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Lack of age-associated telomere shortening in long- and short-lived species of sea urchins

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Abstract The red sea urchin, *Strongylocentrotus franciscanus*, can live in excess of 100 years while the sea urchin *Lytechinus variegatus* has an estimated lifespan of only 3–4 years. In an effort to understand the molecular mechanism underlying the difference in their longevity we characterized telomere biology in these species of sea urchins. Telomerase activity was found throughout early stages of development in *L. variegatus* and is maintained in adult tissues of *L. variegatus* and *S. franciscanus*. Terminal restriction fragment analysis indicated a lack of age-associated telomere shortening. These data suggest that long- and short-lived sea urchins do not utilize telomerase repression as a mechanism to suppress neoplastic transformation.

Keywords: Telomerase; Telomere; Sea urchin; Longevity

1. Introduction

Telomeres, the repetitive sequences of DNA and associated proteins which cap the ends of eukaryotic chromosomes, shorten during cell division due to the inability of the DNA replication machinery to replicate the ends of linear DNA molecules [1,2]. Critically short telomeres trigger a signal for the cell to permanently stop dividing, a process referred to as cellular senescence [1]. The onset of senescence prevents erosion of essential genes located near telomeres and prevents catastrophic genomic instability [1,2]. Cellular senescence is well characterized in cultured human cells and senescent cells have been shown to accumulate in aging tissues *in vivo* [1,3–5]. Immortal cells and cells which require a high replicative potential can prevent or delay senescence by expression of telomerase [1]. Telomerase is a ribonucleoprotein which can synthesize telomeric DNA and maintain telomere integrity [1,2].

In human development, telomerase is present in early embryogenesis and in all tissues examined during the first trimester, however it is repressed at different times in tissues as fetal development progresses [6]. In the absence of telomerase, telomeres shorten in somatic tissues with consecutive cell divisions [1,6]. Telomerase remains active in germ cells and cell types which require a high proliferative capacity (i.e. stem cells) and is reactivated during the progression of most cancers. It is thought that telomerase inactivation has evolved as a 'tumor-protection mechanism' that reduces the incidence of cancer in long-lived organisms such as humans [1,6].

Sea urchins have been a key model for the study of early development. Historically, they were used to help understand a variety of developmental processes including fertilization, egg activation, cleavage, gastrulation and more recently, to investigate the mechanisms of gene regulation in early development [7,8]. Maintenance of telomeres is essential for normal sea urchin development as telomere-interactive agents have been shown to decrease the rate of cell proliferation and induce chromosome destabilization in sea urchin embryos [9]. In this study, we investigated the pattern of telomerase expression throughout development and whether telomerase is active or repressed in later development in sea urchin species with tremendously different lifespans. *Strongylocentrotus franciscanus* is one of the longest-lived species of sea urchins, living for more than 100 years without evidence of age-related disease and no apparent decline in reproductive potential [10–12]. In contrast *Lytechinus variegatus* is short-lived, with an estimated maximum lifespan of 3–4 years [13,14]. We are interested in the underlying molecular mechanisms that determine this disparity in longevity.

2. Materials and methods

2.1. Collection of urchins and preparation of adult tissues

Adult *L. variegatus* (sub-species *atlanticus*) (3.6–7.6 cm in diameter) were collected from sea grass beds in Richardson's Bay Bermuda. Adult *S. franciscanus* (4.8–16.1 cm in diameter, representing ages from 4 to >100 years) were collected offshore from the Bodega Marine Research Laboratory in California. Dissected adult urchin tissues (gonads, intestine, esophagus, Aristotle's lantern muscle, ampullae and tube feet) were briefly rinsed with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and frozen at −70 °C prior to analysis.

2.2. Spawning and collection of early developmental stages of *L. variegatus*

L. variegatus were induced to spawn by the injection of 2–5 ml of 0.53 M KCl. Gametes were collected from the gonophores and examined under a microscope to distinguish eggs from sperm. Eggs were suspended in filtered sea water and sperm were added at a ratio of approximately 1:2000 (egg:sperm). Samples at various developmental

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate; TRAP, telomeric repeat amplification protocol; TRF, terminal restriction fragment

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stages (formation of the fertilization membrane, blastula, gastrula, prism and pluteus) were collected by centrifugation, washed briefly with TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and frozen at −70 °C prior to analysis.

