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PRELIMINARY COMMUNICATION

Preparation, cryopreservation, and growth of cells prepared from the green turtle (*Chelonia mydas*)

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Abstract. Techniques are described for preparing, preserving, and growing cell cultures from 30 to 40-day old green turtle embryos (2.0–3.0 cm length) including cells derived from skeletal muscle, liver, heart, kidney, eye, lung, and brain. Acceptable growth of all cells occurred in all standard cell culture media tested, with optimum growth temperature near 30 °C. These cell cultures will be used in the study of sea turtle viral diseases including fibropapillomatosis, which is currently epidemic in some green turtle populations.

Key words: Cell culture, *Chelonia mydas*, Fibropillomatosis, Reptile, Sea turtle

Abbreviations: FP = fibropapillomatosis; GTE = green turtle embryo; GTEF = green turtle embryo fibroblasts

1. Introduction

The green turtle (*Chelonia mydas*), considered endangered by the International Union for Conservation of Nature (IUCN), is an herbivorous species with circumglobal distribution in warm, semi-tropical ocean waters. Some populations of green turtles are afflicted with fibropapillomatosis (FP), a disease first documented in Florida in this species by Smith & Coates [23]. The disease appears to have been unknown in the Hawaiian Islands prior to 1958 [3]. This disease causes the formation of fibrous tumors on the eyes, mouth, flippers, and sometimes on the internal organs [2, 4, 12, 27]. Since the mid-1980s the incidence of FP has increased to major proportions in both Hawaii and Florida and now represents a significant threat to the biological recovery of the species [4, 25]. The cause of FP remains unknown although a herpesvirus [14, 15] and a retrovirus [7] have been implicated.

There are few diagnostic tools currently available to investigate the etiology of diseases in sea turtles. Microscopic pathology, bacteriology and biochemical analyses are used, but have limitations, especially when involving viral diseases that may leave nonspecific lesions. A major obstacle to the investigation of FP has been the absence of adequate green turtle cell cultures derived from non-diseased tissue for virus isolation attempts. Cell cultures from cutaneous fibropapilloma cells have been established [19, 22], but have thus far proven unsuccessful for virus isolation. A continuous cell line from the heart of a freshwater turtle, *Terrapene carolina*, has been developed [8], but may not be sensitive enough for isolation of green turtle pathogens. Koment & Haines [17] found that skin cells prepared from 8-week old green turtles were the only cell type that proved permissive for replication of the herpesvirus that causes Gray Patch, a disease of captive-reared green turtles.

Preparation of cell cultures from reptiles has not been widely developed. However, Stephenson [24] and Wolf [26] found that standard cell culture media, developed primarily for avian and mammalian species, would support growth of reptilian cells. Cells from embryonic tissue will, generally, survive and grow better in culture than those from an adult [11], and cell cultures made from fetal tissue provide the most sensitive system for isolation of the greatest variety of viruses from that species [9, 10]. This paper describes the preparation, preservation and growth requirements of cell cultures from green turtle embryos (GTE) collected from the Hawaiian Islands.

2. Materials

A. Culture media, solutions, and chemicals
- Basal Medium Eagle (BME), No. 21015-037.[1]

- Dulbecco's Modified Eagle Medium (D-MEM), No. 11995-065.[1]
- F-10 nutrient mix (Ham), No. 11550-043.[1]
- F-12 nutrient mix (Ham), No. 11765-054.[1]
- Leibovitz L-15 medium, No. 11415-064.[1]
- Medium 199 (M 199) with Earle's salts, No. 31100-084.[1]
- Minimum essential medium (MEM), No. 11096-054.[1]
- RPMI medium 1640, No. 11875-093.[1]
- Trypsin, 2.5%, No. 15090-046.[1]
- Versene, 1:5000, No. 15040-066.[1]
- Fetal bovine serum (FBS), No. F2442.[2]
- Sodium bicarbonate, 7.5%, No. 25080-094.[1]
- Trypan blue, 0.4%, No. 15250-061.[1]
- L-glutamine, 200 mM, No. 25030-016.[1]
- MEM non-essential amino acids solution, 100X, No. 11140-050.[1]
- MEM vitamin solution, 100X, No. 11120-052.[1]
- Nystatin, 10,000 Units/ml, No. 15340-052.[1]
- Gentamicin, 50 mg/ml, No. 15750-029.[1]
- Penicillin-streptomycin (10,000 Units/ml penicillin and 10,000 μg/ml streptomycin sulfate), No. 15140-122.[1]
- Dimethyl sulfoxide, No. D-5879.[2]
- Sodium chloride, NaCl, No. 7581.[3]
- Potassium chloride, KCl, No. 6858.[3]
- Sodium phosphate, dibasic anhydrous, Na_2HPO_4, No. 7919.[3]
- Potassium phosphate, monobasic anhydrous, KH_2PO_4, No. 7100.[3]
- Sodium pyruvate, No. P-5280.[2]
- 2-Mercaptoethanol, No. M-7522.[2]
- Iodine, I_2, No. I-3380.[2]
- Potassium iodide, KI, No. P8256.[2]
- Ethyl alcohol, 100%.[4]
- Phenol red solution, 0.5%, No. 15100-019.[1]
- Hydrochloric acid, HCl, No. H7020.[2]
- Sodium hydroxide, NaOH, No. 7708.[3]
- Colcemid solution, No. 15210-040.[1]

