Aging Process in Dermal Fibroblast Cell Culture of Green Turtle (*Chelonia mydas*)

Anggraini Barlian*, Yemima D. Riani

School of Life Sciences and Technology, Bandung Institute of Technology, Indonesia.

*) Corresponding author; e-mail: aang@sith.itb.ac.id

Abstract

Senescence in green turtle is an interesting process to study because until now no clear explanation has been established about senescence at cellular or molecular level in this species. One of common markers used for detecting senescence is telomere shortening. Reduced telomerase activity may also cause senescence. The aims of this research are to establish and identify dermal fibroblast cell culture from green turtle and also to compare telomere length and telomerase activity from the cells subculture 5 and 14. Cells were identified with Rabbit Anti-Vimentin Polyclonal Antibody and Goat Polyclonal Antibody using confocal microscope. Telomere length was obtained using TeloTAGGG Telomere Length Assay (Roche), while telomerase activity was obtained using TeloTAGGG Telomerase PCR ElisaPlus. Primary cell culture from green turtle skin showed fibroblastic morphology and immunocytochemistry results using vimentin antibody proved that the culture was fibroblast cell. Measurement of telomere length and telomerase activity showed that telomere length and telomerase activity of subculture 14 was greater than subculture 5. However, based on morphology, green turtle fibroblast skin cell culture showed senescent morphology. Therefore, possible aging mechanism that the green turtle fibroblast skin cell culture underwent did not go through both telomere shortening and reduced telomerase activity.

Keywords: cell culture, *Chelonia mydas*, telomerase activity, telomere length, senescence.

1. Introduction

Green turtle (*Chelonia mydas*) is one of the most popular long-lived animals whose age may exceed 100 years [1, 2]. Aging in *C. mydas* is still unknown because early studies showed no mortality advances yet increasing reproductive capacity [3]. The study of aging in *C. mydas* is important for further comparison with mammals in the biomedical field. However, since 1999, IUCN recorded *C. mydas* as one of the endangered species, therefore sacrificing this animal for research must be minimized. One solution for this issue is the establishment of *C. mydas* cell culture. The establishment of new cell culture from primary explants may produce one dominant cell type dependent on medium, growth factor, and tissues collected from the donor. Therefore, cell type must be analyzed first before the aging process can be analyzed further. As cells replicate in culture, the cell will experience senescence caused by telomere shortening. Telomeres are repeated sequences at chromosome end and will become shortened at cell replication because of end-replication problem [4]. DNA bases can only be added from hydroxyl 3' end, so replication of lagging strand can only be processed by the presence of RNA primer. Once replication reaches the end of a chromosome, RNA primer at the 3' end degraded and no DNA base can be added at this end. This telomere shortening phenomenon is one of the main causes of replicative senescence which leads to an estimation of Hayflick limit, number of which cells can be passage until cells stop divide, and finally undergo apoptosis [5]. As cells replicate and chromosomes shortened, chromosome fusion, break, and damage could happen to DNA. Thus, cells will activate p53 protein to arrest the cell cycle in the late G1 phase before cells may show senescence phenotype such as decreased protein degradation, housekeeping enzyme expression, energy production, and increased ROS level (reactive oxygen species) [6]. This phenotype may lead to a decrease in normal cell function, after which cell may undergo apoptosis. The presence of telomerase, a ribonucleoprotein, prevents telomere shortening because telomerase adds extra TAGGG base to telomere end [7]. In mammals, telomerase is no longer expressed in adult tissues [8], except in cancer [9] and stem cells [10]. Hence, analysis of telomere length and telomerase activity could be markers of aging in cell culture. Since *C. mydas* may not have a regular aging process such that is found in mammals, we hypothesize that telomere length and telomerase activity in dermal fibroblast cell culture at subculture 14 is higher than subculture 5.

2. Methods

2.1. Cell culture

Explants for cell culture were biopsied from a 2-year old *C. mydas* hindleg skin. Explants were washed in PBS and then cultured in Leibovitz-15 medium (Sigma, cat. number: L5520), supplemented with 100 U/mL (P/S) (Sigma, cat. number: P4333) and incubated at 30 ± 1 ℃ in incubator (BlueM). Medium was changed every three days and subculture was performed by using 0.2% EDTA and 0.02% trypsin (Sigma, cat. number: T4049).

