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In Vitro Thermal Effects on Embryonic Cells of Endangered Hawksbill Turtle *Eretmochelys imbricata*

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The hawksbill turtle is an ectotherm, whose sex is determined by temperature during embryonic development. This study aimed to determine whether embryonic hawksbill turtle cells respond differently to temperature than mammalian cells. Embryonic hawksbill turtle cells were established in culture, and thermal effects on these cells were investigated in vitro. Cells were maintained in Dulbecco's Modified Eagle Medium supplemented with non-essential amino acids, vitamin solution, sodium pyruvate, and 10% fetal bovine serum at 33°C and cell proliferation occurred at 25–33°C. When cells were incubated at 37°C (the temperature of mammalian cell culture) for 24 h, cell growth was completely inhibited. This growth inhibition was evidently recovered by changing the incubation temperature back to 33°C. Expression of heat shock protein was found to increase with elevating culture temperature from 25 to 33°C.

Key words: hawksbill turtle, embryonic cells, temperature sensitivity, growth inhibition, heat shock protein

INTRODUCTION

Primary reptilian cell cultures have been described by several investigators. These include various cells from the heart of the box turtle, *Terrapene carolina* (Clark and Karzon, 1967; Huang and Clark, 1967), the skin of the green sea turtle, *Chelonia mydas* (Koment and Haines, 1982), the heart and lung of the Tokai gecko, *Gecko gecko*, the heart, liver and kidney of the green iguana, *Iguana iguana*, the heart of the side-necked turtle, *Podocnemis unifilis*, and the spleen of the Grecian tortoise, *Testudo graeca* (Clark et al., 1970). Cell lines have also been established from cutaneous fibropapillomas of *C. mydas* (Mansell et al., 1989; Lu et al., 1999). However, embryonic cell cultures have not been established from the hawksbill sea turtle, *Eretmochelys imbrica*.

In 1996, the hawksbill turtle was listed as critically endangered by the International Union for the Conservation of Nature and Natural Resources and by the Convention on International Trade in Endangered Species (CITES) (Mortimer et al., 2007). Bowen et al. (2007) indicated that an effective conservation strategy would depend on understanding key aspects of hawksbill biology, including the degree of isolation among nesting colonies, migratory pathways of juveniles and adults, and the source (nesting colony) of the foraging population. Development of a cell line from this organism and studies utilizing hawksbill cells will be crucial to these efforts.

It is well known that in reptiles, including many turtles, some lizards and all crocodilians, sex determination is dependent on the incubation temperature of eggs during specific periods of embryonic development (Bull, 1980; Raynaud and Pieau, 1985; Dournon et al., 1990; Janzen and Paukstis, 1991; Pieau et al., 1994). At low incubation temperature, hatchlings are of one sex, while at high temperature they are of the opposite sex. At intermediate temperatures, hatchlings of both sexes are produced (Harry et al., 1990). In turtles, low temperature induces males (Yntema and Mrosovsky, 1980; Yntema and Mrosovsky, 1982; Standora and Spotila, 1985), while in lizards and crocodilians, females are induced (Deeming and Ferguson, 1988).

At the cellular level, the response to temperature is known to induce synthesis of a small set of highly conserved proteins called heat shock proteins (hsps). This response is universal, although some features of the response vary from one organism to another. Moreover, Marshall and Harley (2001) indicate that an interaction between hsp70, the major inducible hsp and SOX9, an important developmental transcription factor, may be significant in the process of mammalian sex determination.

The primary aim of this study was to establish hawksbill cell cultures and elucidate hawksbill cellular characteristics in response to culture temperature changes.

