Development and characterization of five rainbow trout pituitary single-cell clone lines capable of producing pituitary hormones

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Abstract

Five single-cell clone lines (mRTP1B, mRTP1E, mRTP1F, mRTP1K, and mRTP2A) have been developed from adult rainbow trout pituitary glands. These cell lines have been maintained in a CO₂-independent medium supplemented with 10% fetal bovine serum (FBS) for more than 150 passages. At about 150 passages, the doubling time of each single-cell clone in a CO₂-independent medium supplemented with 10% FBS at 20 °C was 3.6 ± 0.7 , 2.8 ± 0.7 , 3.2 ± 0.8 , 5.5 ± 0.6 , and 6.6 ± 0.6 days respectively. Each single-cell clone contains 60 ± 2 chromosomes, which is within the range of the 2N chromosome numbers reported for rainbow trout. Reverse transcription-PCR analysis revealed that in addition to expressing *gh*, prolactin (*prl*), and estradiol (E₂) receptor α (*e2ra* or *esr1*) genes, each single-cell clone line also expressed other pituitary-specific

genes such as *tsh*, gonadotropin 1 (*gth-1* or *fshb*), gonadotropin 2 (*gth-2* or *lhb*), somatolactin (*sl* or *smtl*), proopiomelanocortin-B (*pomcb*), and corticosteroid receptor (*cr* or *nr3c1*). Immunocytochemical analysis showed that all the five single-cell clones produced both Gh and Prl. Furthermore, the expression of *gh* and *prl* genes in the single-cell clone lines is responsive to induction by E₂, dexamethasone, and *o,p'*-dichlorodiphenyltrichloroethane. All together, these results confirm that each of the single-cell clones was derived from rainbow trout pituitary glands. These single-cell clone lines not only can be used to study factors that regulate the expression of pituitary hormone genes, but can also be developed as a rapid screening system for identifying environmental endocrine disruptors.

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Introduction

Development of *in vitro* systems is crucial for understanding the mechanism of regulation of pituitary hormone gene expression at the molecular level in fish. Whole pituitary gland cultures (Yada *et al.* 1991, Elango *et al.* 2006) and dissociated pituitary gland primary cell cultures (Le Goff *et al.* 1992) are the two *in vitro* systems that have been used to study factors that regulate the production and secretion of GH and prolactin (PRL) in the pituitary of rainbow trout (*Oncorhynchus mykiss*). Both systems are considered short term, with cultures usually only being able to be maintained for about 1 week. There are several drawbacks associated with these two systems for studying the expression of *gh* and *prl* genes. Since the pituitary gland of fish is very small, pituitary glands from many fish have to be used for establishing any pituitary gland explant culture, and this will lead to a great variability within an experiment or between experiments because of the variability among fish. In addition, setting up pituitary gland explant cultures is further limited by the seasonal availability of sufficient numbers of fish which can receive experimental manipulation. The specific drawback of using primary pituitary cell cultures stems from the step of dissociating

pituitary glands. This step can be difficult to reproduce consistently, and the dissociated cells frequently require a period of time to recover, during which the expression of certain genes could be detrimentally affected. For instance, as reported by Le Goff *et al.* (1992), the expression of *prl* gene in the rainbow trout primary pituitary cell cultures dropped significantly during the first 2 days of the cultures. Additionally, primary pituitary cell cultures can only be maintained for 1 week; new cultures need to be prepared in order to repeat any experiment. Almost all these problems could be overcome if continuous pituitary cell lines that can produce pituitary peptide hormones are available. Unfortunately, very few fish pituitary cell lines are available to date (Ribeiro & Ahne 1982, Bols & Lee 1991, Bols *et al.* 1995).

The first mammalian pituitary cell lines (GH₃ cells, four clonal strains) capable of synthesizing GH and PRL were established from rat pituitary tumors many years ago (Yasumura *et al.* 1966, Tashjian *et al.* 1968, 1970), and these cell lines have been proven useful in a wide range of studies (Ool *et al.* 2004, for review). Furthermore, Chomczynski *et al.* (1988) also have developed a cell line (somatomammotroph cells, rPCO) from the normal pituitary of an adult rat. Although the tumor cell lines can grow indefinitely in

culture, the rPCO line was developed without any immortalizing treatment and yet has been maintained in culture for more than 1 year (over 45 passages).

