CHAPTER I

INTRODUCTION

1.1 STATEMENT AND SIGNIFICANCE OF THE PROBLEM

Most cancers are currently increasing in incidence and it remains a huge treat to public health for many more years. Even with the modern advancements in diagnosis, prevention and therapy, cancer still affects millions of patients worldwide and is one of the leading causes of death. Advances in treatment with surgery, radiotherapy and chemotherapy have had a limited impact on mortality. The majority of human cancers are difficult to treat, especially in their advanced, metastatic forms. There is thus a pressing need for new and effective forms of systemic therapy. The discovery of novel, mechanism-based agents directed against the molecular pathology of cancer offers a huge potential. The evidence that most cancer cells activate telomerase, whereas normal cells are usually devoid of telomerase activity, has led to extensive investigations to detect this protein and its activity for a potential use in cancer diagnosis and prognosis, and to eventually monitor the tumor response to therapy.

Telomerase is a ribonucleoprotein enzyme complex that is responsible for adding the telomeric repeats onto the telomeric ends of chromosomes. It has two major components, human telomerase reverse transcriptase (hTERT) catalytic subunit and a functional or template RNA, the human telomerase RNA (hTR, also known as TERC). Telomerase activity has been found in 80-90% of human tumors but not in

adjacent normal cell. Tumor cells generally have short telomere lengths and show no net loss of average telomere length with successive cell divisions, suggesting that telomere stability might be required for cells to escape from replicative senescence and proliferate indefinitely. Most malignant tumors might need to become immorta to sustain their growth, and telomerase activity could therefore be a rate-limiting step required for the continuing proliferation of advanced cancers. Therefore, telomerase is a target not only for cancer diagnosis but also for study and development for novel therapeutic agents.

Herbs have been an indispensable source of natural products for medicine. Recently, considerable attention has been focused on identifying naturally occurring chemopreventive substances capable of inhibiting, retarding, or reversing the multi-stage carcinogenesis. A wide array of phenolic substances, particularly those present in dietary and medicinal plants, have been reported to possess substantial anti-cancer and anti-mutagenic activities. The majority of these naturally occurring phenolics retain antioxidative and anti-inflammatory properties which appear to contribute to their chemopreventive or chemoprotective activity.

Ginger, the rhizome of *Zingiber officinale Roscoe*, one of the most widely used species of the ginger family. Ginger has long been used in traditional oriental medicine as a cure for some diseases including inflammatory diseases. Ginger contains active phenolic compounds such as gingerol, paradol and shogoal. These phenolic substances have been found to possess many interesting pharmacological and physiological activities such as antioxidant, anti-inflammatory, anti-atherosclerotic, anti-angiogenesis, anti-cancer and anti-mutagenic activities.

The effects of ginger and its components as chemotherapeutic agents have been reported in a wide range of experimental models.

Recently, our group has reported that the ethyl acetate fraction of ginger extract inhibits the expression of two prominent molecular targets of cancer, the human telomerase reverse transcriptase (hTERT) and c-Myc, in A549 lung cancer cells. However, we have not yet identified the active compound that is responsible for these activities. The finding of pure active compound that down-regulates *hTERT* and *c-Myc* expression from ginger extract would be crucial for the use of ginger in cancer treatment.

1.2 LITERATURE REVIEWS

1.2.1 Telomere

The extremities of eukaryotic chromosomes are composed of specialized nucleoprotein complexes termed telomeres (Figure 1.1). Human telomeres consist of a variable number of tandem repeats of the T_2AG_3 sequence, together with a group of specific proteins, and are therefore of variable length. At the 3′ end, the G rich strand of the telomere forms a single stranded extension. Recent evidence in vitro suggests that the telomere repeated sequence folds back on itself to form a duplex loop structure termed T-loop [1]. Both telomeric DNA and telomere-associated proteins have an essential role in stabilizing chromosome ends by forming a cap structure that protects chromosome ends from exonucleolytic degradation and terminal fusions. Some telomere-associated proteins bind directly to the T_2AG_3 DNA repeats, whereas others are associated with the telomere via protein–protein interactions (Figure 1.2).