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MATERIALS AND METHODS

Initiation of cell culture

Eggs of *E. imbricata* were obtained with permission from the Okinawa Commemorative National Government Park, Okinawa, Japan. Whole embryos were removed from the eggs (day 42 post-lay), and sections of muscle were removed from the neck, shoulder and belly regions of the embryos. All sections were placed directly into 35 mm plastic cell culture dishes with phosphate buffered saline (PBS) containing antibiotics (100 U penicillin G/ml and 200 μg streptomycin/ml) and left for 2 h at 4°C. After two additional washes in trypsin/EDTA solution (Invitrogen Corp., Carlsbad, CA, USA), tissue sections were finely minced using sterile instruments and incubated at 30°C for 1 h. The trypsin-digested mixtures were centrifuged at low speed and washed twice with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corp.) supplemented with non-essential amino acids, vitamin solution and 10 mM sodium pyruvate (Invitrogen Corp.). The resulting cell pellet was seeded into 35 mm collagen-coated dishes in DMEM supplemented with non-essential amino acids, vitamin solution, sodium pyruvate, 10% fetal bovine serum (FBS) (Intergen Co., Purchase, NY, USA) and antibiotics. Cell cultures were incubated at 30°C in a 5% CO_2 humidified incubator. The medium was changed twice a week, and on reaching confluence, cells were sub-cultivated at a ratio of 1:4 with growth medium (without antibiotics).

Human embryonic cells were obtained from abdomen tissues of 7–8 week-old human embryos, as described previously (Watanabe et al., 1992). Cells were cultured in Eagle's Minimum Essential Medium supplemented with 0.2 mM serine, 0.2 mM aspartate, 1 mM pyruvate, 10 mM 2-[4-(hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES), 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% FBS at 37°C in a 5% CO_2 humidified incubator. 10^6 cells were inoculated in a 75 cm^2 plastic flask and subcultured every seven days.

Growth studies

Growth characteristics of the newly established embryonic hawksbill turtle cells were evaluated at selected temperatures. To determine the incubation temperature for optimal cell growth, 2×10^4 cells at passage 5–10, were seeded in 35 mm tissue culture dishes and pre-incubated at 30°C for 24 h, prior to further incubation at 25, 27, 30, 33, and 37°C in a 5% CO_2 humidified incubator. On days 2, 4, and 7, cells from three dishes at each temperature were trypsinized, counted with a hemocytometer and average cell counts were calculated. Each experiment was performed in triplicate and the mean and standard error (SE) of the mean were plotted.

Flow cytometric analysis

Cell numbers in the different phases of the cell cycle at 33°C and 37°C were measured by uptake and intercalation of propidium iodide (PI) to DNA. Briefly, on reaching cell confluence, FBS concentration was reduced from 10% to 0.05% in the growth medium and cells were further incubated at 33°C for 24 h (serum starvation). After cell synchronization by serum starvation, cells were harvested by trypsin treatment and were reseeded (5×10^5) onto 90 mm dishes in growth medium. Cells were incubated at 33°C for 5 h and further incubated at 33°C or 37°C for 24 h. Flow cytometric analysis was carried out using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). For flow cytometry, cells were prepared at 1×10^6/ml, washed twice with PBS and fixed in ice cold 70% ethanol for 30 min on ice before the addition of PI.

Determination of hsp70 by western blotting analysis

To detect hsp70 production, turtle cells at passage 10–15 were seeded in 90 mm plastic tissue culture dishes and pre-incubated at 33°C for 24 h. Cells were then transferred to and maintained at either 33 or 37°C in a 5% CO_2 humidified incubator. Human embryonic cells were seeded into plastic cell culture flasks (T-25) and pre-incubated at 37°C for 24 h, and then rapidly warmed in a water bath maintained at 43°C for 24 hr. Under these conditions, 0.1% of human embryonic cells survived.

To determine hsp70 levels at different culture temperatures, cells at passage 10–15 were seeded onto 60 mm tissue culture dishes and pre-incubated at 25°C for 24 h. Cells were then transferred to and maintained at 25, 27, 30, 33 and 37°C in a 5% CO_2 humidified incubator.