2.2. Immunocytochemistry

Cells at passage 2 were plated on cover glass. After cells reached confluent, cells were fixed in methanol/PBS series. 0.05% PBST was used to increase cell membrane permeability. Cells were hybridized with Rabbit Anti-Vimentin Polyclonal Antibody (ab-45939, Abcam) with concentration 4:1000 in PBS. After that first hybridization, fixed cells were blocked with 0.3% BSA-PBS. Fixed cells were hybridized further with Goat Polyclonal Anti-Rabbit IgG-FITC (ab-6717, Abcam) 4:1000 and mounted in glycerol/PBS 1:9. Cells preparation was then analyzed under confocal microscope (Zeiss, LSM 710) with maximum intensity.

2.3. Telomere Length Assay

Cells at subculture 5 and 14 were harvested using 0.02% EDTA and 0.2% trypsin. DNA isolated from these two subcultures with High Pure PCR Template Preparation Kit (Roche). Telomere length analysis was performed with TeloTAGGG Telomere Length Assay (12209136001, Roche). As much as 1μg DNA was restricted with HinfI/RsaI, then separated in 0.8% agarose (Sigma) gel electrophoresis with 1x TAE Buffer for 4 hours at 50 V. DNA was transferred to nylon membrane (11417240001, Roche) with capillary transfer method. Membrane hybridized with telomere probe and *DIG Easy Hyb Granules* was then reacted with *Anti DIG-AP* and substrate solution. Membrane was then analyzed with chemiluminescence assay and transfered to X-Ray film (Fujifilm). Telomere length was measured with ImageJ software.

2.4. Telomerase Activity Assay

Telomerase activity assay was done with TeloTAGGG Telomerase PCR ElisaPlus (120113789001, Roche). Protein from cells at subculture 5 and 14 was extracted with Lysis Buffer and centrifuged at 16.000 x g for 20 minutes. Protein concentration was measured with Bradford method. Telomerase was then added with DNA base to biotin-labeled synthetic primer with Telomeric Repeat Amplification Protocol (TRAP Assay). Furthermore, amplification result was hybridized with hybridization buffer T and hybridization buffer IS microplate module for analysis based on spectrophotometry. The result was analyzed in ELISA

microplate reader (BioRad) at wavelength 450 nm and 590 nm.

