally originated in four independent experiments

Fig. 9. A pair of daughter cultures originated from a single pineal cell. One of them was nourished in the isotonic medium and processed for DOPA histochemistry (A), whereas the other was grown in the hypertonic medium and processed for MM–CK immunostaining (B). Bar, 50 μm.

![Fig. 9](image_url)
Fig. 10. (A) Peptide patterns of pineal cells cultured in the hypertonic medium as revealed by SDS-PAGE. Cultures for 12 days in the medium E with no additional NaCl (lane 1), +25 mM (lane 2), +50 mM (lane 3), +75 mM (lane 4), +100 mM (lane 5) and +125 mM (lane 6). The arrow indicates a peptide with an apparent relative molecular mass of 56×10^3, which is only seen in cells cultured in the medium containing additional NaCl higher than 75 mM (lanes 4 to 6). (B) Autoradiograms showing proteins synthesized by the pineal cells pulse labelled with [35S]menthionine for 2 h in 12 days' cultures. Lanes 1 to 6 correspond to lanes 1 to 6 in A, respectively. The arrow indicates a newly synthesized 56K protein, which is clearly seen in the cells cultured only in media with additional NaCl higher than 75 mM (lanes 4 to 6).

Fig. 11. Comparison of peptide pattern of pineal cells cultured in the hypertonic medium (lane 3) with that in the muscle tissue of 13-day-old quail embryo (lane 1) and cultured muscle cells of 8-day-old quail embryos (lane 2). Pineal cells were cultured for 6 days in the medium supplemented with 100 mM NaCl, whereas muscle cells were cultured for 6 days in the standard medium E. A 56K protein band is seen only in the pineal cells cultured in the hypertonic medium (lane 3, arrowhead).

Then the question arises from which component of pineals, epithelial cells or mesenchymal cells, does the muscle differentiation occur. We suggest that the epithelial cells of follicles are the probable candidate for the following reasons. (1) In the present culture system, the inoculated cells were dissociated from follicles almost freed from adherent mesenchymal cells. (2) In clonal cell cultures, the frequency of muscle-forming colonies is too high to ascribe them to contaminating mesenchymal cells, even if present. (3) MM-CK-positive cells appeared mostly in islands of epithelial cells which are likely derived from follicles. (4) In an unpublished experiment, only mesenchymal cells of the pineals were collected and cultured in the hypertonic conditions. Few muscles were differentiated in these cultures.

The fate map shows that the presumptive regions of pineal are located bilaterally at the neural folds of cephalic region in amphibian neurula (Kamer, 1949). They fuse at the midline of the brain and form a pineal complex (Eakin, 1970). Cephalic neural folds also contribute to cephalic neural crest cells which differentiate into diverse cell types after migration in all vertebrates (Le Douarin, 1982). Therefore, judging from a common developmental origin, the pineal cells of 8-day quail embryos possibly retain a similar differentiation potency to neural crest. It has been revealed that the cephalic neural crest gives rise to muscle cells in normal avian development (Le Lievre & Le Douarin, 1975). Thus, the muscle differentiation in pineal cultures can be interpreted as the
expression of latent potency of neural crest retained in embryonic pineals.

The latent potency of pineals is, however, not only a neural crest one, but it also includes an ocular one. In pineal cultures, as demonstrated in the present experiment and previous ones (Watanabe et al. 1985; Watanabe, 1986), the differentiation into pigmented epithelial cells (considered to be retinal pigmented cells) and lens cells are frequent. Such oculopotent of pineals can be explained as a remnant of ancestral function of pineals; the pineal of lower vertebrates is the photoreceptive organ which is composed of lens cells, pigment cells and photoreceptor cells (Eakin, 1970).

One of the man aims of the present study is to examine whether there are any multipotent precursor cells present in embryonic pineals which give rise to both muscle and ocular cells in their progenies. Clonal culture experiments were conducted for this purpose. It was shown that the frequency of such multipotent precursors was 16% of all clonable cells, whereas those precursors producing MM–CK colonies were 42% (multipotent pairs plus unipotent pairs with MM–CK activity). However, the frequency of such multipotent precursors might be underestimated. As described in the results section, 76% of clonal pineal cells were estimated to be myogenic. If we assume that the frequency of melanogenic cells is similar and that myogenesis and melanogenesis occur independently, the probable frequency of multipotent cells can be calculated to be 58% (approx. 0.76 × 0.76 = 0.58). Usage of suppressive medium (Ham's F12) for the initial step of clonal culture, passage to daughter cultures and the relatively long culture period might interfere with the expression of cell phenotypes in the assay for detecting multipotent cells in the present culture conditions.

It is demonstrated here that pineal cells can be simultaneously myogenic and oculopotent. Another example of such bipotency is the chicken pigmented epithelium of iris (sphincter and dilater of pupil), in which cells are provided with a hybrid character containing both pigmented granules and muscular organelle of skeletal type (Patricia & Koch, 1984).

It is well known that neural crest cells of avian embryos at 2–3 days of incubation have a wide repertoire of differentiation into various cell types including myogenesis (Le Douarin, 1982). However, except for the neural crest origin of iridal striated muscle in normal development of bird (Nakano & Nakamura, 1985), the potency of crest cells to differentiate into ocular cells has not been demonstrated. Anyway, the multipotential nature of neural crest cells is present only in early stages. Retention of multipotency in the pineal cells at stages as late as 8-day-old embryos is thus a unique characteristic comparable only to that of the neural and pigmented retina (Okada, 1983).

Ectopic cytodifferentiation also occurs, though very rarely, in pineals in situ. Some available examples are the striated muscle fibres (Watanabe et al. 1981), melanogenesis (Watanabe et al. 1985), neurones (Ueck, 1979) and calcification like bones (Tapp, 1979). Such ectopic differentiation usually occurs very inconsistently and varies among individuals in situ. However, when the pineal cells are dissociated and cultured in vitro, the ectopic differentiation becomes very extensive. In relation to the multipotency of pineals, it is interesting to mention that this organ is the most frequent site of development of teratoma and germinoma in the brain (Russell & Rubinstein, 1977). It is very conceivable that such multipotent tumours clonally originate from multipotent precursors in the pineals.

The fact that the pineal cells cultured in conditions permissive to myogenesis start to synthesize a new protein with an approximate relative molecular mass of 56 × 103 (P56) seems to provide an initial step for searching key molecule(s) to channel the differentiation of multipotent pineal cells into the ectopic pathways. It has been known that when cells are exposed to adverse conditions, they often produce new proteins including heat-shock proteins (hsp), among which the 25K, 65K and 81K hsp protein have been reported in chicken muscle cultures (Bag, 1983). P56 found in the present system may be produced for adaptation to hypertonicity. Similar observation has been reported, when cultured chicken fibroblasts were exposed to the hyperosmolar medium (Petrini et al. 1986). It remains to be investigated whether such change in the polypeptide pattern of pineal cells in the hypertonic medium is a cue to ectopic differentiation of these cells.

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