To obtain total cell protein, cells were lysed in RIPA buffer (50 mM Tris-HCl buffer, pH 7.2, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 0.1% SDS). After freezing at –20°C, the cell lysate was centrifuged for 30 min at $18{,}500 \times g$. The supernatant was harvested and protein content was measured using bovine serum albumin as a standard. Fifteen micrograms of protein per lane was loaded onto an SDS-polyacrylamide gel and electrophoresis (SDS-PAGE) was performed using the Laemmli method (Laemmli, 1970) at 7.5% gel concentration. Proteins were transferred electrophoretically in transfer buffer (0.1 M Tris, 0.192 M glycine, 20% methanol) onto polyvinyl difluoride filters. The filters were blocked overnight with 10% dried milk. In order to determine cross-reactivity, four monoclonal antibodies recognizing only heat-inducible type hsp72 proteins (W27, Oncogene Science, Inc., Cambridge, MA, USA), constitutive heat shock cognate (hsc)-73 protein (IB5, Stressgen Biotechnologies, Corp., San Diego, CA, USA), hsp72 and hsc73 proteins (N27F, Stressgen Biotechnologies, Corp., and 5A5, Affinity Bio Reagents, Inc. Golden, CO, USA), were used as primary antibodies. To visualize bound primary antibodies, filters were incubated with biotinylated anti-mouse or rat Ig whole antibody (Amersham Japan Co. Ltd., Tokyo, Japan) and streptavidin/biotinylated alkaline phosphatase conjugate (Amersham Japan Co. Ltd.). This was followed by incubation with 5-bromo-4-chloro-3-indolylphosphate *p* -toluidine salt and nitroblue *tetra* -zolium chloride (Invitrogen Corp.) for color development. The amount of hsp70 was determined by densitometric analysis.

Statistical analysis

All data are expressed as mean ± standard error. Statistical analysis for cell growth characteristics was performed using a paired Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

RESULTS

Cell growth characteristics

Cell adhesion from the hawksbill turtle muscle sections to the cell culture dishes was slow. Only muscle sections removed from the neck successfully produced a confluent monolayer by 12 days. However, once initial monolayers were formed, growth became more rapid and maintenance in growth medium enabled approximately 50 subcultures to be made. Cells cryopreservation was carried out in growth medium containing 15% FBS and 10% dimethylsulfoxide at –196°C.

Growth characteristics of the newly established embryonic hawksbill turtle cells were evaluated at selected temperatures. Hawksbill turtle cells showed temperature-dependent cell proliferation between 25 and 33°C, and optimal cell growth was achieved when cells were incubated at 33°C (Fig. 1). At this temperature, a confluent monolayer was reached one week after seeding. The number of confluent cells per 35 mm tissue culture dish was approximately 2.8×10^5 cells. Cell proliferation significantly decreased at 22°C and was negligible at 19°C (data not shown). At 37°C, hawksbill turtle cell growth was completely inhibited (Fig. 1).

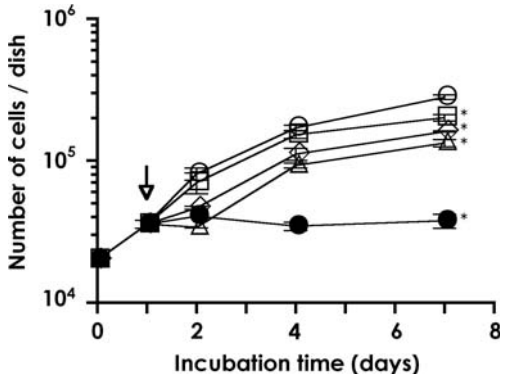

Fig. 1. Growth of hawksbill turtle cells at various incubation temperatures. Cells were plated at 2×10^4 cells/35 mm culture dish in DMEM/10% FBS and incubated at 30°C for 24 h. Cells were then incubated at 25 (△), 27(◇), 30 (□), 33 (○) and 37°C (●) (arrow in the figure) for up to 7 days. The growth curve was determined by cell counts using a hemocytometer. For each time point the mean and standard error of the mean are plotted ($n = 3$). Each experiment was performed in triplicate. Statistical significance ($P < 0.05$) from 33°C is indicated by asterisks.