3. Results and discussion

3.1. Cell Identification

Cells cultured from *C. mydas* skin showed fibroblastic morphology which has spindle shaped-like structure with long cytoplasmic protruding. Cell staining with vimentin antibody and analysis using confocal microscope showed positive result which ensured us the cell type of *C. mydas* skin cell culture is fibroblast (Figure 1).
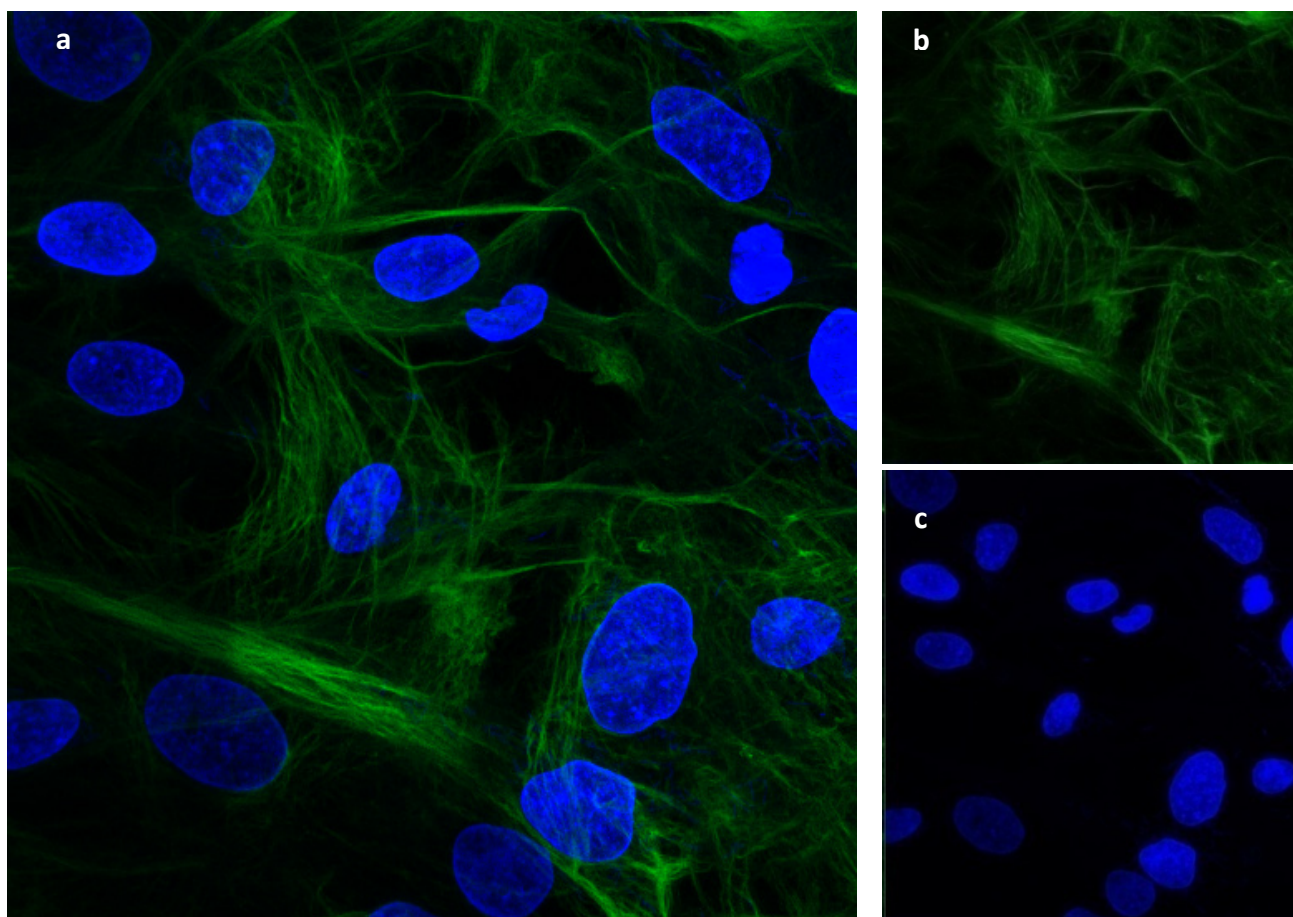

Figure 1 Immunocytochemistry analysis of (a) *C. mydas* skin cell culture (x: 134.69 μm, y: 134.69 μm). (b) Vimentin stained with rabbit anti-vimentin antibody and goat anti-rabbit IgG-FITC.(c) Nuclei stained with DAPI

Although the primary source of tissue culture, skin biopsies, is a mixture of various kinds of cells, skin cell cultures of *C. mydas* was eventually dominated by a particular cell. In terms of morphology, it can be concluded that the type of cell that is successfully cultured were fibroblasts. The FBS used in this research contained abundant growth factors including bFGF (basic fibroblast growth factor) that will support fibroblast growth [11].

Fibroblasts are interstitial cells which build connective tissue. Fibroblasts were derived from mesenchymal cells and produce extracellular matrix such as collagen I and III,

glycosaminoglycans, elastic fibers, and glycoproteins. Therefore, the role of fibroblast cells in the skin tissue is important if it is associated with the aging process. Reduced ability of skin fibroblasts to synthesize extracellular matrix collagen I and III will be associated with the appearance of wrinkles on the skin, which is the most particular symptom in the skin aging process [12]. Fibroblasts as a proponent of the connective tissue must be able to withstand mechanical stress. The ability of fibroblasts to migrate and have been known to withstand mechanical stress is determined by vimentin [13]. Vimentin is type III intermediate filament and is used as a marker of fibroblasts [14].