B. Supplies
- Pressure tank, 10 liter, No. XX6700L10.[5]
- Filter holder, 142 mm, No. YY3014230.[5]
- Prefilter, No. AP2512450.[5]
- Filter membrane, No. GSTF 14250.[5]
- Bottle, aspirator, No. 2534-13000.[6]
- Bottles, 500 ml, glass, No. 16159-889.[7]
- Bottles, 100 ml, glass, No. 16159-845.[7]
- Paraffin wax, 125 × 60 × 12 mm.
- Plastic petri dish, 100 × 15 mm, No. 25373-100.[7]
- Scalpel handle, #4, No. 25601-026.[7]
- Scalpel blades, #21, No. 25860-144.[7]
- Dissecting scissors, 5″, No. 25608-360.[7]
- Forceps, tissue, 5 3/4″, No. 25607-608.[7]
- Needle, 18 gauge × 1 1/2″, No. BD305196.[7]
- Pipet-aid, No. 1225-80000.[6]
- Pipets, glass, 10 ml, No. 1200-10010.[6]
- Pipets, glass, 5 ml, No. 1200-05010.[6]
- Pipets, glass, 1 ml, No. 1200-10001.[6]
- Beaker, glass, 100 ml, No. 13910-165.[7]
- Beaker, glass, 250 ml, No. 13910-201.[7]
- Beaker, glass, 400 ml, No. 13910-223.[7]
- Trypsinizing flask, 250 ml, No. 1986-00125.[6]
- Funnel, glass, No. 30211-027.[7]
- Cheesecloth, cotton mesh.
- Syringe, 10 ml, No. BD309604.[7]
- Centrifuge tubes, 200 ml, No. 3045-00200.[6]
- Centrifuge tubes, 15 ml, No. 21008-678.[7]
- Tissue culture flasks, 75 cm^2, No. 430725.[8]
- Tissue culture flasks, 25 cm^2, No. 3056.[8]
- Cryovials, 1.2 ml, No. 430487.[8]
- Liquid nitrogen, UN 1977.
- Nitrogen gas, UN1060.
- Carbon dioxide gas, UN1030.

C. Equipment
- Laminar flow biological safety cabinet, No. B60-112.[9]
- Freezing controller, model CRC-1.[10]
- Freezing chamber, model CRFC-1.[10]
- Temperature recorder, No. 9991-9C11.[10]
- Canes, aluminum.[1]
- Liquid nitrogen storage tank, Super 30-6.[10]
- Water bath, model 185, No. 66562.[11]
- Stir bar, No. 58948-230.[7]
- Magnetic stirrer, No. 58935-410.[7]
- Carbon dioxide incubator, No. 6300.[12]
- Hi-low incubator, No. 3440.[13]
- Ultra low freezer, Revco No. ULT-2090.[14]
- Refrigerator/freezer, TBF 21DW.[15]
- Egg candler.[16]
- Microscope, inverted, model CK2.[17]
- Hemocytometer, double chamber, No. 15170-172.[7]
- Centrifuge, Superspeed RC2-B.[18]
- Centrifuge rotor, Type HS-4.[18]

3. Procedures

A. Preparation of culture media, solutions and wax-filled dishes

All media and solutions, unless otherwise indicated, are sterilized by positive N_2 pressure through a prefilter and a 0.22 μm filter, collected in an aspirator bottle, and dispensed with a filling bell into sterile containers.