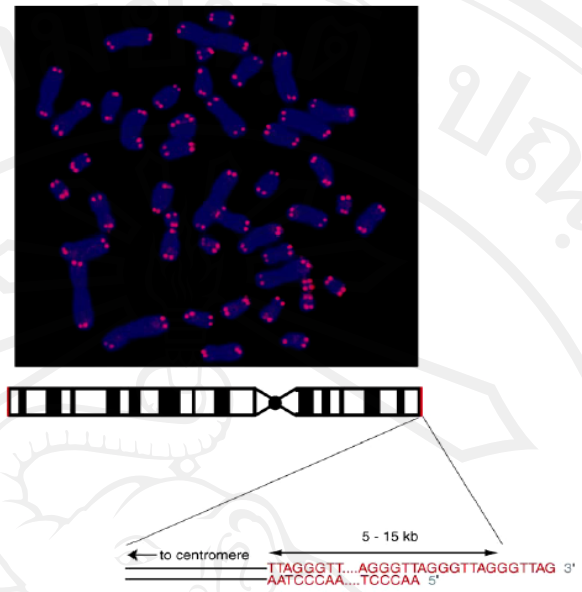

Figure 1.1 Telomere sequence and structure in humans [2]**.**

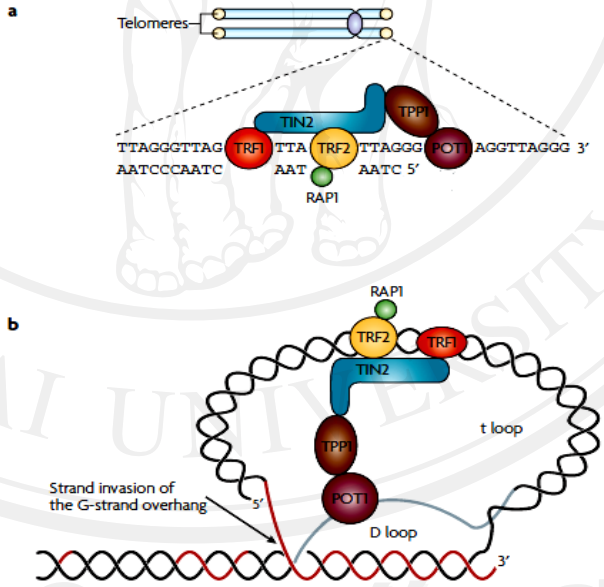

Figure 1.2 The structure of human telomeres. (*a*) Human chromosomes end in an array of TTAGGG repeats that varies in length. (*b*) Schematic of the t-loop and d-loop structure [3].

1.2.2 Telomeric Proteins

Telomeric DNA is complexed with the six telomere-specific proteins, so-called “shelterin complex”, composed of telomeric-repeat binding factor 1 (TRF1), telomeric-repeat binding factor 2 (TRF2), protection of telomeres 1(POT1), TINF2-interacting protein (TPP1), TRF1-interacting nuclear factor 2 (TIN2), and the repressor/activator protein 1 (RAP1) [3], providing a cap over the ends of the chromosomes that protects chromosome termini from degradation, recombination and end-joining reactions. The roles of telomerase-/telomere-associated proteins and their interactions are summarized in Table 1.1.

1.2.3 Human Telomeric DNA

Measurements of average human telomere (TTAGGG)n tract lengths in normal cells have ranged from greater than 20 kb in germline tissues to approximately 2 kb in senescing cells [4]. In a recent study, human germline terminal tract lengths displayed a wide individual specific variation range (from less than 9 kb to greater than 17 kb), consistent with genetic differences in the factors that determine telomere length settings in individuals [5]. In addition to interindividual variation of bulk average telomere lengths, the lengths of (TTAGGG)n tracts vary from telomere to telomere within individual cells [6-8] and between alleles at the same telomere [9, 10]. These individual specific patterns of relative telomere-specific (TTAGGG)n tract lengths are regulated in part by cis-acting factors [11-13], and these patterns appear to be defined in the zygote and maintained throughout life [14].

Table 1.1 Telomerase-/telomere-associated proteins and their interaction [2]**.**

Factors	Name	Function at telomere	Interactions with
Telomere specific protein			
Telomerase catalytic core	hTR	RNA subunit	
	hTERT	Reverse transcriptase subunit	
Telomerase accessory factors	EST1A, EST1B	Stabilizing the complex	Telomerase
G-tail binding factors	POT1	Binds T2AG3 using OB-fold	TRF1, TRF2, PTOP, TIN2, Tankyrase 1
Duplex T2AG3 binding factors	TRF1	Binds telomeres, negative length regulator	POT1, TRF2, TIN2, PINX1, TANK1/2, Ku, BLM, ATM
	TRF2	Binds telomeres, negative length regulator, role in T-loop, chromosome stability	POT1, TRF1, hRAP1, PARP2, TIN2, MRN, ERCC1/XPF, WRN, BLM, Ku, ATM
Proteins indirectly binding telomere	hRAP1	Length regulator	TRF2, MRN
	TANK1/2	PARP activity, TRF1 ribosylation, positive length regulator	TRF1
	TIN2	Positive length regulator	TRF1, TRF2
	PINX1	Telomerase inhibitor	TRF1, TIN2
Others			
DNA repair proteins	Ku70/ Ku80 DNAPKcs Mre11/Rad50/Nbs1 ERCC1/XPF	NHEJ, telomere localisation, negative length regulator, telomere capping, Recombination, NER	Telomerase, TRF1, TRF2
Helicases	WRN and BLM	Recombination, NHEJ	TRF1, TRF2
Checkpoint proteins	ATM	DNA damage signaling	TRF1, TRF2

Cis-acting regulators of allele-specific (TTAGGG)n tract length would be expected to depend upon subtelomeric sequences immediately adjacent to terminal (TTAGGG)n tracts. These sequences have been determined for the reference sequences of some chromosome arms [15], and a model of human terminal and subterminal DNA based upon these completed reference sequences is shown in Figure 1.3. Most of the known human (TTAGGG)n-adjacent sequences are related in some fashion to the telomere-associated repeat (TAR) sequences originally described by Brown and coworkers [16, 17]. Parts of the canonical TAR1 sequence are present within 2 kb of the beginning of nearly all sequenced (TTAGGG)n-adjacent DNA, although the part and degree of similarity can vary substantially. TAR1 similarity can also be found adjacent to many of the internal (TTAGGG)n like sequences in subtelomeric repeat (Srpt) regions, and more distantly related copies of TAR1-like sequence are often found in pericentromere regions [18].