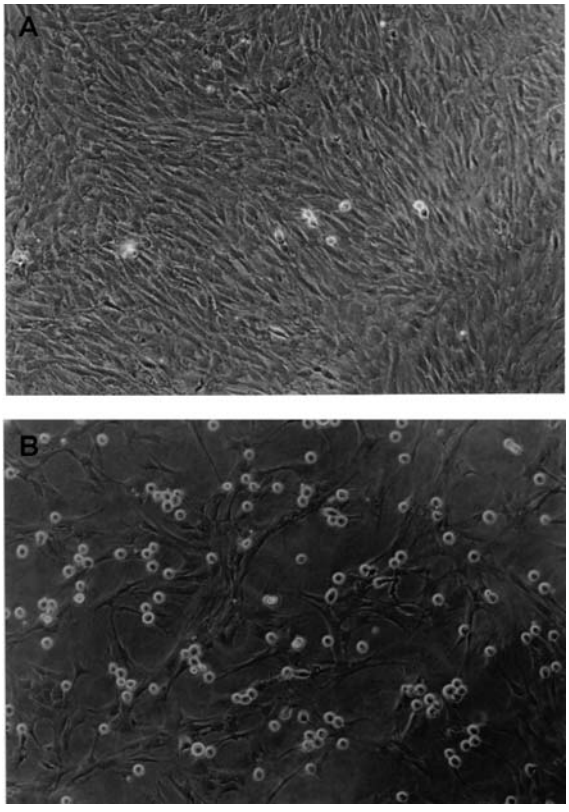

Fig. 2. Phase-contrast micrographs of cultured hawksbill turtle cells (original magnification × 40). **(A)** Hawksbill turtle cell morphology during normal growth, 33°C. **(B)** Change in morphology during growth inhibition, 37°C, 24 h.

Morphological change

Cellular morphologies during optimal growth (33°C) and growth inhibition (37°C) states, were observed in passage 8 cell cultures. Hawksbill turtle cells exhibited fibroblastic morphology and formed monolayers at 33°C (Fig. 2A). When confluent cells were incubated at 37°C for 24 h, morphology dramatically changed. Adherent cells exhibited a broader morphology and abundant floating spherical cells appeared (Fig. 2B).

Table 1. Effect of temperature on cell cycle progression by flow cytometric analysis after synchronization.

Growth Phase	% Synchronized	33°C, 24 h	37°C, 24 h
G_0/G_1	92.4%	39.2%	77.6%
S	3.6%	46.6%	22.1%
G_2/M	4.0%	14.2%	0.3%

Cells synchronized by serum starvation were cultured for 24 h at 33°C and 37°C, fixed, stained with PI and analyzed for DNA content on a flow cytometer (see MATERIALS AND METHODS).

Growth properties at 37°C

Based on the hawksbill turtle cells characteristic growth inhibition and morphology at 37°C, subsequent experiments were aimed at investigating the growth properties in more detail.

To investigate the cell cycle pattern during the growth inhibition state, cell numbers in the different phases of the cell cycle, at 33°C (normal growth temperature) and 37°C (growth inhibiting temperature) after synchronization by serum starvation, were measured by flow cytometry. The DNA histogram of cells immediately after serum starvation was characterized by synchronization in G_0/G_1 phase in 92.4% of cells. When incubated in growth medium at 33°C for 24 h, cells evidently entered the cell cycle (G_0/G_1 phase; 39.2%, S phase; 46.6%, G_2/M phase; 14.2%). Conversely, when the temperature was raised to 37°C, the majority of cells remained arrested in the G_0/G_1 phase even after 24 h (77.6%) (Table 1). These results strongly suggest that hawksbill turtle cell growth is inhibited at 37°C.

Since incubation at 37°C inhibited cell growth, we next investigated whether growth ability could be restored. After inhibition of cell growth by incubation at 37°C for 48 h, cells were incubated at 33°C. Consequently, cell growth was gradually restored and eventually approached control levels (Fig. 3). These results indicate cell growth inhibition at 37°C was a temporary and reversible event, and not affected the rate of exponentially growth phase.

Detection of hsp70

To determine whether the level of hsp expression changes in newly established turtle cells, total protein prepared from normal growth and growth inhibition state cells, was separated by SDS-PAGE and cross-reactivity of hsp70 was determined by immunoblotting. Figure 4 shows that anti-hsp72/hsc73 and anti-hsc73 antibodies specifically recognized respective proteins, and these protein levels increased at 37°C. Chang et al. (2000) reported that available hsp72 antibody failed to bind to the turtle tissue proteins. Our results show that hsp70 (hsp72/hsc73 or hsc73) expressed in the hawksbill turtle cells at 37°C might correspond to heat-inducible hsp72 in human embryo cells. These results indicate that hawksbill turtle cells express low levels of heat-responsible hsp70.

