EFFECT OF CONSUMPTION OF BEE PRODUCTS ON TELOMERE LENGTH AND LONGEVITY OF LIFE IN BEEKEEPERS

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UNIVERSITI SAINS MALAYSIA

2015

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by

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Thesis submitted in fulfillment of requirements

for the degree of

Master of Science

October 2015

ACKNOWLEDGMENTS

First and foremost, I would like to praise Allah S.W.T for His uncounted blessings.

Special appreciation goes to my main supervisor, Assoc. Prof. Dr. T.P. Kannan for his enormous support, expertise and enthusiastic guidance during my study. I had learnt a lot from him, not only about the subject itself but also about life. For that, I am grateful to be supervised under him. He has my full respect, not only as my supervisor, but also as a mentor.

I would like to express my sincere gratitude to my co-supervisor, Assoc. Prof. Dr. Shaharum Shamsuddin for his support as well as for teaching me on how to do hybridization (Southern blot). His help during the trouble-shooting of Southern blot is also appreciated. I would also like to extend my gratitude to my other co-supervisors, Prof. Dr. Siti Amrah Sulaiman and Dr. Azlina Ahmad for their encouragement and effort during this study.

A special thanks to my dear husband, Dr. Che Muhammad Nur Hidayat Che Nawi for his full support and understanding during my study. He has always been with me through thick and thin. Once he told me this when I felt devastated after failing many times in my experiment, "Honey, do you know that Thomas Edison had failed thousands times before he successfully invented the light bulb? Instead of giving up, he said, "I haven't failed. I have just found 10,000 ways that won't work" and that is how I found my courage again.

I take this opportunity to thank my dearest daughter, Che Fatimah Az-zahrah Che Muhammad Nur Hidayat who has always been my strength, my little angel sent by God. She always accompanies me until late night whenever I had to finish writing my manuscripts and thesis and there are times when she would fall asleep on my lap. To mommy's little angel, I always love you.

I am also thankful to my family especially my mother, Mrs. Noriah Mohammed and my siblings who have always supported me during this study period. They helped me to take care of my daughter whenever I had to go home late due to lab work and always there to cheer me up when I lost my way.

I would like to thank my postgraduate friends, Hani, Wani, Siti, Izyan, Han, Yati, Ili, Ain, Dayat, Aizat and Marini for their kindness and moral support during this course of study. Our friendship and memories will always be cherished.

I am grateful to the staff of Human Genome Centre, INFORMM, Craniofacial Science Laboratory (School of Dental Sciences) and Molecular Biology Lab (School of Health Sciences) for their friendly environment and being helpful at times of need.

I sincerely thank the Academic Staff Training Scheme (ASTS) Universiti Sains Malaysia and Kementerian Pengajian Tinggi Malaysia for providing the scholarship to do my Master programme. Last but not least, I thank the USM Short Term Grant (304/PPSG/61312032) for funding this research.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
%	Percent
µl	Microlitre
µg	Microgram
A260/A280	Ratio of 260 absorbance over 280 absorbance
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
BRCT	BRCA1 C-terminus
BTBD12	BTBD12 domain contains protein 12
Buffer AL	Lysis buffer AL
Buffer AW1	Wash buffer 1
Buffer AW2	Wash buffer 2
Buffer TAE	Tris-acetate buffer
CAPE	Caffeic acid phenethyl ester
CAT	Catalase
Cm	Centimeter
cm^2	Centimeter square
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
ddH_2O	Deionized distilled water
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid

Exo1	Exonuclease 1
GAR1	GAR1 ribonucleoprotein
GPx	Glutathione reductase
GSH	Glutathione
G	Gram
H_2O	Water
HCl	Hydrochloric acid
hnRNPA1	Heterogenous nuclear ribonucleoprotein A1
HSCs	Hematopoietic stem cells
iNOS	Inducible nitric oxide synthase
Kbp	Kilo base pair
L_i	Length of TRF at position i
MEFs	Mouse embryonic fibroblasts
Ml	Millilitre
mJ	Millijoule
MreII	Double strand break repair protein MreII
Myb	Myeloblastosis
MW	Molecular weight
NaOH	Sodium hydroxide
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate oxidase
NHEJ	Non-homologous end joining
NHP2	NHP2 ribonucleoprotein
Nm	Nanometer
NOP10	NOP10 ribonucleoprotein

OB	Oligonucleotide/oligosaccharide-binding
OBFC1/Stn1	OB Fold-containing Protein 1/Stn 1
OD_i	Chemiluminescent signals
PARPI	Poly [ADP-ribose] polymerase I
POT1	Protection of telomere 1
RAP1	Repressor/activator protein 1
RCT	C-terminal domain
RNA	Ribonucleic acid
RNP	Ribonucleoprotein complex
ROS	Reactive oxygen species
RPA	Replication protein A
SANT	Swi 3, Ada 2, N-Cor and TFIIIB
SB	Southern blot
SSC	Saline-sodium citrate
SOD	Superoxide dismutase
TERT	Telomerase reverse transcriptase
TERC	Telomerase RNA component
TCAB1	Telomerase Cajal body protein 1
TRF1	Telomere repeat binding protein 1
TRF2	Telomere repeat binding protein 2
TIN2	TRF-1 interacting protein 2
TPP1	Telomere protection protein 1
TRFH	TRF homolog
TERRA	Telomeric repeat-containing RNA
TNF-α	Tumour necrosis factor alpha