2.3. Telomeric repeat amplification protocol (TRAP) assay

Telomerase activity was determined using the telomeric repeat amplification protocol (TRAP) assay [15] using the TRAPeze kit from Chemicon International. Detergent extracts were prepared using the CHAPS lysis buffer and protocol supplied with the TRAPeze kit. The eggs, sperm and early developmental stages were resuspended in CHAPS lysis buffer for extraction while the adult tissues were homogenized in CHAPS lysis buffer. Protein estimations were done using Coomassie Protein Assay Reagent from BioRad and the CHAPS extracts were aliquotted and stored at −70 °C. For all TRAP reactions, the telomerase extension step prior to PCR was increased to 60 min and PCR amplification was performed for 33 cycles. In some instances, the nucleotide dCTP was omitted from the telomerase extension portion of the TRAP reaction and added only prior to the PCR cycling steps. The PCR products were run on 12.5% non-denaturing polyacrylamide gels and visualized by the DNA stain SYBR green (Molecular Probes). The molecular weights of the bands on the gels were calculated using the KODAK Molecular Imaging Software (version 4). In this analysis, the molecular weights of the telomerase-generated bands were normalized to the molecular weight of the internal control bands to compensate for any curvature of the gels.

2.4. Isolation of DNA from sea urchins and telomere length analysis

DNA was extracted from urchin tissues using previously described methods [16]. Telomere length was determined using the terminal restriction fragment (TRF) length analysis using the TeloTAGGG Telomere Length Assay kit from Roche Molecular Diagnostics with 1.5 μg of DNA loaded per lane.

3. Results

3.1. Telomerase activity in early developmental stages of L. variegatus

Telomerase activity was measured using the TRAP assay. In this assay, telomerase activity is detected by its ability to synthesize telomeric repeats onto an oligonucleotide substrate. These elongation products are then amplified by PCR resulting in a ladder pattern of bands incremented by the telomeric repeat unit when analyzed by non-denaturing polyacrylamide gel electrophoresis. Mature eggs were found to have telomerase activity while telomerase could not be detected in mature sperm (Fig. 1). Mature sperm were assayed using an extended range of protein concentrations (0.05–3.2 μg of protein added to the TRAP reaction), but no telomerase activity was detected (data not shown). Each TRAP reaction mixture contains primers and a template for amplification of a 36 bp internal control which helps to identify false-negative samples that contain inhibitors of Taq polymerase. Inhibitors of Taq polymerase would prevent the amplification of telomerase-generated products in the TRAP assay. The presence of the internal control band in the sperm samples indicates the absence of a Taq polymerase inhibitor in these samples (Fig. 1). Following fertilization, samples of early developmental stages were collected at 45 min after the formation of the fertilization membrane and subsequent stages including blastula, gastrula, prism and pluteus. All stages of early development investigated showed telomerase activity and the results from gastrula, prism, and pluteus are shown in Fig. 1. The periodicity of the telomerase-generated bands, calculated using the KODAK Molecular Imaging Software (version 4), indicated a 6 nucleotide repeat unit for *L. variegatus* similar to that generated by the human

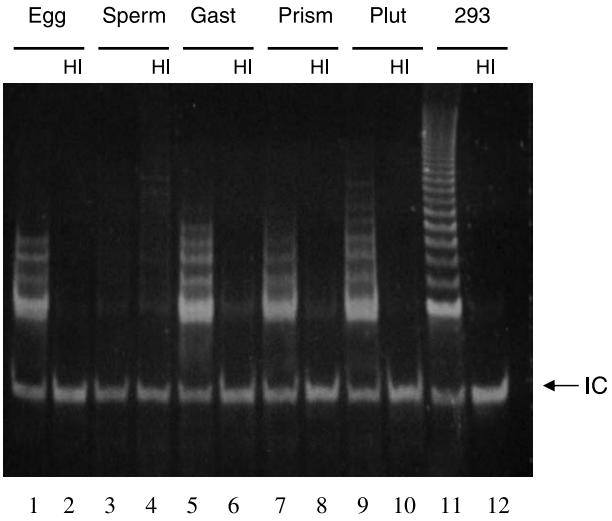

Fig. 1. Analysis of telomerase activity in early developmental stages of *Lytechinus variegatus*. TRAP analyses were conducted using CHAPS extracts with the indicated amount of input protein. Lanes 1–2: eggs (1 μg protein), Lanes 3–4: sperm (1 μg protein), Lanes 5–6: gastrula (1 μg protein), Lanes 7–8: prism (1 μg protein), Lanes 9–10: pluteus (1 μg protein), Lanes 11–12: positive control 293 cells (1000 cell equivalents). In lanes 2, 4, 6, 8, 10 and 12 the CHAPS extracts were heat inactivated prior to TRAP analysis (HI). The internal control for the TRAP reaction is indicated with the arrow (IC).