The amount of hsp70, determined during elevating cul-

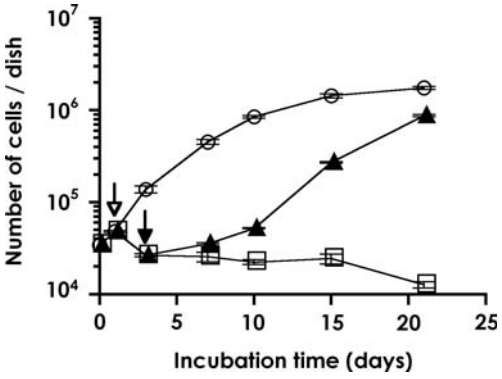

Fig. 3. Growth restoration of hawksbill turtle cells. Cells were plated at 2.5×10^4 cells/35 mm culture dish in DMEM/10% FBS and incubated at 33°C for 24 h. Cells were divided into three groups: incubation at 33°C (○), 37°C (□) for up to 21 days (open arrow in the figure), and post-incubation at 33°C after incubation at 37°C for 48 h (▲, closed arrow in the figure). The medium was changed twice weekly. The curve was determined by counting cells using a hemocytometer. For each time point the mean and standard error of the mean are plotted (n = 3). Each experiment was performed in triplicate.

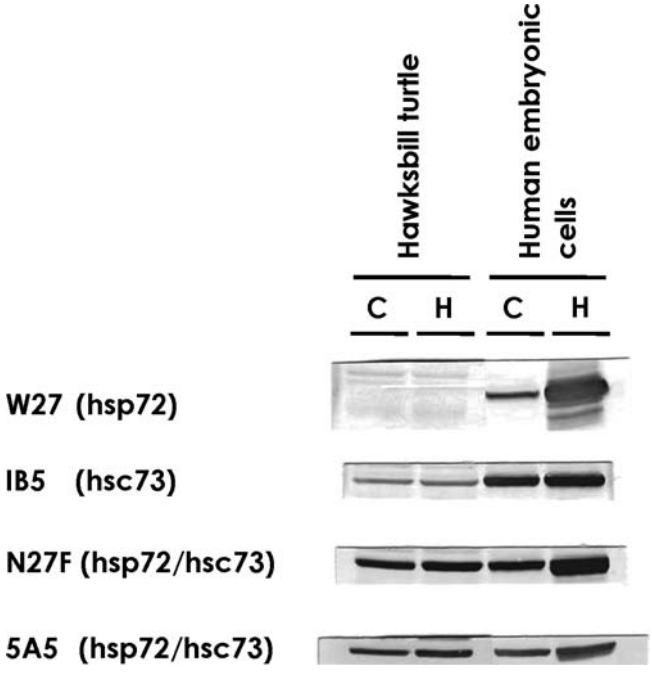

Fig. 4. Cross-reactivity of various anti-hsp70 antibodies to intracellular protein of hawksbill turtle cells. Fifteen micrograms of protein per lane was loaded for electrophoresis in SDS-PAGE. The cross-reactivity of various anti-hsp70 antibodies was analyzed by western blotting. C, control; H, hawksbill turtle cells incubated at 37°C for 72 h, human embryo cells heated at 43°C for 10 h.

ture temperature conditions, was increased in relation to cell growth. The relative amount of hsp70 and the reciprocal of the growth rate were plotted against absolute temperature. Figure 5 shows that increased growth rate leads to a parallel increase in relative amount of hsp70, within the range of growth permitting temperatures for hawksbill turtle cells.