TRFs	Terminal Restriction Fragments
U/µl	Unit per microliter
UV	Ultra violet
V/cm	Volt per centimeter
V	Volt
Wt/vol	Weight over volume

KESAN PENGAMBILAN PRODUK LEBAH TERHADAP PANJANG TELOMER DAN PENINGKATAN JANGKA HAYAT PENTERNAK LEBAH

ABSTRAK

Kepercayaan bahawa penternak lebah hidup lebih lama berbanding orang lain telah wujud sejak berkurun lamanya. Namun, tiada kajian telah dibuat bagi mendalami isu peningkatan jangka hayat penternak lebah. Kajian yang lepas menunjukkan telomer berkait dengan peningkatan jangka hayat. Justeru, kajian ini dibuat untuk menganalisa telomer 30 orang penternak lebah dan 30 orang bukan penternak lebah lelaki dan mengaitkan dengan peningkatan jangka hayat. Analisis Southern Terminal Restriction Fragment Length (TRFs) telah dibuat dengan mencernakan DNA dengan *Hinf*I/*Rsa*I dengan menggunakan kit TeloTAGGG Telomere Length Assay. Menariknya, kajian mendapati panjang telomer penternak lebah lelaki adalah lebih panjang berbanding bukan penternak lebah lelaki dengan nilai p kurang daripada 0.05, mencadangkan bahawa penternak lebah mungkin hidup lebih lama berbanding bukan penternak lebah. Kajian ini juga mendapati bahawa pengambilan produk lebah dalam jangka masa yang lama dan kekerapan pengambilan produk lebah untuk setiap hari berkait dengan panjang telomer. Satu peningkatan tahun dalam pengambilan produk lebah berkait dengan peningkatan panjang telomer sebanyak 0.258 kbp. Di samping itu, setiap peningkatan frekuensi dalam pengambilan produk lebah setiap hari berkait dengan peningkatan panjang telomer sebanyak 2.66 kbp. Hasil kajian ini mencadangkan bahawa produk lebah mungkin memainkan peranan dalam mengekalkan panjang telomer.

EFFECT OF CONSUMPTION OF BEE PRODUCTS ON TELOMERE LENGTH AND LONGEVITY OF LIFE IN BEEKEEPERS

ABSTRACT

The belief that beekeepers live longer than anyone else is present since ages and no research has been done to explore their longevity. Research has shown that telomere is associated with the longevity of life. Hence, this study aimed to investigate the telomere length in 30 male beekeepers and 30 male non-beekeepers and associate them with the longevity of life. Southern blot analysis of terminal restriction fragments (TRFs) was carried out by *Hinf*I/*Rsa*I digestion of human genomic DNA using *Telo*TAGGG Telomere Length Assay. Interestingly, the present study found that the telomere length of male beekeepers was significantly longer than those of male non-beekeepers with a *p*-value of less than 0.05, suggesting that beekeepers may have longer life compared to non-beekeepers. It was further found that the consumption of bee products for a long period and frequent consumption of bee products per day are associated with telomere length. A year increase in consuming bee products is associated with a mean increase in telomere length of 0.258 kbp. In addition, an increase in frequency of consuming bee products per day was also associated with a mean increase of 2.66 kbp in telomere length. These results suggest that bee products might play a role in telomere length maintenance.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

“There is nothing in the world that could beat honey as an aid to defy old age. Keep bees and eat honey if you want to live long. Beekeepers live longer than anybody else.”

-John Anderson

There are many examples in history which confirm the belief that beekeepers seem to live longer than anyone else. One of the examples was Anacreon, who died at the age of 115. He credited his long life to the daily use of honey. Other example includes Johann Dzierzon who was the Father of Modern Beekeeping, lived until he was 95 years old. Lorenzo Lorreine Langstroth, who was described as Father of American Beekeeping, died at the age of 85 years (Health, 2014). This observation is thought to be contributed by the great consumption and inhalation of honey by beekeepers.

Bees have been of human interest for more than 5000 years ago due to the benefits of honey (Association, 2005). Ancient Egypt for example, highly valued the honey and bees. The pharaoh had used the title of Bee King and the Gods were also associated with bees. In addition, bees were also chosen as a symbol for the country. They kept bees and honey in temples and named them as Mansion of Bee (Crane, 1999). These events suggest that the beekeeping activity has existed for a very long time. Interestingly, honey has been suggested as a significant food item in human evolution (Crittenden, 2011; Wrangham, 2011). Recently, it is thought that the ability

of human to climb trees mainly stems from the desire to collect honey (Kraft *et al.*, 2014). Honey is extremely high in energy (~3.0 kcal g-1) and nutritions (Bogdanov *et al.*, 2008). Besides that, it has many functional properties preferred by humans such as long preservation time (Nagai *et al.*, 2006), anti-microbial, antiviral, anti-parasitory, antioxidant effects and anti-inflammatory (Bogdanov *et al.*, 2008). Propolis and royal jelly, which are the other bee products are also widely known for such properties (Viuda-Martos *et al.*, 2008). Hence, it is unsurprising that bee products could play such a vital role in human evolution.