1. M 199 with additives

Component	Amount
– M 199 powder	98.7 g
– MEM non-essential amino acids, 100X	100 ml
– MEM vitamins solution, 100X	100 ml
– L-glutamine	100 ml
– Sodium bicarbonate, 7.5%	200 ml
– Penicillin-streptomycin solution, 10,000 Units/ml penicillin, 10,000 μg/ml streptomycin sulfate	100 ml
– Nystatin (10,000 Units/ml)	50 ml

– Gentamicin, 50 mg/ml 10 ml
– Double distilled water (ddH_2O) to 9 liters
Dissolve M 199 powder in about 3 liters ddH_2O. Add the remaining solutions (660 ml) and mix thoroughly. Bring volume to 4 liters with ddH_2O. Transfer to pressure tank and add another 5 liters water, bringing total volume to 9 liters. Mix and filter sterilize, dispensing into 500 ml sterile, glass bottles. Store at –5 to –20 °C.

2. RPMI medium 1640 with additives
   – 2-Mercaptoethanol 87.5 µl
   – Sodium pyruvate 0.03 g
   Add mercaptoethanol and sodium pyruvate to 10 ml RPMI medium 1640. Filter through a 0.22 µm filter into 240 ml RPMI medium 1640. Mix well. Dispense and freeze in 5 ml aliquots at –20 °C. Just prior to use, add one 5 ml aliquot to 500 ml RPMI medium 1640, containing 10% FBS. Mix well.
3. Phosphate buffered saline (PBS)
   – Sodium chloride, NaCl 80.00 g
   – Potassium chloride, KCl 2.00 g
   – Sodium phosphate, dibasic, anhydrous, Na_2HPO_4 11.46 g
   – Potassium phosphate, monobasic, anhydrous, KH_2PO_4 2.00 g
   – Double distilled water (ddH_2O) to 10 liters
   Dissolve salts in about 4 liters ddH_2O. Bring to 10 liters with ddH_2O. Adjust pH to 7.3 with 1 N HCl or 1 N NaOH. Filter sterilize and dispense in sterile 500 ml glass bottles. Store at 4 °C.
4. Freezing medium
   – Medium 199 with additives 70 ml
   – FBS 20 ml
   – Dimethyl sulfoxide 10 ml
   Aseptically mix ingredients together and store at –5 to –20 °C.
5. Trypsin-Versene
   – Trypsin, 2.5% 10 ml
   – Versene, 1:5000 90 ml
   – Phenol red 0.2 ml
   Aseptically combine ingredients, mix, pH to 7.6 with 7.5% sodium bicarbonate and store at –5 to –20 °C.
6. Trypsin in PBS
   – Trypsin, 2.5% 10 ml
   – PBS 90 ml
   – Phenol red 0.2 ml
   Aseptically combine ingredients, mix, pH to 7.6 with 7.5% sodium bicarbonate and store at –5 to –20 °C.
7. Alcohol-iodine solution
   – Potassium iodide, KI 2.0 g
   – Iodine, I_2 1.0 g
   – Ethanol, 100% 95.0 ml
   – Distilled water 5.0 ml
   Dissolve KI in 5.0 ml distilled water; add iodine, and dilute to 100 ml with ethanol. Store at room temperature.
8. FBS
   Divide 500 ml bottle into 5 × 100 ml aliquots in 100 ml sterile glass bottles. Heat inactivate at 56 °C for 30 min in water bath. Store at –20 °C.

B. Preparation of wax-filled petri dishes
One cake of paraffin wax (125 × 60 × 12 mm) will prepare about five petri dishes. Place two cakes of wax in an autoclavable container and cover loosely with aluminum foil. Autoclave for 15 min at 121 °C, 15 pounds pressure. Remove from autoclave; pour into 10 plastic petri dishes (about 25–30 ml per dish). Allow to harden and store at room temperature.

C. Egg procurement and incubation
Eggs were collected upon oviposition from green turtles at French Frigate Shoals in the Northwestern Hawaiian Islands, and at Sea Life Park Hawaii, where nesting occurs on an artificial sand beach. After collection, the eggs were buried to a depth of 8 to 10 cm of sand in an insulated container and left undisturbed throughout the 30 to 40 day incubation period. Ambient temperature within the sand ranged from 24 to 28 °C. Numerous small holes were made in the container for air exchange and the sand was periodically moistened with a small quantity of fresh water to maintain humidity.