transformed cell line 293 which was used as a positive control and which possesses the telomeric repeat sequence TTAGGG. This telomeric sequence is highly conserved in both vertebrates and invertebrates and has been previously found in the sea urchin species *S. purpuratus* [17]. The specificity of the telomerase reaction was confirmed by the lack of the ladder pattern of bands in the CHAPS extracts that were heat treated (85 °C for 10 min) prior to performing TRAP analysis (Fig. 1). The spawning experiment was repeated three times with identical results.

3.2. Telomerase activity in adult tissues of L. variegatus and S. franciscanus

Tissues from six adult *L. variegatus* (four male and two female) ranging in size from 3.6 to 7.6 cm in diameter and seven adult *S. franciscanus* (four male and three female) ranging in size from 4.8 to 16.1 cm were analyzed for telomerase activity using the TRAP assay. The estimated ages, based on diameter, for the *S. franciscanus* individuals were 4, 10, 20, 30, 30, 50 and >100 years. Telomerase activity was detected in Aristotle's lantern muscle, ampullae, esophagus, intestine, tube feet and both male and female gonads for both species of sea urchins regardless of age (Fig. 2, panel A and B). The periodicity of the telomerase-generated ladder pattern of bands calculated using the KODAK Molecular Imaging Software (version 4) indicated a six nucleotide repeat unit for both *L. variegatus* and *S. franciscanus*. Further, omitting dCTP from the telomerase extension portion of the TRAP reaction did not affect the results indicating that dCTP is not included in the *L. variegatus* or *S. franciscanus* telomeric sequence (data not shown). The specificity of the telomerase reaction was confirmed by the lack of the ladder pattern of bands in the CHAPS extracts that were heat treated (85 °C for 10 min) prior to performing TRAP analysis (Fig. 2).