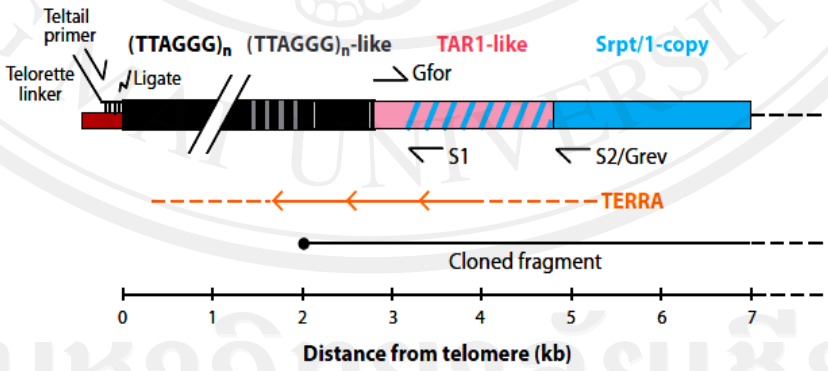

Figure 1.3 Model of terminal and subterminal human DNA [19].

1.2.4 Telomere Shortening and Cellular Senescence [20]

The linear chromosomes created a significant challenge for DNA replication. The problem, referred to as the end replication problem (Figure 1.4), originates from the use of short RNAs to prime DNA synthesis. Replication begins with the separation of the double-stranded molecule so that the replication of each strand is done individually. As the two strands are separated, new bases must be added in the 5′ to 3′ direction. That task is straightforward on the leading strand, whose template is of the opposite polarity, and the bases are added in serial fashion. On the opposing lagging strand, replication must be done in segments, called Okazaki fragments, in order to accomplish 5′ to 3′ addition of bases. A new RNA primer is synthesized and used to initiate the synthesis of each fragment and eventually the fragments are ligated together to create a continuous strand. A problem occurs when the lagging strand nears the end of the chromosome. There is no DNA beyond the end to serve as a template for the next Okazaki fragment to fill in the gap between the last Okazaki fragment and the end of the chromosome. Thus, the extreme end of the chromosome is not replicated and the telomeres progressively shorten.

Fortunately, this problem does not result in the loss of essential genes in that each of the 46 human chromosomes is capped with long repeats of expendable non-coding DNA bases called telomeres (Figure 1.1). Loss of the telomeric DNA continues with successive divisions until the telomeres reach such a critically short length that replication is halted. Human cells are estimated to have the potential

to undergo on average 60–70 divisions, and at this point the cells growth arrest and enter senescence.

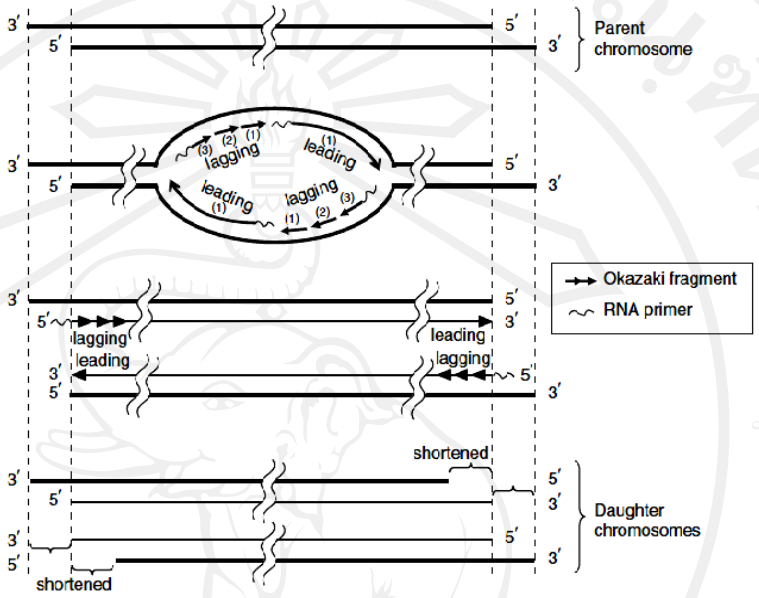

Figure 1.4 Renewed ‘‘end-replication problem” [21]**.**

The shortening of telomeres is responsible for the counting mechanism that Hayflick observed in normal cells in tissue culture in 1961. He found that normal human fibroblasts predictably entered a period where they ceased replication but continued metabolism [22]. The telomere hypothesis is the idea that progressive telomere shortening is a biologic or mitotic clock of the cell, keeping track of the number of replications a cell has used and indicating the time for permanent growth arrest when some of the telomeres are sufficiently short. Later, when telomeres become sufficiently short, cell enters an irreversible growth arrest called “cellular senescence”, or mortality stage 1 (M1). This stage can be bypassed in *vitro* by abrogation of the function of *p53* and *pRB* human tumor suppressor gene. Cells that escape replicative senescence by inactivation of a critical cell cycle

checkpoint gene continue to divide and suffer further telomere loss until they reach a second proliferative block, or mortality stage 2 (M2). The net growth arrest in the M2 is caused by a balance between the cell proliferation and cell date rate. At this stage, when most of the telomeres are extremely short, end-to-end fusion and chromosome breakage-fusion cycles cause chromosomal abnormalities and apoptosis (Figure 1.5).