D. Preparation of fibroblasts. This procedure is a modification of Docherty & Slota [9]

1. After eggs have incubated for 30 to 40 days, remove eggs from incubation container, one at a time, and candle each egg. Age is difficult to determine with one candling, but fertility can be determined by the presence of veins in the egg. Discard any infertile eggs and select four to eight eggs, depending on embryo size, for cell preparation.
2. Place approximately 20 ml alcohol-iodine solution in a sterile petri dish. Place one egg in the solution, rolling it around until covered with solution. Transfer egg to another sterile dish to dry.
3. Hold egg gently, and carefully puncture the parchment-like egg shell with a sterile scalpel, on the lower part of the egg, held away from you. Depending on fertility and age of the embryo, the egg may burst when punctured (infertile eggs and younger embryos are more prone to this as there is more fluid in the egg).
4. Make a 2.5–3.0 cm slit in the egg and fold back the shell. Using sterile forceps, remove the embryo and place in another sterile petri dish. Immediately separate the head with sterile dissecting scissors and place the head

and body in separate sterile 250 ml beakers containing about 100 ml of cold (4 °C) PBS.

5. Continue this process for the remaining embryos. Cover beakers with sterile aluminum foil and place beaker containing the heads at 4 °C and continue working with the remainder of the embryos as quickly as possible.
6. Pour about 10 ml cold PBS in a waxed-filled petri dish. Transfer one embryo to the petri dish, using sterile forceps. Place embryo with the carapace down and pin it to the wax with two sterile 18 gauge needles.
7. Using a sterile scalpel, gently cut the front of the embryo along the mid-line of the plastron and also horizontally across the sternum. Fold back the skin, with sterile forceps, to expose the internal organs. Using the photos in Rainey [20] as a guide, remove and store the heart, liver, kidney, and lungs in separate sterile 100 ml beakers containing cold PBS. Remove and discard any other viscera and place remainder of embryo in 100 ml fresh PBS in a sterile 250 ml beaker.
8. Repeat above procedure with each embryo, combining like tissues in sterile beakers of cold PBS. After all embryos have been dissected, place the organ beakers, covered with sterile aluminum foil, at 4 °C.
9. Transfer each eviscerated embryo, one at a time, to a sterile petri dish containing cold PBS. Mince the embryo into pieces of about 1 cm, using a sterile scissors, and transfer to a sterile 250 ml beaker. Carefully pour off as much of the PBS as possible and discard. Pour tissue pieces and remaining PBS into the top of a sterile 250 ml trypsinizing flask, containing a small, sterile stir bar.
10. Add about 25 ml prewarmed (35 °C) trypsin in PBS. Stir at medium speed for 5 min. Carefully pour off the supernatant through the side arm of the flask and discard.
11. Add 50 to 75 ml fresh trypsin in PBS and stir at medium speed for 15 min. Pour off supernatant, through the side-arm, into a sterile funnel covered with two layers of sterile cheesecloth, draining into a 200 ml sterile centrifuge tube containing 20 ml cold M 199 with additives plus 5 ml FBS. Place centrifuge tube in refrigerator while processing continues.
12. Add 50 ml fresh trypsin in PBS to the remaining cells and stir for another 15 min. Decant supernatant into a second sterile centrifuge tube containing 20 ml cold M 199 with additives plus 5 ml FBS.
13. Divide cell suspension evenly between the two centrifuge tubes. Centrifuge for 30 min at 600 $\times g$ at 4 °C.
14. Carefully pour off supernatant and discard. Add 10 ml of M 199 with additives containing 20% FBS and suspend cells using a sterile 10 ml pipet. Bring volume in each tube to about 100 ml using the same medium. Centrifuge again for 30 min at 600 $\times g$.
15. Pour off supernatant and discard. Suspend both pellets in a total of 60 ml freezing medium. Count the cells in the resulting suspension, using a hemocytometer, diluting 0.1 ml cells into 0.9 ml trypan blue.
16. Dilute cells in freezing medium to approximately 4.0 to 10.0×10^6/ml and dispense into 1.2 ml cryovials (1 ml/vial). Place at 4 °C for 1 hour.