1.2 Problem statement

Although history has proven that beekeepers had lived longer than anyone else, there is dearth of research and information in exploring if this belief is only the “old wives tales” or vice versa? The quest for the ‘miracle’ to longevity of life has been longing by human race since long time ago. The desire for longevity of life can be seen from the market growth of anti-ageing products. According to Global Industry Analyst report, anti-ageing market is projected to be worth USD 291.5 billion by 2015 (WorldHealth.Net, 2009). They continued that consumers spending on anti-ageing products are also expected to reach $291.9 billion by 2015 (Mitteness, 2013). Thus, seeking an answer to this belief might be a good opportunity to probably solve some of the puzzles into longevity of life that might benefit human beings rather than leaving to be a mere belief.

Telomere length has been suggested to be a marker of biological ageing (Mather *et al.*, 2011). Telomeres are the tandem repeat sequence of TTAGGG (Blackburn, 1991; Lu *et al.*, 2013) and associated with telomere-associated proteins called shelterin (Lu *et al.*, 2013). Telomeres shorten with every cell division (Harley *et al.*, 1990). This is because the DNA replication machinery is unable to copy the ends of the linear molecules (Olovnikov, 1970). Shorter telomere length has been associated with ageing as well as human ageing associated diseases like cancer, cardiovascular diseases and obesity (Blackburn, 2010; Codd *et al.*, 2013). In simpler thought, shorter telomere length might indicate shorter life. In this connection, telomere length can be a good indicator of measuring the longevity of life biologically.

In addition, there is lack of research in exploring the association between bee products on telomere length as well. To date, people have studied the antioxidant capacity of honey on cells (Beretta *et al.*, 2007). The study demonstrated that honey may lower the risks and effects of acute and chronic free radical induced pathologies *in vivo* by reducing and lowering reactive oxygen species (ROS). The association between telomere length and other antioxidants such as β-carotene, vitamin C or E and omega 3 had been established (Shen *et al.*, 2009; Paul, 2011). However, there is lack of study on the association between telomere length and bee products. Thus, the focus of this research is to throw light on this problem and to provide the answer to this question.

1.3 Justification of the study

This research is aims to provide an insight into the longevity of life in beekeepers by measuring and comparing the mean terminal restriction fragment length (TRF) of telomere between beekeepers and non-beekeepers and associate with longevity of life. Besides that, we hope to shed some light on the factors that may influence the longevity of life in beekeepers. It is also hoped that this research would offer a base for further studies in identifying independent beekeeping related factors such as bee sting or using bee products as food causative agent for longevity of life and finally, lead to the utilisation of bee products as agents for longevity of life.

1.4 Objectives of the study

1.4.1 General objective

To study the association between telomere length and longevity of life in beekeepers.

1.4.2 Specific objectives

1. To determine the Terminal Restriction Fragment length of telomere among beekeepers and non-beekeepers.
2. To statistically evaluate the Terminal Restriction Fragment length of telomere between the above two groups.
3. To determine the association between consumption of bee products and telomere length variations.

1.5 Research hypothesis

Beekeeping and consumption of bee products influence telomere length variations.

CHAPTER TWO

LITERATURE REVIEW

2.1 Beekeeping

Beekeeping or apiculture is the maintenance and study of honey bee colonies, commonly in hives, by humans (Crane, 2009; Columbia, 2011). A beekeeper or apiarist keeps bees so that they could collect honey and other bee products like propolis, pollen, beeswax and royal jelly. Other purposes of beekeeping are to pollinate crops and to produce bees for sale to other beekeepers (Crane, 2009). Generally, each colony of bees is kept in a hive although some may build their nests in the open. Other type of beekeeping involves certain non-social bees that are reared to pollinate crops (Crane, 2009).

Nowadays, bees are kept in movable-frame hives. This is because the hives need not be destroyed in order to collect the honey. Another reason is because bee products are also in their specific level of frames. These reasons make the work of harvesting honey or other bee products to become more effective (Crane, 2009). During the harvesting of honey or other bee products, the beekeepers smoke the bees (Figure 2.1) to reduce the electroantennograph response of the guard bees, who otherwise would release a volatile alarm odour pheromone (Boch, 1962; Visscher *et al.*, 1995). When the smoke enters the hive, the antennae receptors of the guard bees are dulled and they fail to sound the alarm. When exposed to smoke, bees are dramatically less defensive and aggressive. As a result, the risk for engorgement and the tendency to sting is reduced (Visscher *et al.*, 1995).

Apis mellifera is a species of bees which has been used in most of the world's beekeeping. Other species include *Apis dorsata* and *Apis cerana*. Previously, bees were kept mainly to produce honey and beeswax. Nowadays, beekeeping has been tailored to different purposes like rearing queens or package bees for other beekeepers who produce honey. Some may provide bee colonies for crops pollinations and to produce royal jelly, pollen and bee venom since 1950s (Crane, 2009).