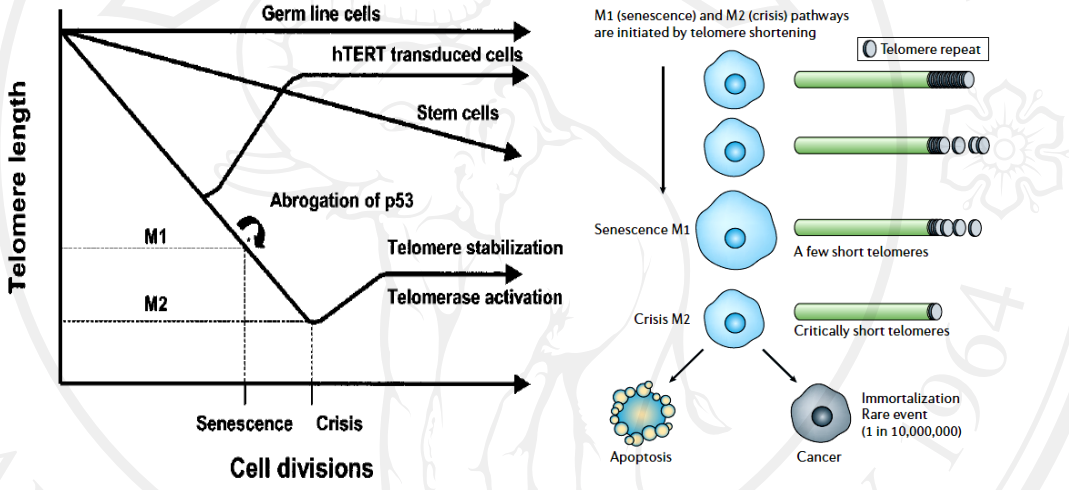

Figure 1.5 The two-stage M1/M2 model of senescence [23,24].

At the cellular level, senescence is generally believed to be a biological mechanism operating to suppress oncogenic conversion of normal cells. Consistent with the two-step hypothesis for senescence and immortalization [25], it has been experimentally shown that senescence is not only a potent defense against cancer [26-31], but that cancer cells that have escaped senescence can be reversed to senescence by restoration of the tumor suppressor *p53* [32,33]. This almost always occurs through the activation of the enzyme telomerase. It is thus widely believed that senescence and immortalization share some common biology, and telomeres and telomerase may be the connection [34].

1.2.5 Telomerase

Telomerase is a unique reverse transcriptase composed of two major components: the telomerase reverse transcriptase (TERT) catalytic subunit and the integral telomerase RNA (TR) component. In human, they are called hTERT and hTR [35-37], respectively (Figure 1.6). The hTR is a member of small nucleolar RNA molecules termed box H/ACA RNAs [38]. The role of the major protein subunit, hTERT, is to catalyze the polymerization of nucleotide. The subunits associates to form a complex tetramer compose of two RNA subunits and two catalytic subunits [39-41] and are sufficient for catalytic activity both in vitro and in vivo [42, 43]. In addition to these core components other proteins that are dispensable for catalytic activity associate with telomerase, including TP1, hsp23 and hsp90 [44-47], hStau, L22 [48] and dyskerin [49]. The heat-shock protein 90 (hsp90) and the chaperonin, *p23* are involved in preserving the folding states of proteins and, consequently, preserving the physiological activities of many enzymes. As both proteins have been shown to bind telomerase directly, it is thought that they enhance telomerase function by facilitating the assembly of the active holoenzyme. Consistent with this role, an inhibitor of hsp90, geldanamycin, has been shown to reduce telomerase activity. More recently, homologs of the yeast Est1p protein, that recruits and activates telomerase at the 3′ end of telomeres, have been identified in human. EST1A and EST1B associate with telomeres and bind telomerase in vitro and overproduction of EST1A affects telomere length and capping.

The processive elongation of telomere by telomerase requires a number of steps, as illustrated in Figure 1.7 First, telomeric DNA terminus is recognized by the telomerase ribonucleoprotein (RNP), which is positioned on an alignment site in TERC such that the 3'-end of the telomere is adjacent to the short (often 6 nt) template sequence. The 3'-end of the DNA forms a hybrid with the RNA template, whereas the more 5' region of the DNA is postulated to interact with the "template-proximal" and "template-distal" anchor site. Next, telomerase reverse transcriptase (hTERT) catalyzes the addition of nucleotides base sequence TTAGGG repeatedly in the 5'-to-3' strand of telomeres. Telomerase moves along the DNA, elongating the telomere until the 5'-end of the template is reached. Telomerase then undergoes the translocation reaction and repositions the 3' end of the DNA in concert with recognition of the 3' template boundary, and another round of nucleotide addition is initiated. Reiterative translocation and nucleotide addition result in the addition of multiple repeats [50].

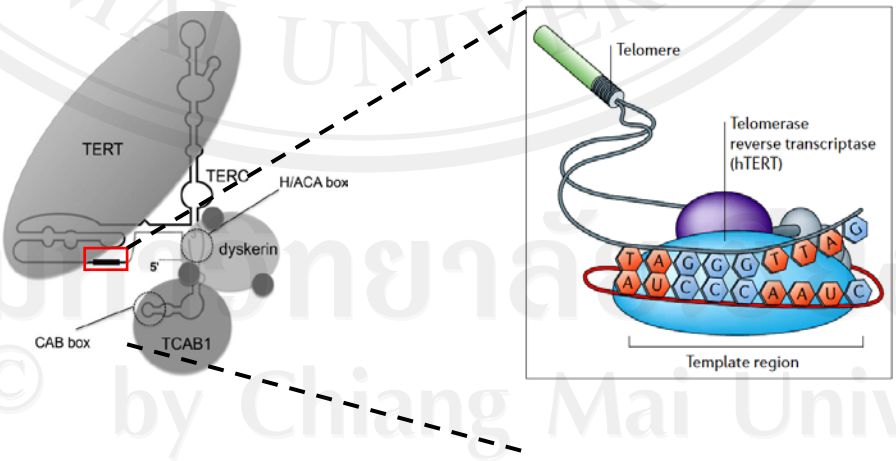

Figure 1.6 Telomerase Components [24].