E. Preparation of heart, liver, kidney, brain, eye, and lung cells (prepare during trypsinization and centrifugation of fibroblasts)

1. Remove beaker containing embryo heads from refrigerator. Transfer heads, one at a time, to a sterile petri dish, containing cold PBS. Carefully remove eyes from head with a sterile scalpel. Using sterile forceps, transfer eyes to a 100 ml sterile beaker containing cold PBS. Remove the brain through the translucent area on top of the head with the edge of the scalpel and place in another 100 ml sterile beaker containing cold PBS.
2. Place all organ beakers in the refrigerator except the one being processed. Working with one organ type at a time, carefully transfer all of the tissue from the beaker to a sterile petri dish containing 10 ml freezing medium (use 20 ml medium for liver). Trim fat or extraneous material from the tissue (especially the eyes) and discard. Cut remaining tissue into small pieces (0.5 cm or smaller) with sterile scalpel or scissors. Using a 10 ml sterile syringe, gently draw tissue and medium into syringe and expel into sterile petri dish. Repeat this process until pieces of tissue are well broken up. Transfer suspension to sterile cryovials (1 ml/vial). Place at 4 °C and record time.

F. Cryopreservation of cells

The fibroblasts and organ-derived cells are frozen by different methods because of limitations in time and cell freezer space. The controlled-rate biological freezing method is preferred but either method will produce viable cells.

1. Fibroblasts

   After cells have remained at 4 °C for 1 hour, transfer to the controlled-rate biological freezer. We recommend a freezing rate of 0.6 to 1.0 °C/min down to –4 °C [9]. The chamber is then filled with liquid nitrogen for 16 s, followed by a freezing rate of 10 °C/min down to –90 °C. The following settings are used: Rate 1 = 12; Trip = –4 °C; Time = 16 s; Rate

2 = 76. After freezing, transfer vials as rapidly as possible to the liquid phase of a liquid nitrogen storage tank.

2. Organ-derived cells
Cryovials, held in styrofoam racks for insulation, are placed at the following temperatures for 1 hour each: 4 °C, –20 °C, and –70 °C. Transfer vials as quickly as possible to the liquid phase of a liquid nitrogen storage tank.

G. Growth and characterization of GTE cells

1. GTEF and GTE organ-derived cells in M 199 medium with additives
Remove one cryovial of each cell type and thaw at room temperature. Place contents of cryovial in sterile 25 cm^2 flask containing 4 ml M 199 with additives and 1 ml FBS. Incubate at 30 °C with 2% CO_2, media changing flask at day three and seven. Harvest cells when confluent (7 to 14 days) and start new flasks with 2.0×10^6 cells and 4.5 ml M 199 with additives and 0.5 ml FBS. Subculture cells until passage 10, recording cell counts for each passage.

2. GTEF growth in various media
Remove eight cryovials of GTEF from liquid nitrogen and thaw at room temperature. Combine all vials in a 15 ml centrifuge tube and mix gently. Add 1 ml of cell suspension to each of eight flasks (25 cm^2) containing 1 ml FBS and 4 ml of one of the following media: BME, D-MEM, F-10 nutrient mix (Ham), F-12 nutrient mix (Ham), Leibovitz L-15, MEM, M 199 plus additives, and RPMI medium 1640 plus additives. Incubate at 30 DEGC with 2% CO_2. Media change and subculture as above to passage five. Observe flasks for days to confluency, total cell yield upon harvest, pH of medium, and general appearance of cells.

3. Preferred growth temperature of GTEF
Remove four GTEF cryovials from liquid nitrogen and thaw at room temperature. Combine all vials in a 15 ml centrifuge tube and mix gently. Add 1 ml of cell suspension to each of four flasks (25 cm^2) containing 1 ml FBS and 4 ml Leibovitz L-15 medium. Incubate flasks, without CO_2, at the following temperatures: 20, 25, 30, and 37 °C. Media change and subculture as above, if growth occurs.

4. Karyotyping of cells
GSTEF and GTE organ-derived cells are grown in 75 cm^2 flasks to 50 to 70% confluency in RPMI medium 1640 plus additives with 10% FBS. Colcemid solution is added to each flask to a final concentration of 0.5 µg/ml to arrest cells in metaphase, followed by incubation at 30 °C for 3 hours. The remaining steps in the slightly modified standard procedure [21] include the use of 0.075M KCl hypotonic solution, preparation of slides, and G-banding of chromosomes. Homologous chromosome pairs are arranged according to standard human chromosome techniques (Cytogenetics Laboratory, Waisman Center, University of Wisconsin – Madison) using computer imaging.