Figure 2.1: Smoking the hive to reduce the electroantennograph response of the guard bees (Adapted from http://www.sabah.gov.my/kpd/oldoldweb/Projek-LebahMadu.html).

2.2 Telomere and its regulation

2.2.1 Telomere

Telomeres are long repetitive DNA sequences located at the end of the linear chromosomes (Blackburn, 1991) and bound by shelterin proteins or telosomes (Palm and de Lange, 2008). Telosomes are the proteins which act as protection for the telomere loop structure. This protection prevents the chromosome ends uncapped, resemble a DNA break and activates DNA repair mechanism (Gomez *et al.*, 2012).

In mammalian cells, telomere comprises double-stranded tandem repeats of TTAGGG (Palm and de Lange, 2008). These repeat sequences do not encode for proteins (Hodes, 1999). However, it consists of G-rich hexanucleotide repeats which enable the single-stranded telomere G overhangs to form G-quadraplexes (Palm and de Lange, 2008; Lipps and Rhodes, 2009), where each G base serves as both donor and acceptor for hydrogen bond formation. In humans, telomeric G-quadraplex structure is thought to contribute in telomere protection, suppression of recombination and inhibition of telomerase-dependant telomere extension (Lipps and Rhodes, 2009).

It is thought that telomere adopts the T-loop structure, where the telomere end folds back on itself and the 3′ G strand overhang invades into the double-stranded DNA. This structure formation is called D-loop (Palm and de Lange, 2008). Besides that, it is believed that telomere structure can switch between a closed, protected state and an open, extendable state, which allows the DNA terminus to undergo replication. The protected state is necessary to safeguard the integrity of genomic material,

whereas the extendable state allows telomerase to extend short telomeres (Stewart *et al.*, 2012).

Telomeres protect the ends of linear chromosomes from breaking down and degradation and in avoiding recognition and processing as double-strand breaks (Kobryn and Chaconas, 2001). Studies carried out in yeast and other single organisms have shown that the functions of telomeres include protection from the chromosomal recombination, end-to-end fusion and recognition as damaged DNA, determination of chromosomal localization within the nucleus and to regulate the cell capacity for replication (Hodes, 1999).

Telomere length varies between chromosomes and between species. For instance, mice have longer telomere length as compared to human. The shortest telomere length is estimated to be 10 kbp. In human chromosomes, the telomere length is between 0.5 and 15 kbp. In addition, telomere length is also dependent on the type of tissue, age of the donor and the replicative history of the cells. For example, chromosome 17p has shorter telomere length as compared to other chromosome ends. Besides, it was observed that the average telomere length declines significantly with increasing age in human nucleated blood cells (Aubert and Lansdorp, 2008). Interestingly, rate of telomere attrition also varies markedly at different ages (Frenck *et al.,* 1998). An *in vitro* analysis of human fibroblast revealed that the telomere loss is 50-100 bp per cell division (Allsopp *et al.*, 1992).

2.2.2 Telomerase structure

Telomerase is a unique eukaryotic ribonucleoprotein (RNP) complex (Greider and Blackburn, 1985; Blackburn, 1992; Bryan and Cech, 1999), which aids in the stabilization of telomere length in human stem cells, reproductive cells (Wright *et al.*, 1996) and cancer cells (Kim *et al.*, 1995; Shay and Bacchetti, 1997) by adding TTAGGG repeats onto chromosomes ends. This addition is achieved using its intrinsic RNA as a template for reverse transcription (Feng *et al.*, 1995). There are two conserved components of telomerase which are essential in the addition of telomere repeat sequences. The first one is the core telomerase protein called telomerase reverse transcriptase (TERT) and telomerase RNA component (TERC) which complexes with TERT and provides the template for telomeric sequence synthesis (Greider and Blackburn, 1989; Feng *et al.*, 1995; Lingner *et al.*, 1997). It is thought that the human telomerase holoenzyme is assembled in the Cajal body, where TERT and TERC form a RNP enzyme complex (Podlevsky and Chen, 2012). While TERT and TERC are sufficient for the telomerase activity *in vitro*, other proteins are also required for its assembly, trafficking and regulation (Blackburn and Collins, 2011; Podlevsky and Chen, 2012).

Dyskerin is the most characterized mammalian telomerase accessory component (Mitchell *et al.*, 1999b). Dyskerin forms a core complex with three smaller proteins NHP2 ribonucleoprotein (NHP2), NOP10 ribonucleoprotein (NOP10) and GAR1 ribonucleoprotein (GAR1). Dyskerin binds to an H/ACA box RNA structural motif within TERC and to small nucleolar RNAs. This binding is essential for TERC stability and telomerase function *in vivo* (Mitchell *et al.*, 1999a; Mitchell *et al.*,

1999b; Chen *et al.*, 2000). Another protein called telomerase Cajal body protein 1 (TCAB1) binds to TERC and regulates its trafficking (Tycowski *et al.*, 2009; Venteicher *et al.*, 2009).

2.2.3 Interplay between telosome and telomerase in telomere maintenance

Telomere maintenance involves the interaction between telosome and telomerase. It is the interplay between both which helps to maintain and protect the telomeres. Any fault in either one would affect both the protection and maintenance of telomeres.