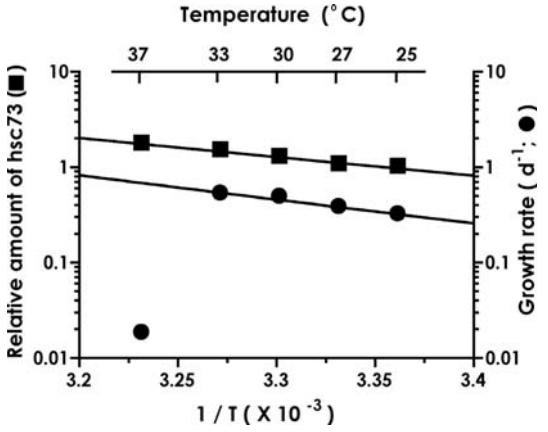

Fig. 5. Arrhenius plots of the growth rate and the relative amount of hsp70 of hawksbill turtle cells against culture temperature. The growth rate (●) was obtained from the slope of the exponential growth phase in Fig. 1. The amount of hsp70 (■) was analyzed in 7-day-old cultured cells at each temperature by western blotting. Lines were drawn by the method of least squares.

DISCUSSION

This is the first study to report the successful culture of embryonic hawksbill turtle cells and to demonstrate they differ in terms of thermal influence compared to mammalian cells. Hawksbill turtle cells were sub-cultured more than 50 times using a 1:4-split culture protocol and were optimally maintained at 33°C. At low temperature (19°C), cells were unable to replicate (data not shown). Koment and Haines (1982) reported that normal epithelioid skin cells of the green sea turtle, *Chelonia mydas*, were successfully grown at 30°C, although replication occurred between 16 and 37°C. At 37°C however, the cells were noticeably ragged and granulated, resulting from acceleration of metabolism by rapid change in pH. Lu et al. (1999) also reported that cells from *C. mydas* with fibropapillomas, exhibited optimal growth at 30°C and were able to proliferate at incubation temperatures between 20 and 25°C. However cell growth was not observed when cultures were incubated at 37°C or 15°C. *E. imbricata* nesting areas are located at lower latitudes compared with *C. mydas* (Uchida, 1982). The individual differences in permissible low culture temperature for cells from these two sea turtle species may reflect their respective habitats.

Hawksbill turtle cell growth was found to be completely inhibited when culture temperature rose from 33 to 37°C (Fig. 1). At this temperature, cells went through morphological changes, with increased numbers of floating cells and increased numbers in G_1 arrest (Fig. 2, Table 1). In addition, appearance of retained the progression between S and G_2/M phase may cause inhibition of DNA synthesis after incubation at 37°C. Wang et al. (1989) reported that ovarian cells of the warm water *Tilapia* ceased to proliferate when moved from normal growth temperature of 31 to 37°C, and became arrested in G_1 and G_2 phases of the cell cycle. Moreover, the ability of the arrested cells to re-enter the cell cycle when restored to a temperature of 31°C is likely attributable to the presence of certain proteins that fulfill the serum require-

ments.

When culturing hawksbill cells at 37°C, there was no further increase in the number of floating cells and attached cells after 24 h. Although cells attaching on the surface of the culture dish were no growth at 37°C, they were grown to a slight extent by changing the incubation temperature to 30°C. Furthermore, floating cells were able to resume growth under normal culture conditions (data not shown), indicating that cells detaching from the surface of the culture dish were in a growth inhibition state and retained their cellular proliferation potential. Hence hawksbill turtle cells had temporally growth inhibition, but not gained heat resistance at 37°C. As expected, cell growth hindered by slight heat-stress was restored by transferring the cultures to normal growth conditions (Fig. 3). A similar phenomenon was reported by Bols et al. (1990) using reptilian IgH-2 cells from *Iguana iguana*. These cells are routinely grown at 36°C, but at temperatures above 42°C cytoplasmic vacuoles appeared, cell bodies rounded with extended cell processes, shriveling became apparent, and cells eventually detached from the growth surface. Return of these cells to 36°C for 24 h enabled a variable portion to recover to their normal attachment potential and morphology.