1.2.6 Telomerase Activity in Various Cells

In most human normal somatic cells, telomerase activity is undetectable. The hTR is highly expressed in all tissues, regardless of telomerase activity, but cancer cells generally having five fold-higher expression than normal cells. In contrast, the expression (mRNA) of the human catalytic component hTERT is estimated at less than 1 to 5 copies per cell and is closely associated with telomerase activity in cells. The hTERT is generally repressed in normal cells and up-regulated in immortal cells, suggesting that hTERT is the primary determinant for the enzyme activity. However, lymphocytes and most, but not all, stem/progenitor cells in self-renewal tissues can express telomerase upon mitogenic stimulation. These cells have elongated lifespan so that humans can retain immune reactivity to each antigen and maintain the function of important organs, such as the bone marrow, intestine, skin, etc. The phenomenon is consistent with the telomere hypothesis, of which the high proliferative potential of these normal tissues would entail a special need for telomerase to maintain telomere length and genetic stability.

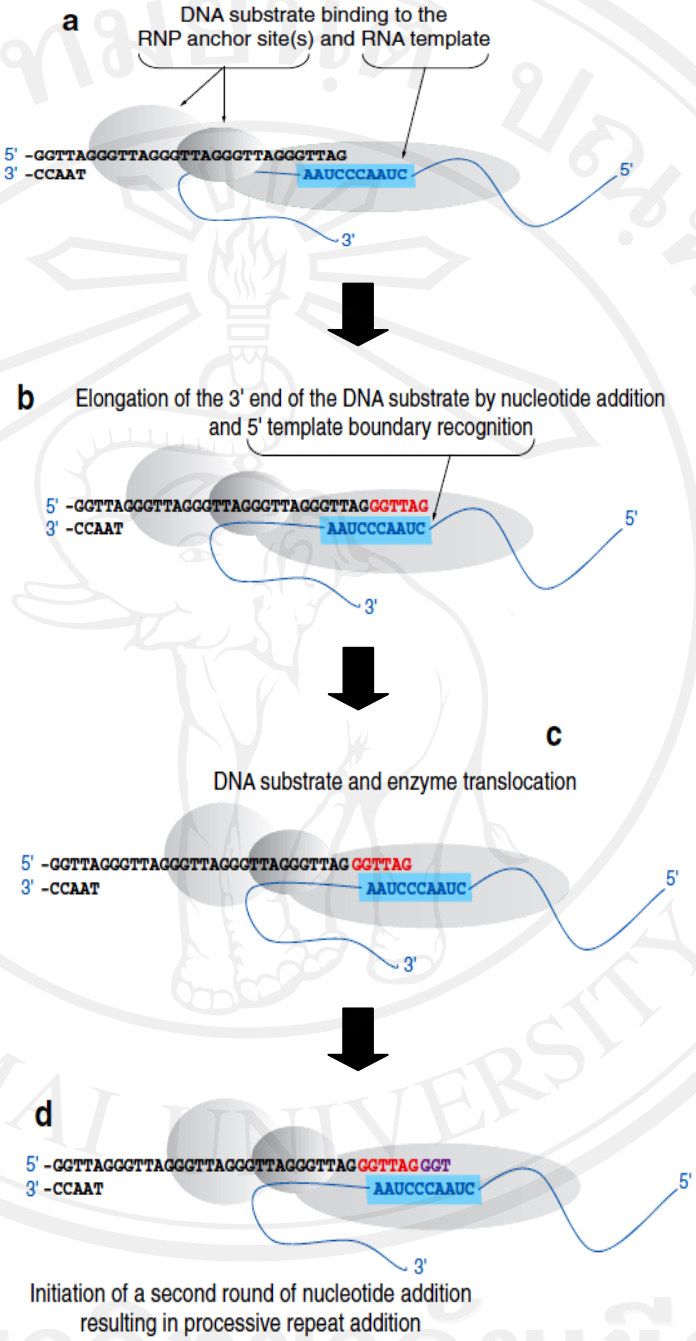

Figure 1.7 Function of telomerase [50].

1.2.7 Regulation of Telomerase Activity

The regulation of telomerase activity occurs at various levels, including transcription, mRNA splicing, maturation and modifications of hTR and hTERT, transport and subcellular localization of each component, assembly of active telomerase ribonucleoprotein, and accessibility and function of the telomerase ribonucleoprotein on telomeres. Telomerase activity is modulated under particular physiological conditions during tissue development and homeostasis. In addition to growth-related regulation, telomerase activity is subject to regulation by differentiation [51-54] and by extra- and intracellular signals such as UV irradiation [55], alpha interferon (IFN-α) [56], and estrogen [57, 58].