2.2.3.1 Telosome

The maintenance of telomere depends on the massive network of protein complexes at the telomere. In this regard, telosome is central to this process. Telosome is composed of six protein complexes which include telomeric repeat binding protein 1 and 2 (TRF 1 and TRF 2), the TRF-1 interacting protein 2 (TIN2), Repressor/activator protein 1 homolog (RAP1), protection of telomeres 1 (POT1) and telomere protection protein 1 (TPP1) (Liu *et al.*, 2004a; de Lange, 2005). TRF1 and TRF2 have similar domain structure consisting of a C-terminal SANT/Myb domain and an N-terminal TRF homology (TRFH) domain. These domains have high binding specificity for the half site 5´-ÝTAGGGTTR-3´ in telomeric double-stranded DNA (dsDNA) (De Lange, 2005). The two N-terminal oligonucleotide/oligosaccharide-binding (OB) folds of POT1 are highly specific for the 5´-TAGGGTTAG-3´ sequence of single-stranded G-overhangs (Lei *et al.*, 2004). TIN2 functions as a hub by binding to TPP1/POT1 heterodimer, TRF1 and TRF2

(Xin et al., 2008). TPP1 can also act together with POT1, TIN2 and telomerase (Wang *et al.*, 2007; Xin *et al.*, 2008). Mammalian RAP1 is targeted to telomeric DNA by directly interacting with TRF2. These six core proteins can act together as a platform that recruits players from various pathways to the telomeres for maintenance and protection (Lee *et al.*, 2011). The details on the functions of each telosome protein are described below.

2.2.3.1.1 Telomere repeat binding factor 1

TRF1 is the first double-stranded telomere DNA binding protein identified (Zhong *et al.*, 1992). It functions as a negative regulator for telomere length (Van Steensel and de Lange, 1997). Study showed that the homozygous deletion of TRF1 in mice was lethal to embryo during blastocyst stage with severe growth defects (Karlseder *et al.*, 2003). Apoptosis process was also accompanied these events suggesting that TRF1 plays vital roles that may be independent of telomere length regulation (Karlseder *et al.*, 2003). TRF1 expression is tightly regulated. As a consequence, it will lead to the telomere homeostasis (Zeng *et al.*, 2010).

2.2.3.1.2 Telomere repeat binding factor 2 and repressor/activator protein 1 homolog

TRF2 has appeared as an important player in maintaining the telomere length. It acts as negative regulator for telomere length and contributes to telomere protection (Smogorzewska *et al.*, 2000). It acts as a hub by recruiting various factors for telomere regulation. One of the ways of which TRF2 regulates telomere length is through Ataxia telangiectasia mutated (ATM) mediated non-homologous end joining (NHEJ) pathways. Study showed that TRF2-deficient mouse embryonic fibroblasts (MEFs) had severe proliferation defects caused by enormous end-to-end fusions facilitated by the NHEJ pathway (Celli and de Lange, 2005). Similar to TRF1, homozygous inactivation of TRF2 in mice was embryonic lethal and cannot be rescued by p53 abrogation. This means that different mechanisms are applied by TRF1 and TRF2 to ensure survival during embryonic development (Celli and de Lange, 2005).

The structure of RAP1 is highly conserved. It has a C-terminal (RCT) domain, a BRCA1 C Terminus (BRCT) domain and Myb domain(s). Since mammalian RAP1 lacks telomere-binding capacity, it interacts with TRF2 for telomere localization (Li *et al.*, 2000; Palm and de Lange, 2008). Studies suggested that RAP1 repressed homologous recombination (HR) at telomere. They found that TRF2/RAP1 complexes with DNA repair factor BTBD12 domain-containing protein 12 (BTBD12) and facilitates DNA damage response and Holliday junction processing. In addition, number of DNA repair proteins has been found in the RAP1/TRF2 complex such as Rad50, Mre11, Poly [ADP-ribose] polymerase 1 (PARP1), and

Ku86/Ku70. In contrast to TRF1 and TRF2, RAP1-deficient mice appeared viable, although with increased telomere recombination and fragility (Martinez *et al.*, 2010).

2.2.3.1.3 Protection of telomeres 1

There are three main functions of POT1. The first one is to protect telomere ends from ataxia telangiectasia and Rad3-related protein (ATR) dependent DNA damage response. Other functions include to regulate telomerase-dependent telomere elongation and controlling 5´-end resection at telomere termini (He *et al.*, 2006; Hockemeyer *et al.*, 2006; Wu *et al.*, 2006). Recently, Zou and team discovered that TERRA (telomeric repeat-containing RNA), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1), and POT1 could act together to remove replication protein A (RPA) from telomeric ssDNA after DNA replication. RPA exclusion is performed to support telomere end protection by inhibiting ATR-mediated DNA damage signals (Flynn *et al.*, 2011). Other than that, TPP1 can interact directly with POT1 to enhance POT1 affinity for telomeric ssDNA (Wang *et al.*, 2007; Xin *et al.*, 2007). Interestingly, TPP1 interacts directly with telomerase for its recruitment to telomeres (Wang *et al.*, 2007; Xin *et al.*, 2007).