3.2.   Telomere Length Analysis

Telomere length in *C. mydas* is greater than telomere length in mammals [15]. Telomere length in human ranges from 10 to 15 kbp [16]. Marker used in this assay ranged from 0.8 to 21.2 kbp. However, Figure 2 showed that *C. mydas* telomere length is beyond 21.2 kbp. Therefore, measurement of telomere length was not accurate. However, the increase or decrease in telomere length can still be estimated.
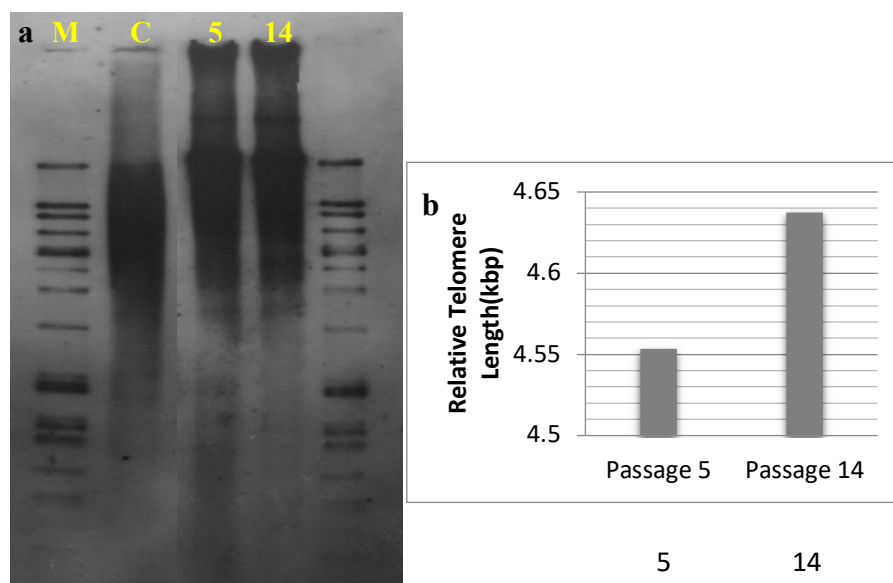

Figure 2 Telomere length in cultured skin fibroblasts of green turtles (a) The results of the analysis of telomeres in skin fibroblasts cultured from green turtle (M = marker, C = control DNA (human dermal telomere), DNA subculture 5 = 5, 14 = DNA subculture 14). (b) Comparative graph of telomere length on DNA subculture 5 and 14

Figure 2.b showed that telomere length at subculture 14 was greater than subculture 5. This result was different than what is commonly found in human. In human fibroblast cell culture, telomere length decreases as the subculture increases [17]. In human endothelial cells culture [18], telomere shortening can be observed between passage 4 and 13.

No-telomere shortening phenomenon is also found in *Emys orbicularis* [19], which shows that the length of telomere from embryo blood cells was no different with the adult organism's. In *Dermochelys coriacea,* another member of Cheloniidae family, a study also shows similar results in which telomere shortening does not occur in blood cell embryo or adult organism [20]. However, conflicting results were found in *Caretta caretta*, which comes from the same family with *Chelonia mydas*. Telomeres in blood cells and adults epidermis of *Caretta caretta* experience telomere shortening with age. It can be concluded that the relationship between age and telomere length could be tissue and species-specific.

3.3.   Telomerase activity analysis

Telomerase activity assay result in *C. mydas* aligns with the telomere length result, which is higher in subculture 14 than 5 (Figure 3). This phenomenon is also different if we compare the result to human. In human, telomerase activity cannot be found in adult tissues. Based on telomere length and telomerase activity, our *C. mydas* dermal fibroblast cell culture from subculture 5 to subculture 14 did not undergo senescence.

Previous research [15] showed that telomere elongation occured and increased telomerase activity in several organs of newly hatched, as compared to 7-month old, *C. mydas*. In this research, telomere length and telomerase activity in cells at subculture 14 was higher than in subculture 5 (Figure 2 and Figure 3). This consistent phenomenon occurred both in vivo and in vitro may conclude that *C. mydas* fibroblast skin cell culture can be used as a good material for further research on the aging process.

In all human organs, except reproductive organs such as testis, telomerase activity does not exist after birth [8], as well as in cultured condition [21]. Allegedly green turtles have a special mechanism that does not exist in human to increase telomere length and telomerase activity with age. The phenomenon of increased telomerase activity with age

occurs not only in *Chelonia mydas* but also in several other species of the order Testudinae, such as in *Chrysemis picta* and *Chelydra serpentine* [22]. *Chelydra serpentine* cell culture indicated the presence of telomerase activity in subculture 157 and continued to increase until subculture 191. Thus, in the future, green turtle can be developed as an animal model for the aging process in the field of biomedicine.