The advances in recombinant DNA and protein purification technologies in the past 20 years have generated abundant knowledge on the structures of GH, PRL, somatolactin (SL or SMTL), one of the GH family proteins found only in fish (Ono *et al.* 1990, Rand-Weaver *et al.* 1991), and other pituitary hormones in fish. While some of the hormonal and environmental factors that regulate the expression of pituitary hormone genes have been identified in mammals, factors that regulate the expression of these genes in fish remain to be fully determined due to lack of reliable pituitary cell lines. Although a cell line has been developed from rainbow trout pituitary glands (Bols & Lee 1994, Bols *et al.* 1995), this cell line has a few drawbacks. First, instead of being a clonal strain, this cell line contains a mixed population of pituitary cell types, which will bring serious variability from experiment to experiment. Secondly, upon continued culturing passing passage 50, this cell line was shown to have lost its ability to express *gh*, *prl*, and *sl* (*smtl*) genes. Thus, there is still an urgent need to develop a permanent clonal fish pituitary cell line.

Shambloott & Chen (1992) reported the existence of four isoforms of pro-insulin-like growth factor 1 (IGF1) that encode identical mature IGF1 but four different forms of pro-peptides, namely Ea1, Ea2, Ea3, and Ea4. In our laboratory, Tian *et al.* (1999) showed that recombinant rainbow trout E-peptides (rtEa4 and rtEa2) of the pro-IGF1 exerted mitogenic activity in BALB/NIH3T3 cells, human embryonic kidney cells (293GP), and caprine mammary epithelial cells (CMECs). It is conceivable that rtEa2 or rtEa4 may serve as an effective mitogenic agent in rainbow trout primary pituitary cells. Elango *et al.* (2006) showed that while the expression of *gh* gene in the trout pituitary organ culture was induced by 17 β -estradiol (E₂), dexamethasone, and *o,p'*-dichlorodiphenyltrichloroethane (*o,p'*-DDT), the expression of *prl* gene was induced only by E₂ and *o,p'*-DDT. Therefore, we reasoned that a functional trout pituitary cell line should be responsive to induction of *gh* and *prl* gene expression by these factors. In this report, we describe the development and characterization of five single-cell clone lines capable of expressing *gh*, *prl*, thyroid-stimulating hormone (*tsh*), gonadotropin 1 (*gth-1* or *fshb*), gonadotropin 2 (*gth-2* or *lhb*), corticosteroid receptor (*cr* or *nr3c1*), proopiomelanocortin-B (*pomcb*), and *sl* (*smtl*) genes from rainbow trout pituitary glands.

Materials and Methods

Establishment of cell lines

Whole pituitary glands including pars intermedia were collected from five female rainbow trout of 1 year of age under sterile conditions, and were rinsed three times in

Dulbecco's PBS (Gibco-BRL) containing penicillin (1000 U/ml), streptomycin (1000 μ g/ml), and fungizon (25 μ g/ml; Gibco-BRL). These glands were minced together into fine pieces (smaller than 0.5 mm cubes), and seeded in a T-25 flask in a CO₂-independent medium (Gibco-BRL) supplemented with fetal bovine serum (FBS, 10%; Gibco-BRL), bovine growth factor (bGF, 5 ng/ml; Gibco-BRL), penicillin (250 U/ml), streptomycin (250 μ g/ml), and fungizone (2.5 μ g/ml), and the culture was incubated in an incubator at 20 °C. After tissues and cells attached to the substratum, the old medium was carefully removed and replaced with fresh CO₂-independent medium containing 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 5 ng/ml bGF, and the culture was left in the incubator. Cells grown out from the tissue were trypsinized in a PBS solution containing 0.25% of trypsin and 0.02% of EDTA, and the detached cells were recovered by gentle centrifugation and divided into two aliquots. Cells from one aliquot (5×10^5 cells) were seeded in a T-25 flask in CO₂-independent medium supplemented with 10% FBS, 5 ng/ml bGF, 100 U/ml penicillin, and 100 μ g/ml streptomycin, and about the same number of cells from another aliquot were seeded in a T-25 flask in the same type of medium but supplemented with 4 μ g/ml recombinant rtEa4-peptide of pro-IGF1 (Tian *et al.* 1999). While cells cultured in the medium without trout Ea4-peptide supplement died within 2 weeks, cells cultured in the medium with trout Ea4-peptide supplement continued to grow. Cells were subcultured in 1/4 dilutions in the same type of medium once a week for 15 passages, and at this time, the penicillin–streptomycin mixture was excluded from the culture medium.

Single-cell clones were isolated from the culture at 15 passages using the method described by Chen *et al.* (2004). Cells were diluted to 3 cells/ml in a conditioned CO₂-independent medium supplemented with 10% FBS and 4 μ g/ml recombinant trout Ea4-peptide. To each 96-well plate, 0.2 ml of the diluted culture was plated in each well, and the plates were incubated at 20 °C. The 96-well plates were examined under an inverted microscope, and wells that contained a single cell were marked. Once the single-cell clone grew to cover more than half of the well, cells were recovered and seeded into T-25 flasks in CO₂-independent medium supplemented with 10% FBS for further propagation. Each single-cell clone was subcultured once a week over 150 passages. Seed stocks were prepared by freezing 3×10^6 cells/vial in a CO₂-independent medium supplemented with 10% FBS and 5% dimethyl sulfoxide, and were stored in liquid nitrogen.