2.2.3.1.4 Telomere protection protein 1

Human TPP1 interacts with both TIN2 and POT1 (Liu *et al.*, 2004b) by binding to the c-terminus of POT1. In addition, TPP1 is required for POT1 to localize telomere (Liu *et al.*, 2004b; Kibe *et al.*, 2010; Tejera *et al.*, 2010). TPP1 interacts directly with POT1 and enhances POT1 affinity for telomeric ssDNA (Wang *et al.*, 2007; Xin *et*

al., 2007). Besides, TPP1 is recruited to telomere through its interaction with telomerase (Wang *et al.*, 2007; Xin *et al.*, 2007). It has been shown that TPP1 null MEFs and mice had decreased telomerase binding to telomeres and short telomeres (Tejera *et al.*, 2010). It had been suggested that telomere length is regulated through the interaction between TPP1 OBFC1/Stn1, an OB-fold protein that directly binds to ss-telomeric DNA (Wan *et al.*, 2009). In support of this notion, recent study discovered that OBFC1/Stn1-containing CTC1, STN1 and TEN1 (CST) complex is involved in 5´-end resection for 3´-overhang generation. It was also found that the depletion of OB Fold-containing Protein 1/Stn1 (OBFC1/Stn1) leads to telomere elongation (Chen *et al.*, 2012; Wu *et al.*, 2012). Hence, these results showed that TPP1 is crucial in both telomere end protection and length regulation.

2.2.3.1.5 TRF1-interacting protein 2

TIN2 interacts directly with TRF1, TRF2, and TPP1 (Xin *et al.*, 2008) and acts as the central component in the telosome complex (O'Connor *et al.*, 2006). The disruption of TIN2 leads to accumulation of RPA binding to telomere termini, significantly decreased telomere localization of all telosome components, and increased ATR-mediated DNA damage responses, similar with the results in POT1a/1b double knockout mice (Takai *et al.*, 2011). Presently, the only identified mutation in telosome component in human diseases is TIN2. Patients with dyskeratosis congenita (DC) have dysfunction in TIN2-dependent telomere length control. It is also believed that TPP1-mediated telomerase recruitment might be interrupted. DC patients had been found to express TIN2 with missense mutations which might justify the

telomere shortening phenotype observed in patients (Yang *et al.*, 2011). Hence, TIN2 could be a possible target for therapeutic and diagnostic studies.

2.2.3.2 Telomere replication

Telomere replication involves multi-step processes (Figure 2.2). Firstly, the telomere duplex is replicated via the conventional replication form machinery. In lagging strand, telomere generated will gain a 3´ overhang automatically because RNA primer has been removed on the terminal Okazaki fragment. In contrast to lagging strand, overhang is not form on the telomere replicated by leading strand. Therefore, telomere is synthesised by a series of DNA processing reactions. In budding yeast, the processing steps are similar to those used to resect double-strand breaks during DNA repair (Longhese *et al.*, 2010). Initiation of resection requires recognition of the DNA terminus by the Mre11-Rad50-Xrs2 (MRX) complex (MRN in humans) and subsequent recruitment of the nucleases, exonuclease 1 (EXO1) and/or DNA replication helicase/nuclease 2 (DNA2). These act in accordance with the helicase Sgs1 to cleave the DNA 5´ strand thus creating the 3´ overhang. Although it is unclear whether EXO1 and DNA2 play a similar role in human telomeres, genetic analysis in mice has shown that another repair nuclease, Apollo/Snm1b is involved in the overhang generation on leading strand telomeres (Chen *et al.*, 2008; Lam *et al.*, 2010). Apollo can associate specifically with telomeres through an interaction with TRF2 (Chen *et al.*, 2008). DNA-processing to generate G-overhangs occurs regardless of whether a cell expresses telomerase (Hemann and Greider, 1999). In telomerase positive cells, the overhang is elongated by the addition of new repeats on to the DNA terminus. Although the recruitment of telomerase to human telomere is

not fully understood, it is thought that the recruitment involves the trafficking of telomerase to the telomere in association with Cajal bodies and interaction between telomerase and TPP1 (Cristofari *et al.*, 2007; Venteicher *et al.*, 2009; Abreu *et al.*, 2010; Tejera *et al.*, 2010). After this event, the complementary C-strand is filled-in to leave an overhang that ranges in length from ~40 to 400 nt (Huffman *et al.*, 2000; Zhao *et al.*, 2009). Finally, the telomeres are rebound by telosome/shelterin and the t-loop reforms.