Importantly, among the core components of human telomerase, only the catalytic component hTERT seems to be the limiting determinant of telomerase activity, as other components are usually expressed ubiquitously. In most cases, hTERT expression is closely correlated with telomerase activity and with cancer initiation and progression. It is transcriptionally repressed in many normal cells and is reactivated or upregulated during immortalization. Substantial experimental data demonstrate that the transcriptional regulation of hTERT expression represents the primary and rate-limiting step in the activation of telomerase activity in most cells [52, 59-61].

Telomerase activity is extinguished in many tissues during embryonic development [62]. The correlation between hTERT mRNA and telomerase activity indicates transcriptional regulation of the hTERT gene. Soon after the hTERT code became available, the genomic organization of hTERT was established [59, 63].

In 1999, the cloning and characterization of the hTERT promoter structure was published. These materials have provided essential reagents for the molecular study of the transcriptional regulation of hTERT, which has been a major focus in the field of telomerase regulation.

1.2.7.1 Transcriptional Regulation [21]

Regulation of the hTERT subunit seems to be extremely important for carcinogenesis, as the RNA subunit (hTR) is constitutively expressed in most cells, while the catalytic subunit (hTERT) is suppressed in normal somatic cells but expressed in tumor cell types. Ectopic expression of hTERT results in functional telomerase, telomere elongation, and extension of lifespan in a variety of cell types. Since then, many groups have undertaken the task of elucidating the genes involved in hTERT activation and regulation to provide a better understanding of telomerase's role in tumorigenesis and extension of cellular life span. This field of research has led to the discovery of many promising transcription factors; however, none of the proposed factors alone have proven to be clear on/off switches for hTERT expression. The confounding problem is mainly due to the intrinsically complicated regulation of the hTERT gene. The transcriptional regulators, both positive and negative, include a variety of proteins that could potentially serve as chemotherapeutic targets, as many are oncogene or involved in tumor suppressor pathways. The fact that so many of the proteins that participate in hTERT also participate in tumor formation (or suppression) further implicates telomerase in tumor pathogenesis.

In human diploid cells, the hTERT gene is present as a single copy on chromosome band 5p15.33 the most distal band on the short arm of chromosome 5p. The mapping of the hTERT gene to the subtelomeric region led to the speculation that telomere positional effect may contribute to the repression of hTERT gene expression. Telomere position effect, which results in the reversible silencing of a gene near the telomere, has been well characterized in the yeast *S. cerevisiae* and was recently observed in human cells. However, the complete genomic sequence of hTERT indicates that the hTERT gene is more than 2 Mb away from the telomere on the short arm of chromosome 5. This is much farther away from a telomere than previously thought.

Transcriptional regulation of hTERT is believed to be the major mechanism of telomerase regulation in human cells. Transient transfection experiments with hTERT promoter-luciferase reporters show that the hTERT promoter is inactive in normal and transformed preimmortal cells but, like telomerase, is activated in immortal cells. Deletional analysis suggests that the minimum sequence requirement for promoter activity is contained within the 330 bp upstream of the start codon. However, it is possible that sequences further upstream of the minimal promoter may also be necessary for promoter activity in vivo and in certain physiological conditions. The differential activity of the hTERT promoter in mortal and immortal cells opens up the possibility of using this promoter for the selective expression of toxic genes in cancer cells for cancer therapy.

The hTERT gene consists of 16 exons and 15 introns and extends over 40 kb [59, 63]. The core promoter region of hTERT is representation with two E-boxes (c-Myc-recognitions sites) located upstream and downstream of the transcriptional start site. The putative Sp1/Sp3 binding sites on the promoter are also illustrated in Figure 1.8 The bottom panel contains the DNA sequence of the core promoter from -279 to -5. Various other transcription factors, such as Ap1 or MZF-2, associate with the hTERT promoter DNA at distant sites and are also involved in regulating its activity.

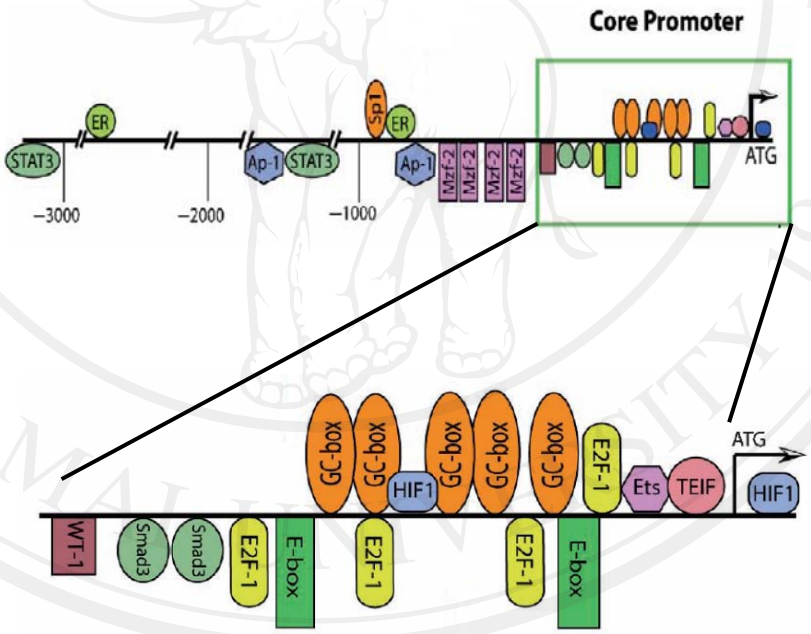

Figure 1.8 The core promoter region of hTERT [21].