Numerous studies on hsps have been published. In all organisms, the induction of hsps is remarkably rapid and intense, keeping with the notion that this is an emergency response. Moreover, there is a striking relationship between the induction temperature and the organism's environment (Lindquist, 1986; Lindquist and Craig, 1988; Maio, 1995). Bols et al. (1992) reviewed the use of fish cell cultures to study three temperature phenomena, including hsp synthesis, temperature acclimation and heat resistance. In the RTG-2 cell line from rainbow trout, a cause and effect relationship between the synthesis of the major hsps and the development of thermotolerance is suggested (Mosser and Bols, 1988). In reptiles, several in vivo studies relating to hsps have been reported to date. Hsp68 is the major component of the heat shock response in lizards and this protein is constitutively synthesized at high levels at normal temperature in thermophylic lizards (Ulmasov et al., 1992). In comparison the constitutive hsp70 is present in tissue homogenates from the red-eared slider, *Trachemys scripta* (Yu et al., 1994). Chang et al. (2000) have quantified hsp60 and hsp72/73 in myocardial tissue from two turtle species, the western painted turtle (*Chrysemys picta belli*, anoxia-tolerant) and the softshell turtle (*Trionyx sponifer*, anoxia-sensitive), during normoxia and anoxia. Ramaglia and Buck (2004) also reported that hsp72, hsp73 and hsp90 expression increases in *C. picta belli* during a forced dive. One unique report demonstrated that bacterial infection causes a tissue-specific increase in the expression of hsp73, hsp72 and hsp90 in *C. picta belli* (Ramaglia et al., 2004). In a reptilian cell line from *Iguana iguana*, synthesis of hsps comprising polypeptides of 87, 76, 70, 29, 28, 22, and 21 kDa were identified (Bols et al., 1990).

In this report, we demonstrate for the first time that hsp70 exists in cultured hawksbill cells. Furthermore, increased levels of hsp70 were detected in these cells after exposure to slight heat-stress (at 37°C). Ulmasov et al. (1992) reported that elevated levels of hsp70-like proteins, found in cells of thermophilic species at normal temperature, likely represent one adaptive mechanism. In addition, they reported a strong positive correlation between the content of hsp70-like proteins in nine lizard species under normal, non-heat-shock conditions and average temperature of each animal's ecological niche. This finding is consistent with the data presented in this study. Replication of hawksbill cells occurred between 25 to 33°C and the level of hsp70 expression increased, dependent on elevating culture temperature. Furthermore, the data demonstrate a significant positive correlation between the ability to proliferate and the level of hsp70 expression under normal growth conditions (correlation coefficient; $r^2 = 0.977$), although the level of hsp70 expression also increased at 37°C. Human embryonic cell proliferation is completely inhibited at 43°C, however it is able to restore by changing the incubation temperature back to 37°C. In this case, the main factor inhibiting cell proliferation is denaturation of some cellular proteins; hsp is induced to repair denatured proteins. The same mechanism may occur in hawksbill turtle cells. Interestingly, increase of hsp70 expression promote cell proliferation at allowable temperature, alternative to that induced at unallowable temperature (Suzuki and Watanabe, 1994). This slight heat-stress may positively regulate proliferation of hawksbill cells, although the exact mechanism remains elusive.

Conservation concerns for the critically endangered hawksbill turtles are immense because populations are severely reduced in every ocean basin (Bowen et al., 2007). An effective conservation strategy depends on understanding key aspects of hawksbill turtle biology, including the degree of isolation among nesting colonies, migratory pathways of juveniles and adults and the source for foraging populations. Corresponding conservation investigations have been reported. Park et al. (2003) found that a variety of hatchling sex ratios can be produced by hawksbills depending on the location and timing of nesting. Sea turtles, similar to many reptiles, possess temperature-dependent sex determination (Bull, 1980; Raynaud and Pieau, 1985; Standora and Spotila, 1985; Dournon et al., 1990; Janzen and Paukstis, 1991; Pieau et al., 1994; Pieau et al., 1999). Lin et al. (2008) indicate that newly developed microsatellites will facilitate the study of genetic diversity, population structure and species relationship of the hawksbill and other marine turtle species. Velez-Zuazo et al. (2008) carried out the largest focal genetic study of hawksbill turtle aggregation and were the first to examine mitochondrial DNA haplotype differences among different life-history stages and demographic segments, and across consecutive breeding seasons. However, these were all demonstrated in the absence of direct investigations using hawksbill cells.

In summary, we established hawksbill cell cultures and elucidated a number of their cellular properties influenced by culture temperature. Our newly established cell line will facilitate future studies to provide both information and conservation. As advocated by Bowen et al. (2007), like many marine conservation issues, international cooperation is the last and best hope for ensuring the hawksbills survival.

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