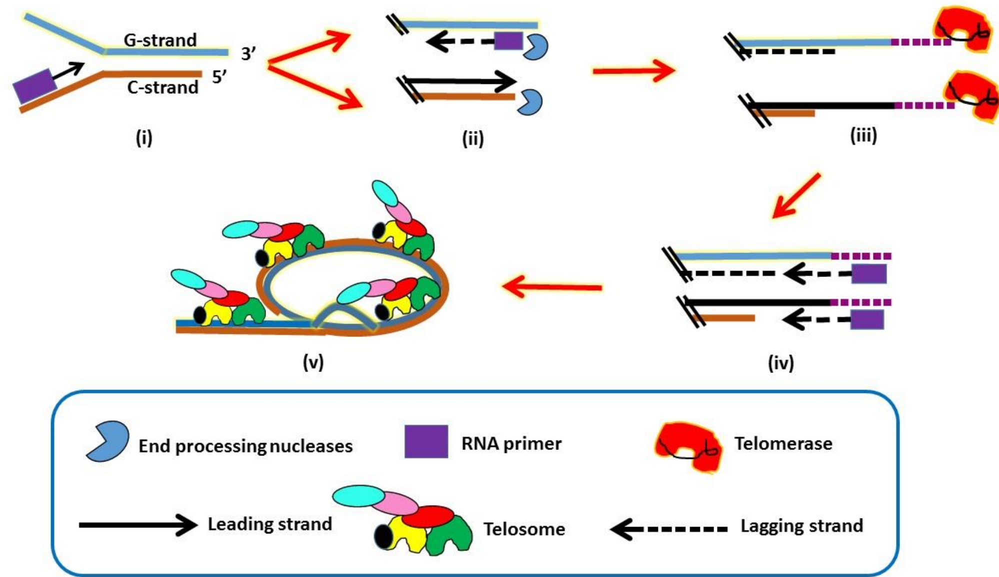

Figure 2.2: Telomere replication. (i) Telomere duplex is replicated via conventional replication form machinery. (ii) Nucleases cleave the C-strand to generate G-overhang (iii) Telomerase elongated the G-strand and creating short G-overhang. (iv) Shelterin rebound to telomeres and t-loop reforms

2.2.3.3 Telomere elongation by telomerase is tightly regulated

Telomere elongation by telomerase is tightly regulated. Telomerase elongates telomeric DNA during S phase and into M phase. This means that the elongation is cell-cycle-regulated (Diede and Gottschling, 1999; Marcand *et al.*, 2000). Telomerase favourably elongates the shortest telomeres. This is in accordance to cis-regulatory mechanisms mediated through the telomere DNA–protein complex. As a result, only a subset of telomeres may be elongated in any cell cycle (Teixeira *et al.*, 2004). Telomere elongation extent is very sensitive to the level of telomerase in cells. This event is obvious in the study of the haploinsufficiency for genes encoding telomerase components in yeast, mouse and human cells (Vulliamy *et al.*, 2001; Erdmann *et al.*, 2004; Armanios *et al.*, 2005; Hao *et al.*, 2005; Yamaguchi *et al.*, 2005; Mozdy and Cech, 2006; Strong *et al.*, 2011)

The reason behind this is probably due to imbalance stoichiometry between telomerase and its substrates, in addition to other telomerase independent processes. Although haematopoietic stem cells (HSCs) are naturally enriched with telomerase, the effect of multiple cell division and ageing can be seen on their telomere length (Vaziri *et al.*, 1994; Chiu *et al.*, 1996). Interestingly, telomere length in human male germ cells remains stable or even elongate with age (Allsopp *et al.*, 1992). Even so, the mechanisms by which telomeres are maintained in germ cell lineages, which are enriched for telomerase (Kim *et al.*, 1994), are not fully understood.

Short telomeres have been suggested as having protective role as an innate tumour suppressive mechanism in long-lived, multicellular organisms (Greider, 2006). The reason behind this is thought to stem from the observation that in most cancer cells, telomerase is upregulated to maintain their sustainability (Kim *et al.*, 1994). Besides that, limiting telomerase levels may also prevent unwanted telomere addition at DNA double-strand-break sites, which could happen if telomerase competes with appropriate DNA repair mechanisms (Zhou *et al.*, 2000; Makovets and Blackburn, 2009).

2.3 Telomeres and ageing

2.3.1 Replicative ageing

While it is true that telomere shortening plays a protective role against cancer cells, it appears that this decision has resulted in ageing consequence. Ageing is defined as a process associated with the gradual decline in the performance of organ systems. This decline has resulted in the loss of reserve capacity which in turn leads to an increased chance of death (Gompertz, 1825). In some organ systems, this loss of reserve capacity with increasing age can be attributed to the loss of cell function (Martin *et al.*, 1970).

The process by which most normal human cells "count" the number of times they have divided and eventually undergoing a growth arrest, cellular senescence is defined as replicative ageing. This process is dependent on telomere shortening (Wright and Shay, 2005). The first observation that suggests the existence of internal

counting mechanism within the cell came from Hayflick. Hayflick observed that cultured human fibroblasts have limited number of cell divisions (Hayflick and Moorhead, 1961). The subsequent study then revealed that the telomeres shorten with every cell division, suggesting that telomere loss is the molecular clock that drives ageing (Harley *et al.*, 1990; Hastie *et al.*, 1990; Harley, 1991; Allsopp *et al.*, 1992).

To understand the reason for this limitation, it is best to appreciate the disposable soma theory (Kirkwood, 1998). The disposable soma theory proposes that the rate at which the species age is the balance between the energy devoted to reproduction versus somatic repair. This means that if too much energy is invested in the repair of somatic cells, less energy is left for reproduction and vice versa. Species that are unable to survive very long due to the high mortality rate must invest most of their energy in reproduction rather than cell repair.