4. Results and discussion

All GTE and GTEF cells have been subcultured through passage 10. All cell types grow very slowly during the first passage after removal from liquid nitrogen, taking from two to three weeks to become confluent. Thereafter, cells are typically confluent by seven to 10 days and yield 3 to 9×10^6 cells per 25 cm^2 flask, depending on cell type and medium used. Fibroblast preparations give the highest yield, followed by lung, eye, heart, brain, kidney, and liver cells. There has been no decrease in days to confluency or in yield during the time of testing. Cells from the liver, kidney, and eye have been subcultured to passage 24, with representative samples of this passage preserved in liquid nitrogen. Additional subculturing is continuing.

Fibroblast cells are able to grow well in all media tested, particularly after the first two passages. Organ-derived cells have not been tested due to the small number of vials that were prepared. Appearance of cells is similar in all media. Days to confluency for the first passage after thawing range from about 16 days (M 199 with additives, F-10 nutrient mix, F-12 nutrient mix, MEM, RPMI medium 1640 plus additives, BME, and Leibovitz L-15 at 30 °C) to 26 days (DMEM and Leibovitz L-15 at 25 °C). After the first passage, all flasks become confluent in seven to nine days. Average cell yields per 25 cm^2 flasks (all passages are similar) are 3.0×10^6 (Leibovitz L-15 at 25 and 30 °C), 7.0×10^6 (F-10 nutrient mix, F-12 nutrient mix, DMEM, and MEM), and 9.0 to 10.0×10^6 (M199 with additives, BME, and RPMI medium 1640 with additives). The pH is low in flasks containing MEM and Leibovitz L-15 at 30 °C, and high in flasks with DMEM. RPMI medium 1640 with additives has been chosen as the medium for future growth of green turtle cells.

The preferred temperature for growth of GTEF cells, of the temperatures tested, is near 30 °C. A few cells attach at 20 °C but no spreading occurs, even after two months. Cells grow well at 25 °C and 30 °C, with growth somewhat slower at 25 °C. Cells attach to the flask at 37 °C but are not able to spread, nor is it possible to harvest and subculture these cells, either at 37 or 30 °C. Wolf [26] suggests an optimal growth temperature slightly above that preferred by the intact animal, usually 23 to 25 °C for temperate

reptiles and near 30 °C for tropical reptiles. However, in exploratory work with green turtle cells prepared from whole embryos, Stephenson [24] found the optimum temperature to be near 37 °C with some growth occurring as high as 43 °C. It is possible that the optimum growth temperature may be partially determined by the incubation temperature of the eggs as well as the temperature that the adult animal experiences in its environment. Stephenson doesn't give incubation conditions of the eggs he used, but this may explain the differences he reported. Generally, virus isolation is attempted at a temperature equal to that experienced by the tissues of the organism where the virus normally multiplies. In the case of the green turtle fibropapilloma, most of the tumors develop on the skin, in water at 21 to 28 °C [16]. The temperature optimum for our GTE cells appears to be near the expected temperature needed for virus isolation.

The first reported green turtle karyotype was by Makino [18]. The species was said to be heterogametic, with a diploid number of 55 for females and 56 for males. Koment & Haines [17] and Herbst [13] found a diploid number of 55, while Bickham et al. [5] and Bickham [6] reported a diploid number of 56 and refuted the heterogametic claim. Since green turtles exhibit temperature dependent sex determination [1], where the nest temperature determines the sex of the turtle, it is reasonable to assume they would not have genotypic sex determination, and would therefore not be heterogametic. Bickham has recently again confirmed a diploid number of 56 in green turtles and in most of the other sea turtle species as well (J. Bickham, personal communication, 1997). In our study, the chromosomes in 30 cells have been G-banded and counted, and each cell was found to contain a diploid number of 56 (Figure 1).

Figure 1. G-banding and metaphase of *Chelonia mydas* chromosomes with diploid number of 56.

The fibroblast preparation of cells and the lung, brain, and eye cells are fibroblast-like, while the cells derived from the heart, kidney, and liver are epithelial-like (Figure 2). There also appears to be a tendency for the fibroblast preparation cells to become more epithelial-like at passages above passage 12. This may be, in part, because the cells are grown at a higher temperature (30 °C) than the turtle experiences in nature (21 to 28 °C). It has been found that a temperature near the upper limit of the growth range favors the growth of epithelial cells over fibroblast cells in reptilian, mammalian, and avian cell cultures [24].