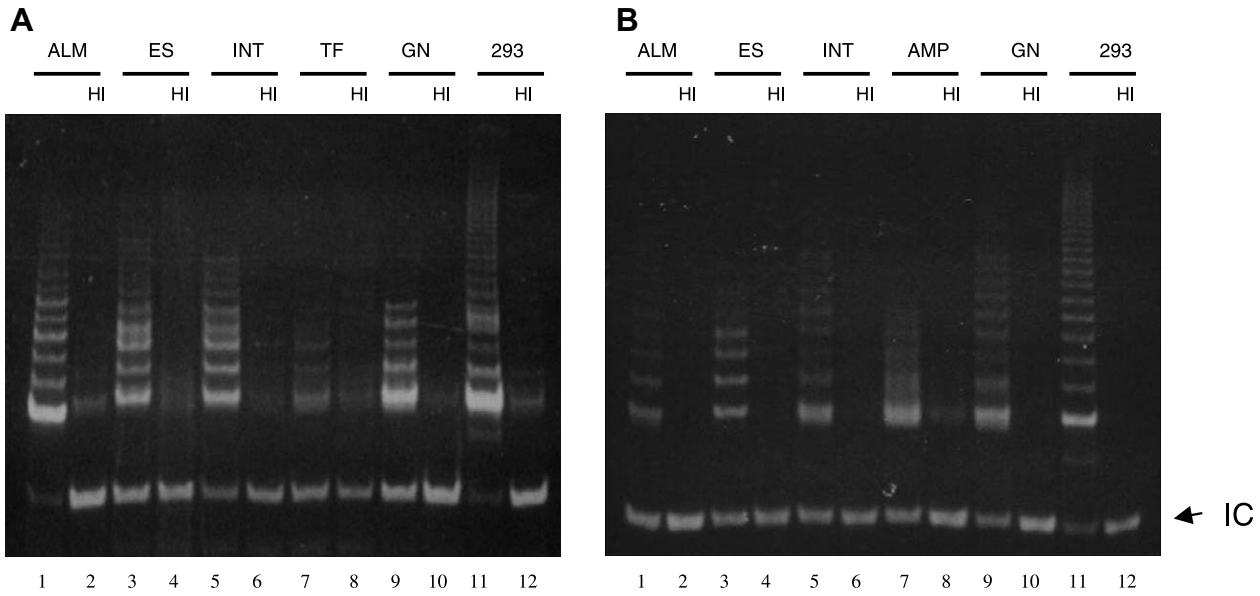

Fig. 2. Analysis of telomerase activity in adult tissues of *Lytechinus variegatus* and *Strongylocentrotus franciscanus*. TRAP analyses were conducted on tissue homogenates using the indicated amount of input protein. Panel A: *L. variegatus*: Lanes 1–2: Aristotle's lantern muscle (2 μg protein), Lanes 3–4: esophagus (2 μg protein), Lanes 5–6: intestine (2 μg protein), Lanes 7–8: tube feet (2 μg protein), Lanes 9–10: gonad (0.4 μg protein), Lanes 11–12: positive control 293 cells (1000 cell equivalents). In Lanes 2, 4, 6, 8, 10 and 12 the CHAPS extracts were heat inactivated prior to TRAP analysis (HI). Panel B: *S. franciscanus*: Lanes 1–2: Aristotle's lantern muscle (2 μg protein), Lanes 3–4: esophagus (2 μg protein), Lanes 5–6: intestine (2 μg protein), Lanes 7–8: ampullae (2 μg protein), Lanes 9–10: gonad (0.4 μg protein), Lanes 11–12: positive control 293 cells (1000 cell equivalents). In Lanes 2, 4, 6, 8, 10 and 12 the CHAPS extracts were heat inactivated prior to TRAP analysis (HI). The internal control for the TRAP reaction is indicated with the arrow (IC).

3.3. Telomere maintenance in L. variegatus and S. franciscanus

DNA was isolated from adult tissues of *L. variegatus* and *S. franciscanus* and telomere length was investigated using terminal restriction fragment (TRF) analysis. In this analysis, genomic DNA was digested with restriction enzymes that digest the genome at frequent sites, but do not digest the DNA within the telomeric repeat sequence. The digestion products were then used for Southern blots that were probed with oligonucleotides complementary to the telomeric sequence TTAGGG. The TRF values for all somatic and germ tissues of *L. variegatus* were beyond the limit of resolution of the TRF analysis (>20 kb). Although telomeres appear to be maintained, small changes in TRF length between somatic and germ tissues or between young and old urchins may not be distinguished using conventional TRF analysis for *L. variegatus*. The results for the oldest urchin examined (>3 years old, 7.6 cm in diameter) are shown in Fig. 3, Panel A. The TRF analysis for *S. franciscanus* tissues demonstrated maintenance of telomeres between somatic and germ tissues and between young and old urchins. The mean TRF values were 5.2 ± 0.8 kb for Aristotle's lantern muscle, 5.3 ± 0.6 for ampullae, 5.2 ± 0.7 for esophagus, 5.0 ± 0.6 for intestine and 5.6 ± 0.7 for gonads. The TRF analysis for the youngest (4 years) and the oldest (>100 years) urchins are shown in Fig. 3, Panel B.

4. Discussion

Telomerase activity was found throughout early embryonic development and is maintained in adult tissues of the sea urchin *L. variegatus*. The lifespan of *L. variegatus*, estimated by size-frequency analysis of natural populations and also by counting the annual growth bands on the demipyramids of Aristotle's lantern, is only 3–4 years [13,14]. Although tagging experiments have not been done for *L. variegatus* to confirm these estimates, it is generally agreed that it is a fast-growing, short-lived species [13,14]. It has been argued that telomerase repression may not be necessary in smaller, shorter-lived species as a tumor protection mechanism [1,6]. Since the total accumulation of mutations would be related to both lifespan and cellularity, it is presumed that other tumor protection mechanisms (i.e. DNA repair, anti-oxidant protection, cell cycle checkpoints, immune surveillance) would be adequate for small, short-lived species and therefore telomere shortening would not have evolved [6].

Analysis of adult tissues of the long-lived species, *S. franciscanus*, indicated sustained telomerase expression and maintenance of telomeres. Although the amount of cell proliferation in tissues of adult sea urchins has not been reported, it has been suggested that continuous telomerase expression is necessary to maintain telomeres in animals that grow throughout their lifespans [18]. It is also possible that telomerase plays an additional role in these cells. For example, it has been shown that telomerase expression can protect cells from apoptosis [19–21].