For example, a mouse that sufficiently repaired itself for 20 years is making bad investment since most mice will be eaten by its predators within 3 months. Therefore, it is better for the mice to invest more energy in the early reproduction and less in maintenance and repair (Wright and Shay, 2005). As humans have longer average survival, we have been evolutionarily selected to invest more energy on tissue maintenance and repair as compared to reproduction unlike mice. However, the variety of tissue maintenance and repair processes such as the efficiency of DNA repair, protection against oxidative damage and others limit the amount of energy invested and contribute to ageing (Wright and Shay, 2005).

Apoptosis of damaged cells and replacing them with new ones are efficient ways of keeping cells healthy. Besides that, replacing dying cells with new healthy cells can dilute the build-up of ‘unrepairable and indigestible’ products that can contribute to ageing. Nonetheless, using cell turnover to repair tissues may carry risk since mistakes can occur during DNA replication. These mistakes can lead to harmful mutations which will then lead to cancer (Vogelstein and Kinzler, 1993). Therefore, by limiting the total number of times a cell could divide provides a powerful barrier for the body from cancer formation (Wright and Shay, 1995).

There has been mounting evidences that the progressive loss of telomeric ends of chromosomes is an important intrinsic timing mechanism in the ageing process, both in cell culture and *in vivo* (Harley *et al.*, 1990; Hastie *et al.*, 1990). Based on the analysis of cultured human fibroblasts and lymphocytes, the rate of loss of telomeres is 50-100 bp per cell division (Allsopp *et al.*, 1992). Short telomeres can induce anti-proliferative signals that result in cellular senescence (Harley, 1991; Shay, 1995; Zou *et al.*, 2004). These events are discussed in detail below.

2.3.2 Replicative senescence

Telomere shortening can induce anti-proliferative signals which result in cellular senescence (Harley, 1991; Shay, 1995; Zou *et al.*, 2004). Cellular senescence triggered by telomere shortening is termed replicative senescence. Replicative senescence is caused by the ‘uncapping’ of critically shortened telomeres. This happens when telomere-binding proteins are no longer protecting telomeres, making telomeres recognized as single and lead to the breaking of the double-strand DNA.

As a result, DNA damage response pathway is activated like p53 pathway (Vaziri and Benchimol, 1996; Takai *et al.*, 2003) which will then lead to the growth arrest of cells, apoptosis and senescence (Chin *et al.*, 1999; Wong *et al.*, 2003; Ferrón *et al.*, 2004; Flores and Blasco, 2009).

Interestingly, these senescent cells can remain viable for years (Shay and Wright, 2007). The accumulation of senescent cells is recognized as one of the two mechanisms which probably contribute to ageing. The production of different constellation of proteins as compared to those that are non-senescent but quiescent adjacent cell during the accumulation of senescent cells is believed to change the homeostasis of that tissue and lead to ageing (Shay and Wright, 2007). Studies reported abundant senescent cells in telomerase null mice (Satyanarayana *et al.*, 2003). The senescent cells are usually marked using beta galactosidase staining and these cells are always associated with changes in p53, p16 and p21 expression (Dimri *et al.*, 1995; Shelton *et al.*, 1999; Oeseburg *et al.*, 2009). The accumulation of senescent cells may also lead to another mechanism of ageing which is the loss of stem cell function (Collado *et al.*, 2007). Stem cells are important because they maintain the homeostasis of tissues by replenishing senescent and apoptotic cells. Besides that, they repair damage that occurs throughout life (Rando, 2007). Various studies reported the loss of stem cell function through telomere shortening in a variety of tissues and experimental systems (Flores *et al.*, 2006). The loss of stem cell functions impair tissue repair and hence weaken the tissue functions and lead to ageing (Collado *et al.*, 2007).

2.4 Telomeres and longevity of life

It has been suggested that long telomeres may provide protection against cellular senescence (Herbig *et al.*, 2006). They could also be an indicator for unique genome stability and cellular health (Epel *et al.*, 2004). During the last 20 years, there are rising evidences suggesting that telomere attrition may function as a key timing mechanism during the ageing process in various species (López-Otín *et al.*, 2013). Shorter telomere length in humans is associated with many age related diseases such as cardiovascular diseases, cancer, cognitive decline, diabetes and overall mortality (Armanios, 2013). Interestingly, gender has also played its role in longevity of life (Barrett and Richardson, 2011). Women are thought to live longer than men because of oestrogen. Oestrogen has been shown to be associated with telomere length (Vina et al., 2005).

Telomere attrition is negatively correlated with organismal life span (Haussmann *et al.*, 2003). Telomere length appeared to increase across its life span in long-lived seabird, *Oceanodromo leucorhoa* (Haussmann et al., 2007). It has also found that this species show little or no accumulation of short telomeres over time (Haussmann and Mauck, 2008). Thus, the study on this species might offer the secret to longevity and reproductive success.

The link between telomere length and human lifespan has been reported (Gomes *et al.*, 2011; Barrett *et al.*, 2013). Study in the elderly aged more than 60 years showed that telomere attrition is significantly associated with higher mortality rates, both from infectious and cardiovascular diseases (Cawthon *et al.*, 2003). Moreover,