The hTERT promoter has no TATA or CAAT boxes but is highly GC-rich. The GC-rich region forms a large CpG island around the ATG, suggesting that methylation may be involved in regulation of hTERT expression. The transcription initiation sites is mapped at 60 to 120 bp upstream of the translational start site, depending on the methods and cell lines used in different laboratories. The hTERT promoter contains binding sites for many transcription factors that may be involved in its regulation. The abundance of these potential transcription factor binding sites suggests that the regulation of hTERT expression may be subject to multiple levels of control by different factors in different cellular contexts. Several transcription factors are known to participate in hTERT gene expression [26] (summarized in Table 1.2).

The most extensively studied activator/repressor is the Myc/Mad/Max system; while Myc-Max heterodimer binds to E-box domains and activates hTERT expression, Mad-Max heterodimer binds to the E-box domains and prevents the subsequent binding of Myc-Max heterodimer, thus repressing hTERT's activity. Mad/Max may be in a ''switched'' role where the functions of Myc/Max are in competition for the Mad/Max complex to regulate telomerase activity. This function has direct implications for both tumorigenesis and regulation of cellular differentiation. Specifically, in proliferating cells, c-Myc is bound to hTERT at the E boxes, whereas in differentiated cells, Mad is bound at the promoter. This switch may be the key to the on/off regulation of telomerase in somatic cells.

Table 1.2 Transcription factors shown to regulate hTERT gene expression [64]**.**

Transcription factors	Role
AP-1	Repressor
BRCA-1	Repressor
Mad 1	Repressor
Mdm2	Repressor
Menin	Repressor
MZF-2	Repressor
P53	Repressor
RAK/BRIT1	Repressor
SIP-1	Repressor
Tax	Repressor
TGF-β	Repressor
Wt-1	Repressor
E2F-1	Repressor in cancer cells
E2F-1	Activator in normal cells
Estrogen	Activator
Sp1	Activator
STAT3	Activator
c-Myc	Activator
U2F1/2	Activator
Survivin	Activator

1.2.8 Telomerase Inhibition

In contrast to normal cells, tumor cells generally have short telomere lengths and show no net loss of average telomere length with successive cell divisions, suggesting that telomere stability might be required for cells to escape from replicative senescence and proliferate indefinitely. Most malignant tumors might need to become immortal to sustain their growth and telomerase activity could therefore be a rate-limiting step required for the continuing proliferation of advanced cancers. The telomere/telomerase hypothesis of aging and cancer is based on the findings that most human tumors have telomerase activity whereas adjacent normal human somatic cells do not. Therefore, a therapeutic window exists in which cancer cells can be efficiently targeted by telomerase inhibitors, while normal telomerase-expressing cells, such as stem and germline cells, remain unaffected as a result of their longer telomere lengths and slower rates of cell division (Figure 1.9).

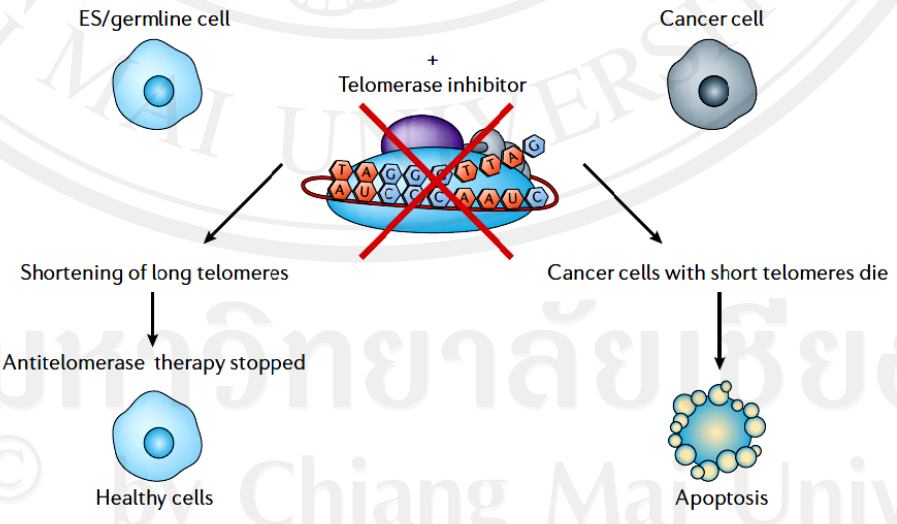

Figure 1.9 Comparing telomerase inhibition in normal versus cancer cells [24].