Preparation of recombinant rtEa4-peptide

Recombinant rtEa4-peptide was prepared according to the method described by Tian *et al.* (1999) and modified by Kuo & Chen (2002), and was stored at 4 °C.

Karyotyping

Metaphase chromosomes were prepared from cells arrested in metaphase with 0.2 µg/ml colcemid (Gibco-BRL) for 3 h following the method described below. Metaphase-arrested cells were treated with 5 ml of 0.25% trypsin until cells dissociated from the surface of the culture flasks. Cells were then resuspended in 5 ml of fresh CO₂-independent medium supplemented with 10% FBS, and were collected by centrifugation at 300 g for 5 min (Beckman GS-6R). The cell pellet was treated with 5 ml of 75 mM KCl and gently resuspended in an additional 25 ml of the same concentration of KCl solution. The cell suspension was incubated at 20 °C for 30 min and spun at 300 g for 5 min. The cell pellet was resuspended in 7 ml of a fixative (methanol–acetic acid, 3:1) followed by centrifugation. The treatment with the fixative was repeated four times, and the cell pellet was resuspended in the fixative in a final volume of 2 ml. These cells were then broken on glass slides by gravity to release chromosomes by dripping the cell mixture from a suitable distance above the slides. Chromosomes were stained using the Giemsa stain method (Gustasaw *et al.* 1994), and were observed at ×1000 magnification with an oil immersion objective under a light microscope (Olympus 1×50). About 500 mitotic figures from each single-cell clone were counted, and the chromosome number was in the range of 60 ± 2.

Determining the doubling time

About 1 × 10⁴ cells of each single-cell clone were resuspended in the CO₂-independent medium supplemented with 10% FBS and plated in each well of 96-well plates, and the cultures were incubated in an incubator at 20 °C. At different time intervals, cultures were removed from the incubator, medium was removed, and the cultures were kept in a –80 °C freezer. The DNA content in each well was determined by staining the DNA with Hoechst dye (H33258) and was quantified in a fluorescence multiwell plate reader, Cytofluor II (Framingham, MA, USA). The doubling time of each single-cell clone was determined from a plot of DNA content versus culturing time.

Colony formation in soft agar medium

Colony formation assay was conducted following the method described by Yang (1975). About 5 × 10⁴ cells from each single-cell clone at log phase were suspended in 0.5 ml of CO₂-independent medium containing 10% FBS and 0.4% purified agar (Difco Laboratories, Detroit, MI, USA) and plated in 24-well culture chambers that contained a solidified layer of agar (0.5%) dissolved in CO₂-independent medium. After the medium had solidified, each well was overlaid with 1.5 ml of CO₂-independent medium with 10% FBS. The plates were incubated in an incubator at 20 °C for 4–5 weeks. Colonies were observed under an Olympus inverted microscope (1×50) equipped with phase contrast objective

lenses (final magnification ×100). Colonies with sizes >50 µm were scored. The viability of cells at the end of the experiment was confirmed by the dye exclusion assay with trypan blue. Four wells were prepared for each single-cell clone, and the experiment was repeated twice.

Detection of Gh and Prl by immunocytochemical staining

Cells of mRTP1E₅₂ were cultured in an eight-well glass culture chamber with CO₂-independent medium supplemented with 10% FBS to 80–90% confluence, and were fixed with 4% paraformaldehyde. Nonspecific binding was blocked by incubating cells in PBS containing 1% dry milk powder and 0.2% Triton X-100 at 4 °C overnight. Cells were incubated with a 1:10 dilution of monospecific universal anti-Gh or anti-Prl sera raised in rabbits (Gonzalez-Villasenor & Chen 1999) at 4 °C overnight. Following washing in PBS containing 0.2% Triton X-100, the cells were subsequently incubated in PBS containing 0.2% Triton X-100 and 6.5 µg/ml of FITC-conjugated mouse-antirabbit IgG at 4 °C overnight. The immunostained cells were observed by fluorescence microscopy (Olympus 1×50 microscope) at 400×, and images were taken using a MicroMAX CCD camera (Princeton Instrument, Bozeman, MT, USA). Negative control slides were also prepared by the same procedures as described, but without incubation with primary antibodies.