We have found several differences between these reptilian embryo cell cultures and the avian and mammalian cells we have worked with. GTE cells generally grow more slowly upon removal from liquid nitrogen, and do not yield cell concentrations as high as most avian and mammalian cells. Stephenson [24] also found this to be true in his work with reptilian cells. However, once growing, GTE cells require extremely little maintenance. We have kept cells at 30 °C in several standard media for five months with no additional maintenance, and then successfully subcultured these cells. In addition, cell cultures derived from green turtle embryonic organs can be frozen, thawed, and then subcultured many times, unlike those from avian embryonic organs, which we have not been able to pass beyond passage two. We do not known if these differences are characteristic of GTE cells or of reptilian cells in general. Further work will be necessary in order to answer this question. These positive features of reptilian GTE cell cultures will, however, be beneficial in their use as a potential host for virus isolation. The procedures

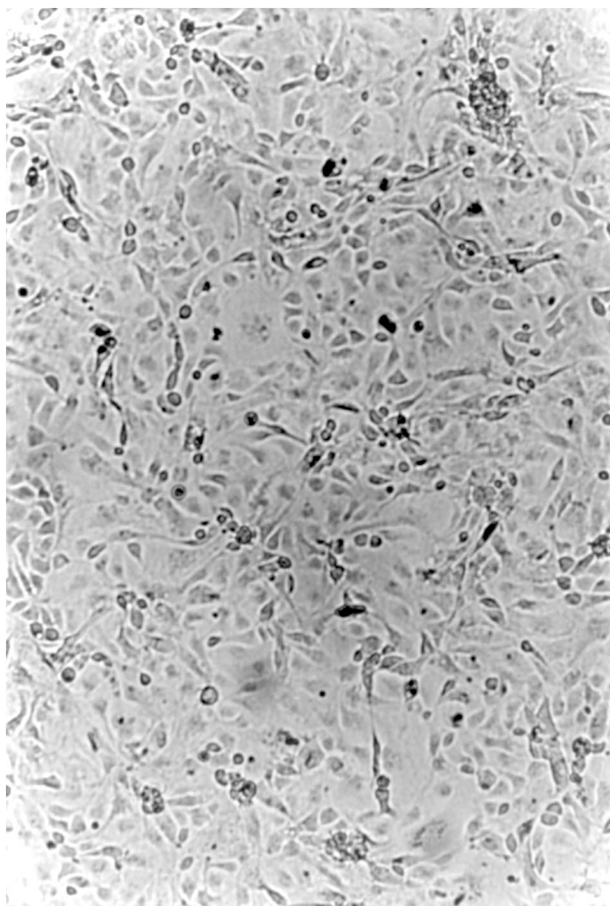

Figure 2. Green turtle embryo liver cells at passage 10, showing epithelial growth characteristics.

described here have been successfully repeated in preparing GTEF and GTE organ-derived cell cultures. We expect that these procedures may also be used to prepare cell cultures from many other reptilian species and therefore aid the study of cell function, toxicology, and pathology in these species.

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Notes on suppliers

1. Life Technologies, Baltimore, MD 21279-0464, USA
2. Sigma Chemical Co., St Louis, MO 63178, USA
3. Mallinckrodt Chemical Company, Paris, KY 40361, USA
4. US Industrial Chemical Co., Tuscola, IL 61953, USA
5. Millipore Corporation, Bedford, MA 01730, USA
6. Bellco Glass Inc., Vineland, NJ 08360-0017, USA
7. VWR Scientific Products Corporation, McGraw Park, IL 60085, USA
8. Corning Costar, Corning, NY 14831, USA
9. Baker Company, Sanford, ME 04073, USA
10. Union Carbide Corporation, Danbury, CT 06817-0001, USA
11. Precision Scientific Group, Chicago, IL 60647, USA
12. National Appliance Company, Tualatin, OR 97062, USA
13. Lab Line Instruments Inc., Melrose Park, IL 60160, USA
14. Revco Inc., Asheville, NC 28804, USA
15. General Electric, Jamaica, NY 11423, USA
16. SpeedKing, Schlueter Co., Janesville, WI 53545, USA
17. Olympus Corporation, Lake Success, NY 11042-1179, USA
18. Sorvall, Newton, CO 06470, USA

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