3.4. Morphology analysis

Morphology of the cell differs between subculture 5 and 14 (Figure 4). Cells at subculture 14 had enlarged nucleus, more vacuoles, and showed multinuclei structure. This result showed some phenotype of aging cells and also cells doubling time was longer as the subcultures get higher.
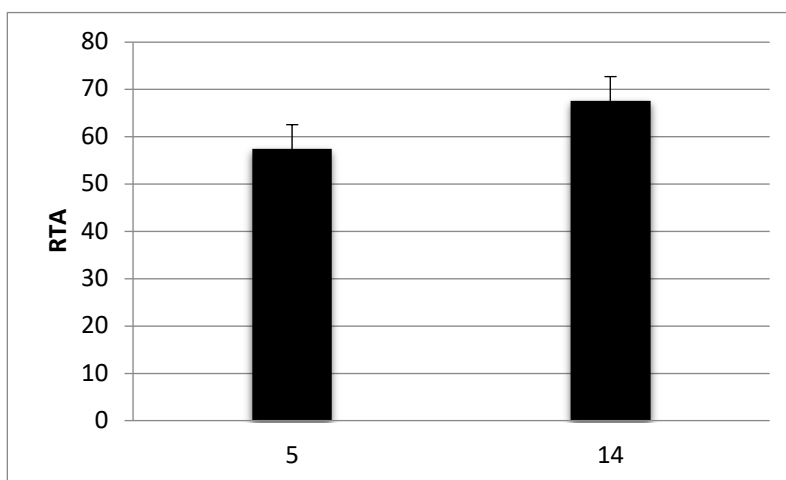

Figure 3 Comparative graph of telomerase activity in 5 and 14 subcultures. Relative telomerase activity (RTA) subculture 14 is higher than 5
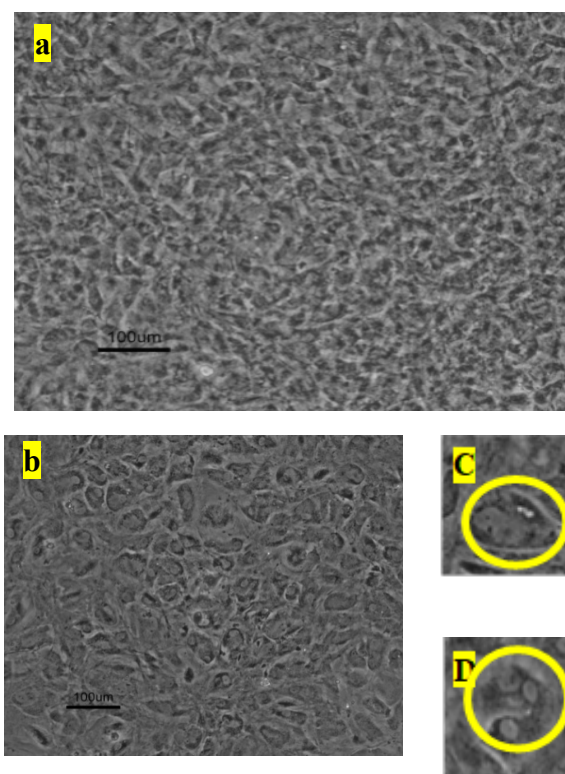

Figure 4 Cell morphology subculture 5 and 14. (a) *C. mydas* skin fibroblast cell culture subculture 5 (45x). (b) *C. mydas* skin fibroblast cell culture subculture 14 (45x). (c) Enlargement of vacuoles in the cell at 14. (d) Multinuclei cell at subculture 14