To determine whether the same single-cell clone was synthesizing both Gh and Prl, an immuno-double staining was conducted as described below. Cells of five single-cell clones were cultured in an eight-well glass culture chamber with CO₂-independent medium supplemented with 10% FBS to 80–90% confluence, induced with 1 × 10^{–8} M of E₂ for 4 h, treated with 2.5 µg/ml of brefeldin A (Fujiwara *et al.* 1988) for 45 min to inhibit the export of Gh or Prl from the cells, and fixed with 4% paraformaldehyde. Nonspecific binding of the cells was blocked by the method described above. Monospecific anti-Gh and anti-Prl were labeled with DyLight₄₈₈ and DyLight₅₉₄ respectively following protocols provided by the supplier (Thermo Scientific, Waltham, MA, USA). The fixed cells were incubated with a 1:100 dilution of the DyLight-labeled monospecific universal anti-Gh and anti-Prl sera at 4 °C overnight. Following washing in PBS containing 0.2% Triton X-100, the immunostained cells were observed by fluorescence microscopy (Olympus 1×50 microscope, at 493–518 nm for Gh and at 593–618 nm for Prl) at 1000× magnification, and images were taken using a MicroMAX CCD camera.

Detection of mRNA by RT-PCR analysis

Total RNA was isolated from each mRTP single-cell clone or pituitary glands according to the acid-guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi 1987). The total RNA was further treated with RNase-free DNase to remove any trace

Table 1 Annealing conditions and amplification oligonucleotide primer sequences used in reverse transcription (RT)-PCR (*) and real-time RT-PCR

	Primer sequence	Annealing (temperature/s)	Product size (bp)	Reference
Gene				
<i>gh1</i>	F: 5'-ACATACTCAACCGACCACCGC R: 5'-GCCAATCTGTATCTGGGA	48 °C/15 s	756	Genbank M22731
<i>gh2</i>	F: 5'-CTGTAACCTGACTCCATCGTG R: 5'-CCCACTTTGAGGTCGCTGAG	50 °C/15 s	200	Genbank M24684
<i>rtE2Rα (esr1)</i>	F: 5'-CCCTTCTTCCCCTCCACCCC R: 5'-GCCGCCCCACCACTCCGC	57 °C/15 s	248	Genbank AJ242740
<i>prl</i>	F: 5'-TCACTCAGCACTTCCCTCACC R: 5'-CCAGGAGGAGGGAGCGAGCC	59.5 °C/15 s	178	Genbank M24738
<i>gth-1β (fshb)</i>	F: 5'-ATCGTCACGGAAGCATCACC R: 5'-CGGGTATGAGAGAGGGCTCG	58.5 °C/15 s	177	Genbank M27153
<i>gth-2β (lhb)</i>	F: 5'-GATCTCCCTGCTTCTGTGC R: 5'-GCTCCTTGGTGACGCACTGG	58.5 °C/15 s	161	Genbank AB050836
<i>tshβ</i>	F: 5'-AATGTGACTTCTGCGTGGC R: 5'-ACGGGGTAGGTGAAGACAGG	58.5 °C/15 s	193	Genbank D14692
<i>pomcb</i>	F: 5'-GATGGGTCACTTCCGCTGG R: 5'-CTGCTGCCCTCCTCTACTGC	59.5 °C/15 s	146	Genbank X69808
<i>cr (nr3c1)</i>	F: 5'-ACTGTCTCTGTTCTGGTCTTCG R: 5'-GGCTCTCTGCTCCCTTTCGC	59.5 °C/15 s	229	Genbank NM_001124730
<i>sl (smtl)</i>	F: 5'-AGAAGGACGCCACAAGATG R: 5'-GGCACCAGAAGCAGGGAGG	59.5 °C/15 s	195	Genbank D10640
<i>β-Actin</i>	F: 5'-GAGCAGGAGATGGGCACC R: 5'-CCGCAAGACTCCATACCGA	59.5 °C/15 s	149	Genbank AF157514

contamination by genomic DNA. First-strand cDNA was synthesized from each RNA sample using Superscript II (Invitrogen) according to the manufacturer's recommendation with modification. Briefly, 3–5 µg of total RNA were incubated with 100 ng oligo (dT)_{12–15} in an 11-µl volume at 70 °C for 5 min, followed by a quick chill on ice. Subsequently, 4 µl of 5× first-strand buffer (Invitrogen), 1 µl of 0.1 M dithiothreitol, 1 µl of RNasin (40 unit/µl, Promega), and 1 µl of 10 µM dNTP mixed stock were added

and incubated at 42 °C for 2 min, followed by addition of 200 units of Superscript III. The reaction was allowed to proceed at 42 °C for 90 min and terminated by heating at 70 °C for 15 min. A Tris-EDTA buffer was added to bring the final volume of the reaction mixture up to 200 µl, and the cDNA was stored at –20 °C until further use.