Fluorescent tagging and ^{14}carbon incorporation have shown that *S. franciscanus* grows indeterminately during its lifespan which can exceed 100 years [11,12]. If telomerase repression serves as a tumor suppressor mechanism, one might expect an increased incidence of neoplasm in long-lived species that do not repress telomerase activity. Remarkably, there are few reported cases of neoplasm in sea urchins including only four potential cases listed in the Registry of Tumors in Lower Animals (RTLA) (www.pathology-registry.org) [22]. These include 2 cases in *S. droebachiensis* (RTLA numbers 83 and

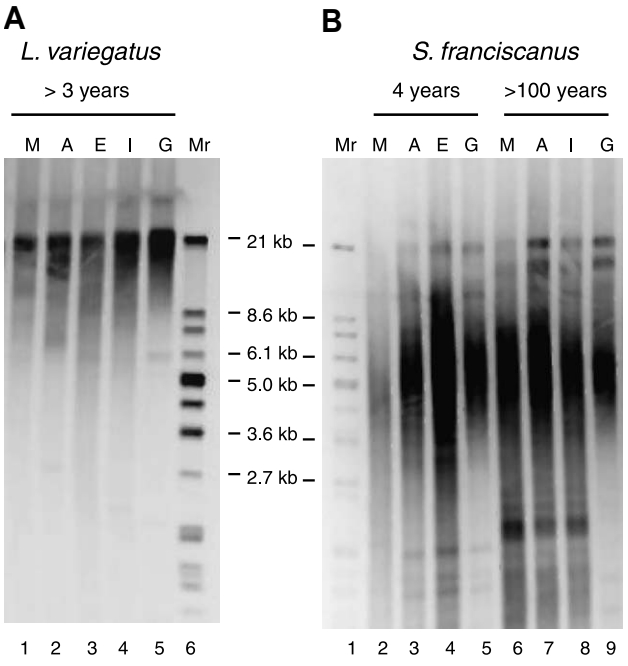

Fig. 3. Terminal restriction fragment length analysis of DNA isolated from adult tissues of *Lytechinus variegatus* and *Strongylocentrotus franciscanus*. Panel A: TRF analysis from *L. variegatus*. Lane 1: Aristotle's lantern muscle (M), Lane 2: ampullae (A), Lane 3: esophagus (E), Lane 4: intestine (I), Lane 5: gonad (G), Lane 6: Molecular weight markers (Mr). The samples shown are from the oldest urchin examined (>3 years old with a diameter of 7.6 cm). Panel B: TRF analysis of young and old *S. franciscanus*. Lane 1: Molecular weight markers (Mr), Lane 2: Aristotle's lantern muscle (M), Lane 3: ampullae (A), Lane 4: esophagus (E), Lane 5: gonad (G), Lane 6: Aristotle's lantern muscle (M), Lane 7: ampullae (A), Lane 8: intestine (I), Lane 9: gonad (G). Samples in Lanes 2–5 are from the youngest urchin (4.8 cm in diameter and an approximate age of 4 years), samples in lane 6–9 are from the oldest urchin with a diameter of 16.1 cm representing >100 years of age.

4290), one case in *Echinometra mathaei* (RTLA number 1104) and one case in *Heterocentrotus mammillatus* (RTLA number 236). This limited number is surprising given the intensity to which sea urchins have been studied as model organisms for the last century and the fact that some species, including *S. franciscanus*, form the basis of a commercial fishing industry. This suggests that sea urchins rely on other mechanisms to prevent the formation of neoplasm such as efficient cellular defense and repair mechanisms or efficient mechanisms to replace damaged cells.

Our results indicate a lack of age-associated telomere shortening in two species of sea urchins, the short-lived *L. variegatus* and the long-lived *S. franciscanus*. Little is known of telomere biology in the most widely studied species of sea urchin, *S. purpuratus*. The sequence of their telomeres has been determined to be identical to that of humans (TTAGGG) and embryos have a telomere length of 6 kb [17]. They are known to have a moderately-long lifespan with approximately 1% of the population living to greater than 50 years of age [23,24]. Further studies could uncover the genetic factors determining longevity in different species of sea urchins and may establish sea urchins as a unique model for understanding the process of aging and resistance to acquiring age-related pathologies such as cancer.

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