Based on morphology analysis, it was shown that *C. mydas* fibroblast skin cell culture underwent aging. Cells in subculture 14 are relatively larger than subculture 5 and also have epithelial-like morphology. According to Baba and Catoi [23], enlargement of cell size and changes of shape can be caused by two things, the cells had started to age or cells transformed into cancer cells. Characteristics of transformed normal cells into cancer cells is increasing the size of the nucleus, growing ratio of nucleus and cytoplasm, irregular cell shape, formation of multilayer cells, and increasing the rate of cell proliferation. Because cells in this research did not form multilayer nor increasing the rate of proliferation, therefore cell culture in this research presumably underwent aging. According to Phipps *et al.* [24], cells that undergo aging may increase in size, nucleus, nucleoli. Furthermore, cell phenotypes that were abundantly occurring in this cell culture and strengthening the hypothesis were multinuclei formation, increasing the number of vacuoles, as well as the increasing number of microfilaments and decreasing rate of proliferation. Characteristics that occurred in this research might be due to the increased amount of RNA and protein in cells. Protein and RNA degradation rate might also be decreased while transcription and translation activities still exist. Cells will be held in the G1 phase so that the cells size continues to increase without undergoing fission. This process may lead to the formation of the enlarged vacuoles and multinuclei. In conclusion, based on the morphology, it is observed that cells underwent aging. However, based on telomere length and telomerase activity analysis, aging did not occur because telomeres and telomerase activity increased as subcultures go higher. *C. mydas* dermal fibroblast cells aging process could be induced by stress through cell culture conditions, but not through the telomere shortening. Two main pathways that might occur in cultured dermal fibroblasts of green turtles which also occur in mammalian cells is through the p53 pathway and pRB [25].

p53 pathway can be activated through telomere shortening and increased ROS (reactive oxygen species). However, in this study, telomere shortening in *C. mydas* dermal fibroblast cell culture did not occur, thereby supposedly cells underwent aging through ROS-induced p53 pathway. ROS accumulate in cells due to incomplete redox reactions in the mitochondria (mitochondrial dysfunction) as well as environmental influences [26]. In addition, due to mitochondrial dysfunction, it is known that the amount of ROS in cells is also enhanced by the activity of Ras (oncogene) [27]. ROS causes DNA damage that triggers DNA damage response which induced p53 expression, which consequently render the cell cycle to be held in G1 phase.

Aging in cultured skin fibroblasts of green turtles can also occur through the pRB. pRB pathway is activated by the p16

cell cycle regulatory proteins. P16 protein is induced by stress stimuli experienced by *C. mydas* fibroblast skin cell culture as activated Ras by ROS and suboptimal culture conditions. pRB activation would lead to a reorganization of chromatin into heterochromatin. This means that proteins that play a role in inducing cell cycle sustainability such as G1/S cyclin and G1/S CDK (cyclin dependent kinase) could not be expressed [25]. Consequently, cells are arrested at G1 phase and cause enlargement of cell size as observed in cells at subculture 14.

4. Conclusions

In this study, we suggested that dermal fibroblast of green turtle experienced aging that is caused by stress from culture conditions that give rise to the increase of ROS in cells. Increasing amount of ROS in the cells of green turtle skin fibroblasts might induce p53 pathway and aging through the PRB. To prove the aging pathway experienced by dermal fibroblasts cell culture of green turtle, further study through the analysis of p53 and pRB gene expression is needed.

References

[1] Gibbons JW, Semlitsch RD. Survivorship and longevity of a long-lived vertebrate species: how long do turtles live?. The Journal of Animal Ecology. 1982 Jun 1:523-7.

[2] Hirth HF. Synopsis of the biological data on the green turtle Chelonia mydas (Linnaeus 1758). Fish and Wildlife Service, US Department of the Interior; 1997.

[3] Girondot M, Garcia J. Senescence and longevity in turtles: what telomeres tell us. In9th Extraordinary Meeting of the Societas Europaea Herpetologica. Université de Savoie, Le Bourget du Lac, France 1999 (pp. 133-137).

[4] Allsopp RC, Vaziri H, Patterson C, Goldstein S, Younglai EV, Futcher AB, Greider CW, Harley CB. Telomere length predicts replicative capacity of human fibroblasts. Proceedings of the National Academy of Sciences. 1992 Nov 1;89(21):10114-8.

[5] Hayflick L, Moorhead PS. The serial cultivation of human diploid cell strains. Experimental cell research. 1961 Dec 1;25(3):585-621.

[6] Rattan, S.I.S. 50 Aging of skin cells in culture. In Textbook of Aging Skin. Oleh M.A.Farage, K.W.Miller, H.I.Maibach. Springer-Verlag : Berlin. 2010

[7] Aubert G, Lansdorp PM. Telomeres and aging. Physiological reviews. 2008 Apr;88(2):557-79.