PCR was conducted with 3 µl of the diluted first-strand cDNA in a total volume of 50 µl using standard buffer conditions as described by the manufacturer (Promega),

Figure 1 Morphological characteristics of five single-cell lines, mRTP1B, mRTP1E, mRTP1F, mRTP1K, and mRTP2A. Sub-confluent cells (panel A) and confluent cells (panel B) were observed by light microscopy with an Olympus 1×50 microscope. Subscript indicates the passage number of each single-cell clone.

Table 2 Doubling time of pituitary single-cell clones

	Doubling time (days)
Single-cell clones	
mRTP1B ₁₅₅	3.6±0.7
mRTP1E ₁₇₆	2.8±0.7
mRTP1F ₁₅₁	3.2±0.8
mRTP1K ₁₅₃	5.5±0.6
mRTP2A ₁₂₅	6.6±0.6

supplemented with 1.5 mM MgCl₂, 200 μM dNTP, 400 nM each gene-specific primer (Table 1), and 1 unit of Taq polymerase (Promega). The following PCR profile was used: denaturation at 94 °C for 3 min, followed by 40 cycles consisting of 94 °C for 20 s for denaturation, 48–60 °C for 15–20 s (depending on the primer set) for annealing, and 72 °C for 30 s for synthesis. The PCR products were resolved on 1.5% agarose gels stained with 0.1 μg/ml ethidium bromide.

Levels of *gh*, *prl*, *tshβ*, *gth-1β*, *gth-2β*, *pomcb*, *cr*, and *sl* mRNAs were determined by relative quantitative real-time reverse transcription (RT)-PCR following the protocol provided by the supplier (Roche Applied Science). The amplification primers used in the PCR are described in Table 1. First-strand cDNA was prepared by RT as described earlier, and 2 μl of the first-strand cDNA were used for PCR in 16 μl of reaction volume. PCR conditions were as follows: denaturation at 95 °C for 10 min and followed by 50 cycles of amplification (95 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s). A melting curve program (95–55 °C) with a heating rate of 0.5 °C/10 s was also included to confirm the

specificity of the amplification. PCR efficiencies of all the reactions were between 95 and 100%. Relative expression levels were calculated as $2^{-(\Delta CT_s - \Delta CT_c)}$, where ΔCT_s = critical cycle number (CT) of hormone treated – CT of β-actin and ΔCT_c = CT of hormone untreated – CT of β-actin. All measurements were performed in triplicate and repeated at least three times. The data were analyzed using iCycler Thermal Cycler analysis software (Optical System Interface version 2.3).

Induction of *gh* and *prl* mRNAs by E₂, dexamethasone, and o,p'-DDT

Cells of mRTP1B were cultured in a CO₂-independent medium supplemented with 10% FBS to 80–90% confluence, starved in a CO₂-independent medium supplemented with 2.5% charcoal–dextrin–stripped FBS for 24 h, and then incubated in the fresh CO₂-independent medium supplemented with 2.5% charcoal–dextrin–stripped FBS and various concentrations of E₂ (10^{–8} and 10^{–7} M), dexamethasone (10^{–8} and 10^{–7} M) or o,p'-dichlorodiphenyltrichloroethane (o,p'-DDT; 5 × 10^{–7} and 1 × 10^{–6} M) for another 24 h. Control cells were cultured in a CO₂-independent medium supplemented with 2.5% charcoal–dextrin–stripped FBS and the equivalent amount of vehicle used to dissolve the hormones. Total RNA was isolated from treated and control cells following the method described in the previous section, and was used as a template for first-strand cDNA synthesis. Double-stranded cDNA of specific mRNA was amplified by PCR using first-strand cDNA as a template. Conditions for RT-PCR were the same as described in the previous section, and nucleic acid sequences for PCR amplification primers are listed in Table 1.

Figure 2 Metaphase chromosomes of rainbow trout pituitary single-cell clones. Subscript indicates the passage number of each single-cell clone. Magnification: ×1000.

Table 3 Colony formation activity of trout pituitary single-cell clones determined in a soft agar medium. Mean ± s.d.

	Number colonies/view ^a
Single-cell clones	
mRTP1B ₁₅₅	8 ± 2
mRTP1E ₁₇₆	10 ± 1
mRTP1F ₁₅₁	39 ± 3
mRTP1K ₁₅₃	16 ± 1
mRTP2A ₁₂₆	1 ± 1

^aNumber of colonies were counted per view under a microscope; each data point is the average of four independent counting.

Results

Several single-cell clones were isolated from primary pituitary cells by the serial dilution cloning method (Chen *et al.* 2004). After single-cell clones were isolated, rtEa4-peptide was no longer included in the culture medium. Five single-cell clones (mRTP1B, mRTP1E, mRTP1F, mRTP1K, and mRTP2A)

were selected for further characterization based on their morphological characteristics (Fig. 1). At a lower cell density, mRTP1E₁₅₄ displayed a fibroblast-like spindle structure with protruding extensions often longer than the main body of the cell. The other four mRTP cell lines, although elongated, were more epithelium-like in morphology. At confluence, the five mRTP cell lines were highly diverse in morphology. Visibly, mRTP1E₁₅₄ cells were fibroblast-like, whereas mRTP1K₁₃₂ cells were epithelium-like. Both mRTP1B₁₃₃ and mRTP2A₁₂₉ cells displayed elongate epithelium-like morphology. In contrast to the generally uniform morphology observed in the other mRTP single-cell clones, mRTP1F₁₂₉ cells were highly pleomorphic and basophilic, features that are often associated with malignant tumors.

Each of these single-cell clones had gone through at least 150 passages, and the doubling time for each single-cell clone at passages 126–176 was determined to be 3.6 ± 0.7 d for mRTP1B₁₅₅, 2.8 ± 0.7 d for mRTP1E₁₇₀, 3.2 ± 0.8 d for mRTP1F₁₅₁, 5.5 ± 0.6 d for mRTP1K₁₅₃, and 6.6 ± 0.6 d for mRTP2A₁₂₆ (Table 2). On the basis of their morphologies and different doubling times, we suggest that each of these five single-cell clones represents a different origin. The chromosome number of each single-cell clone was determined by karyotyping, and the results (Fig. 2) show that the chromosome numbers for these five single-cell clones ranged from 59 ± 1 to 60 ± 2, within the range of the 2N chromosome numbers reported for rainbow trout (Philips 2001). The *in vitro* colony formation activities determined in the soft agar medium for all the single-cell clones are summarized in Table 3, with mRTP1F₁₅₁ showing the highest colony formation activity and mRTP2A₁₂₆ the lowest. We had noted that mRTP1F cells exhibited highly pleomorphic and basophilic morphology and high activity of colony formation in soft agar medium, and that these features are often associated with malignancy.

Cells from all the five single-cell clones were stained positive with antisera to Gh and Prl (see representative figures in Fig. 3A and B), suggesting that these cells produce Gh and Prl simultaneously. Furthermore, total RNA samples

Figure 3 Representative data of Gh and Prl produced in mRTP single-cell clones by immunocytochemical staining. (A) mRTP1E₅₂ cells stained with FITC-labeled monospecific GH antisera or monospecific Prl antisera; (B) mRTP1E₁₆₄ and mRTP1F₁₆₀ cells co-stained with DyLight₄₈₈-labeled monospecific GH antisera and DyLight₅₉₄-labeled monospecific PRL antisera. Protocol of the immunostaining is described in ‘Materials and Methods’, and the stained cells were observed under an Olympus 1 × 50 microscope equipped with an epifluorescence attachment. NS Ab, nonspecific antisera; Gh Ab, monospecific Gh antisera; Prl Ab, monospecific Prl antisera; PhC, phase contrast objective.

Figure 4 RT-PCR determination of *gh*, 17β-E₂ receptor, and *prl* mRNAs in mRTP single-cell clones. Total RNA was isolated from mRTP single-cell clones and used as a template for first-strand cDNA synthesis. Double-stranded cDNA of specific mRNA was amplified by PCR using first-strand cDNA as a template. Conditions for RT-PCR are described in ‘Materials and Methods’, and nucleic acid sequences of gene-specific primers for PCR amplification are listed in Table 1. PRL, prolactin; rtE2Rα, trout E₂ receptor α; 1B, mRTP1B; 1K, mRTP1K; 1F, mRTP1F; 2A, mRTP2A; 1E, mRTP1E; +, cDNA prepared from pituitary mRNA; –, no template added.

Figure 5 RT-PCR determination of *gh*, *prl*, *tshβ*, *gth-1β*, *gth-2β*, *pomcb*, *cr*, *sl*, and β -actin mRNAs in mRTP single-cell clones. Total RNA was isolated from mRTP single-cell clones and used as a template for first-strand cDNA synthesis. Double-stranded cDNA of specific mRNA was amplified by PCR using first-strand cDNA as a template and gene-specific oligonucleotides as amplification primers. Conditions for RT-PCR are described in 'Materials and Methods', and sequences of oligonucleotides for PCR amplification are listed in Table 1. *prl*, prolactin; *tshβ*, thyroid-stimulating hormone β subunit; *gth-1β*, gonadotropin 1 β subunit; *gth-2β*, gonadotropin 2 β subunit; *pomcb*, proopiomelanocortin-B; *cr*, corticosteroid receptor; *sl*, somatolactin; 1B, mPRT1B; 1E, mPRT1E; 1F, mPRT1F; 1K, mPRT1K; 2A, mPRT2A. Numbers in the parentheses indicate passages of each single-cell clone used in this study.