[8] Sorochynska K, Sych N, Duda A, Kulebyakina K, Krasnienkov D, Vaiserman A, Vatlitsov D. Dynamics of Telomere Length and Telomerase Activity in the Human Fetal Liver at 5–12 Weeks of Gestation. Stem cells international. 2018;2018.

[9] Shay JW, Wright WE. Telomeres and telomerase in normal and cancer stem cells. FEBS letters. 2010 Sep 10;584(17):3819-25.

[10] Marión RM, Blasco MA. Telomeres and telomerase in adult stem cells and pluripotent embryonic stem cells. InThe Cell Biology of Stem Cells 2010 (pp. 118-131). Springer, Boston, MA.

[11] Puck TT, Cieciura SJ, Fisher HW. Clonal growth in vitro of human cells with fibroblastic morphology: comparison of growth and genetic characteristics of single epithelioid and fibroblast-like cells from a variety of human organs. The Journal of experimental medicine. 1957 Jul 1;106(1):145-58.

[12] Varani J, Dame MK, Rittie L, Fligiel SE, Kang S, Fisher GJ, Voorhees JJ. Decreased collagen production in chronologically aged skin: roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. The American journal of pathology. 2006 Jun 1;168(6):1861-8.

[13] Strutz F, Okada H, Lo CW, Danoff T, Carone RL, Tomaszewski JE, Neilson EG. Identification and characterization of a fibroblast marker: FSP1. The Journal of cell biology. 1995 Jul 15;130(2):393-405.

[14] Chang HY, Chi JT, Dudoit S, Bondre C, van de Rijn M, Botstein D, Brown PO. Diversity, topographic differentiation, and positional memory in human fibroblasts. Proceedings of the National Academy of Sciences. 2002 Oct 1;99(20):12877-82.

[15] Nalasetya, R. Analisis panjang telomere dan aktivitas telomerase pada organ penyu hijau (*Chelonia mydas*). Skripsi S1 Sarjana Biologi ITB. Institut Teknologi Bandung. 2012

[16] Kong CM, Lee XW, Wang X. Telomere shortening in human diseases. The FEBS journal. 2013 Jul 1;280(14):3180-93.

[17] Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature. 1990 May;345(6274):458-60.

[18] Vasa M, Breitschopf K, Zeiher AM, Dimmeler S. Nitric oxide activates telomerase and delays endothelial cell senescence. Circulation research. 2000 Sep 29;87(7):540-2.

[19] Girondot M, Garcia J. Senescence and longevity in turtles: what telomeres tell us. In9th Extraordinary Meeting of the Societas Europaea Herpetologica. Université de Savoie, Le Bourget du Lac, France 1999 (pp. 133-137).

[20] Plot V, Criscuolo F, Zahn S, Georges JY. Telomeres, age and reproduction in a long-lived reptile. PloS one. 2012;7(7).

[21] Yokoyama Y, Takahashi Y, Shinohara A, Lian Z, Xiaoyun W, Niwa K, Tamaya T. Telomerase activity is found in the epithelial cells but not in the stromal cells in human endometrial cell culture. Molecular human reproduction. 1998 Oct 1;4(10):985-9.

[22] Christiansen JL, Johnson JC, Henderson ER, Budke BJ, Lynch M. The relationship between telomeres, telomerase, reptilian lifespan, and reptilian tissue regeneration. Proceedings of the Iowa Space Grant Consortium. 2001;2001:1-0.

[23] Baba AI, Cătoi C. Comparative oncology. Bucharest: Publishing House of the Romanian Academy; 2007.

[24] Phipps SM, Berletch JB, Andrews LG, Tollefsbol TO. Aging cell culture. InBiological Aging 2007 (pp. 9-19). Humana Press.

[25] Campisi J. Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. Cell. 2005 Feb 25;120(4):513-22.

[26] Kregel KC, Zhang HJ. An integrated view of oxidative stress in aging: basic mechanisms, functional effects, and pathological considerations. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology. 2007 Jan;292(1):R18-36.

[27] Lee AC, Fenster BE, Ito H, Takeda K, Bae NS, Hirai T, Yu ZX, Ferrans VJ, Howard BH, Finkel T. Ras proteins induce senescence by altering the intracellular levels of reactive oxygen species. Journal of Biological Chemistry. 1999 Mar 19;274(12):7936-40.