isolated from cells of all the five single-cell clones were analyzed for the presence of *gh*, *prl*, *pit-1* (*pou1f1*), and estrogen receptor (*rtE2R α*) mRNAs by RT-PCR analysis. The results of the RT-PCR analysis revealed that while all the five single-cell clones produced mRNAs of *gh*, *prl*, and *rtE2R α* , no *pit-1* mRNA (data not shown) was detected (Fig. 4). To address the question whether each of these five single-cell clones also produces other pituitary hormones, mRNA from each cell clone was analyzed for the production of Tsh β , Gth-1 β , Gth-2 β , Pomcb, Cr, and Sl by RT-PCR and relative quantitative RT-PCR, and the results are presented in Figs 5 and 6. To our surprise, while clones 1B and 1F produced *gh*, *prl*, *tshβ*, *gth-1β*, *gth-2β*, *pomcb*, *cr*, and *sl* mRNAs, clones 1E, 1K, and 2A produced all mRNA species except *gth-1β* mRNA. Furthermore, levels of *sl* mRNA in clones 1B and 1K were much lower than those in clones 1E, 1F, and 2A.

It was shown previously that while levels of *gh* mRNA in pituitary glands of rainbow trout maintained in organ culture were modulated by E₂, dexamethasone, and *o,p'*-DDT, levels of *prl* mRNA were only modulated by E₂ and *o,p'*-DDT (Elango *et al.* 2006). To determine whether the single-cell clones established in this study are also responsive to induction by E₂, dexamethasone, and *o,p'*-DDT, mRNA was isolated from mRTP1E clone following treatment with various doses of E₂, dexamethasone, or *o,p'*-DDT and was

subjected to semi-quantitative RT-PCR analysis. As shown in Fig. 7, while levels of *gh* mRNA were induced by E₂, dexamethasone, and *o,p'*-DDT, levels of *PRL* mRNA were only induced by E₂ and *o,p'*-DDT. Furthermore, the responsiveness of the mRTP1E clone to induction of *GH* and *PRL* mRNAs by E₂ at passages 35 and 125 was indistinguishable (Fig. 8).

Discussion

Intensive efforts over several decades by scientists to develop continuous fish pituitary cell lines capable of expressing pituitary hormone genes have achieved only limited success. Although a trout pituitary cell line (RTP-2) was developed by Bols & Lee (1994) and Bols *et al.* (1995), this cell line contained a mixture of different pituitary cell types. While this cell line was shown to express *gh* and *prl* genes at passage 50 (Bols *et al.* 1995), it failed to maintain this characteristic upon prolonged culturing. In our study, five single-cell clone lines were developed from normal appearing primary cultures of rainbow trout pituitary glands without treatment with any known immortalization agent. Since each of these single-cell clones has gone through at least 150 passages, these clones should be considered as permanent cell lines. Karyotype analysis showed that each of these five single-cell clones contains from 59 ± 1 to 60 ± 2 chromosome complements, which are in good agreement with the reported 2N chromosome number for rainbow trout (Philips 2001). It is interesting to note that while primary trout pituitary cells in the presence of recombinant trout

Figure 6 Levels of *gh*, *prl*, *tshβ*, *gth-1β*, and *gth-2β* mRNAs in mRTP single-cell clones determined by relative quantitative real-time RT-PCR analysis. Total RNA was isolated from mRTP single-cell clones and used as a template for first-strand cDNA synthesis. Double-stranded cDNA of specific mRNA was amplified by PCR using first-strand cDNA as a template and gene-specific oligonucleotides as amplification primers following conditions described in 'Materials and Methods', and sequences of oligonucleotides for PCR amplification are listed in Table 1. Relative expression levels were calculated as $2^{-(\Delta C_{T_s} - \Delta C_{T_c})}$. 1B, mPRT1B; mPRT1K, mPRT1F; 2A, mPRT2A; mPRT1E. Numbers in the parentheses indicate passages of each single-cell clone used in this study. Each data point indicates mean $\pm$ S.D. ($n=3$).

Figure 7 RT-PCR determination of *gh* (A) and *prl* mRNA (B) in mRTP1E single-cell clone induced by 17β-estradiol, dexamethasone, or o,p'-DDT. Cells of mRTP1E were cultured in CO₂-independent medium supplemented with 10% FBS to 80–90% confluence, starved in CO₂-independent medium supplemented with 2.5% charcoal–dextrin-stripped FBS for 24 h, and then incubated in the same medium type supplemented with various concentrations of 17β-estradiol, dexamethasone, or o,p'-DDT for another 24 h. Total RNA was isolated from treated cells and used as a template for first-strand cDNA synthesis. Double-stranded cDNA of specific mRNA was amplified by PCR using first-strand cDNA as a template. Conditions for RT-PCR are described in 'Materials and Methods', and nucleic acid sequences of PCR amplification primers are listed in Table 1.

Ea4-peptide (the pro-peptide sequence of the pro-IGF1) supplement grew well and led to the development of permanent single-cell clones, primary pituitary cells died in 2 weeks after their establishment without the supplementation of trout Ea4-peptide in the culture medium. Together with the fact that recombinant trout Ea4-peptide exerted mitogenic activity in nontransformed NIH3T3 and primary CMECs (Tian *et al.* 1999), it is suggested that trout Ea4-peptide may serve as an immortalization agent for the development of single-cell clones in this study.

A useful pituitary cell line should maintain its ability to produce pituitary-specific proteins such as Gh, Prl, and

other peptide hormones. Immunocytochemical analysis with monospecific antisera of Gh and Prl showed that cells of all the five single-cell clones produced both Gh and Prl. This observation was further supported by results of RT-PCR analysis that mRNAs of *gh*, *prl*, *tsh*, *gth-1*, *gth-2*, *sl*, *pomcb*, *cr*, and *rtE2Ra* genes were detected in each of these five single-cell clones. Immunocytochemical studies of fish pituitary glands showed that there are seven to eight types of distinct secretory cells in the adenohypophysis, each of which produces different hormones: Prl, Gh, Tsh, Gth-1, Gth-2, Acth, melanophore-stimulating hormone, and Sl (Doerr-Schott 1976, Follenius *et al.* 1978, Nagahama *et al.* 1981, Farbridge & Leatherland 1986, Saga *et al.* 1999). On the basis of these results, it is believed that each cell type of the fish adenohypophysis *in vivo* produces only one type of the pituitary hormone. To our surprise, results of RT-PCR and relative quantitative real-time RT-PCR analyses revealed that each single-cell clone was able to produce Gh, Prl, Tsh, Gth-1, Gth-2, Pomcb, Cr, and Sl. A similar observation was made earlier in GH₃ cells, a cell line derived from a rat anterior pituitary tumor (Tashjian *et al.* 1968, 1970, Yasumura *et al.* 1966) and somatomammotrophs derived from adult rat pituitary glands (Chomczynski *et al.* 1988), which are capable of synthesizing Gh and Prl. The reason for this

Figure 8 Induction of *gh* and *prl* mRNAs in mRTP1E cells by 17β-estradiol at different passages. Cells of mRTP1E were cultured in CO₂-independent medium supplemented with 10% FBS to 80–90% confluence, starved in CO₂-independent medium supplemented with 2.5% charcoal–dextrin-stripped FBS for 24 h, and then incubated in the same medium type supplemented with various concentrations of 17β-estradiol for another 24 h. Total RNA was isolated from treated cells and used as a template for first-strand cDNA synthesis. Double-stranded cDNA of specific mRNA was amplified by PCR using first-strand cDNA as a template. Conditions for relative quantitative RT-PCR are described in 'Materials and Methods', and nucleic acid sequences of PCR amplification primers are listed in Table 1. Numbers in the parentheses indicate passages of each single-cell clone used in this study. Each data point indicates mean ± s.d. (n=3).

phenomenon remains to be investigated. However, it is possible that the characteristic of cell type-specific production of pituitary hormones by different pituitary cell types in the rainbow trout pituitary gland is maintained only when cells are arranged in the specific location in the intact pituitary gland. Once the pituitary cells are dissociated and maintained in culture, due to changes in the extracellular environment, pituitary cells may lose their cell type specificity with respect to the production of pituitary hormones. This hypothesis may be supported by the finding in this study that none of the five single-cell lines expresses *pit-1* mRNA.

Another important characteristic for a useful pituitary cell line is its response to factors that are known to regulate the expression of pituitary hormone genes. Studies reported by Elango *et al.* (2006) showed that the production of Gh and Prl in trout pituitary explant cultures was induced by E_2 and *o,p'*-DDT. In the current study, we have shown that addition of E_2 , dexamethasone, or *o,p'*-DDT resulted in a significant increase in the levels of *gh* and *prl* mRNAs in mRTP1E cells. Furthermore, the capacity of the single-cell clone lines to respond to induction of *gh* mRNA by E_2 is independent of passage numbers. These results suggest that the mRTP single-cell clones that we developed in this study are fully functional. These single-cell clones can be used not only to determine factors that regulate the expression of pituitary hormone genes, but also as an *in vitro* rapid screening system for detecting environmental endocrine disruptors.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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