The evidence that most cancer cells activate telomerase whereas normal cells are usually devoid of telomerase activity has led to extensive investigations to detect this protein and its activity for a potential use in cancer diagnosis and prognosis, and to eventually monitor the tumor response to therapy. Multuples targets of telomerase inhibition are illustrated in Figure 1.10. These targets includes:

- Inhibiting upstream regulators of hTERT expression
- Immunotherapy vaccines targeting hTERT
- RNA inteference (RNAi) to hTERT and hTR
- Hammerhead ribozymes
- Reverse transcriptase inhibitor
- hTR template antagonist
- Stabilizing G-quadruplexes
- Inhibiting recruitment of telomerase to teleomere

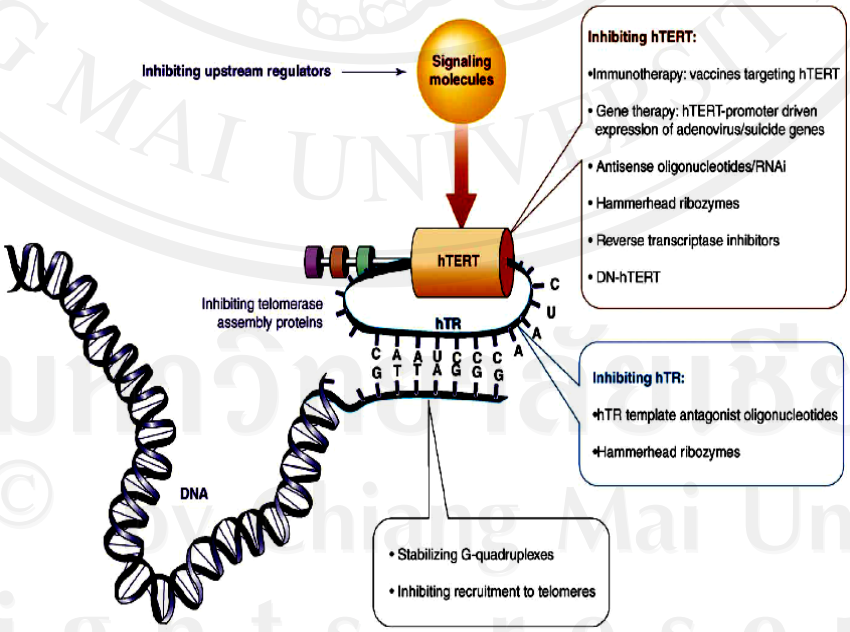

Figure 1.10 Multiple molecular targets of telomerase inhibition [65].

1.2.9 c-Myc and Cell Proliferation [66]

c-Myc is the prototype for oncogene activation by gene amplification and chromosomal translocation. In contrast to the tightly regulated expression of *c-Myc* in normal cells, *c-Myc* expression is frequently deregulated in human cancers. The c-*Myc* gene produces an oncogenic transcription factor that affects many cellular processes including cell growth, cell proliferation, apoptosis, cellular metabolism and tumorigenesis. Hence, *c-Myc* acts as an integrator and accelerator of cellular metabolism and proliferation.

1.2.9.1 MYC Structure and Function

The human *c-Myc* gene is located on chromosome 8q24. It consists of three exons, and transcription may be initiated at one of three promoters. The translational AUG start site is located in the second exon.

The gene encodes for a major 439 amino acid, 64 kDa c-Myc protein. Alternative translational initiation start sites result in both longer and shorter forms of the protein, termed p67 Myc and MycS, respectively. The c-Myc protein is glycosylated and phosphorylated, and these modifications may have an effect on function. The N-terminal region of Myc could function as a transcriptional activation domain and that the C-terminal region contains a helix–loop–helix leucine zipper (HLH LZ), possessed homology to bHLHZ proteins, it was widely assumed that Myc proteins would form homo- or heterodimers, bind DNA, and function as transcriptional activators. The c-Myc dimerization domain is necessary for cellular transformation, and the bHLH LZ protein Max and Mad were identified as a c-Myc-binding protein. Max was shown to interact specifically with all Myc family

proteins, and the resulting heterocomplexes recognize the hexameric DNA sequence CACGTG (belonging to the larger class of sequences known as E-boxes, CANNTG) at concentrations at which binding by either partner alone is undetectable. Importantly, Myc requires Max to activate transcription of genes containing E-box binding sites. Furthermore, Myc has been shown to repress transcription at certain target promoters. The transcription activation function of Myc is mediated at least in part by recruitment of a histone acetyltransferase (HAT). While Mad–Max heterodimers bind the E box to antagonize c-Myc transactivation (Figure 1.11).

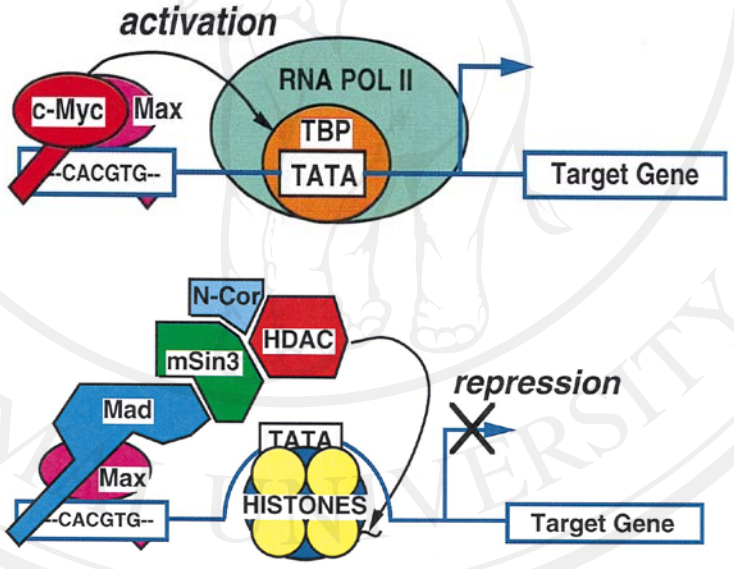

Figure 1.11 Models of c-Myc/Max and Mad/Max in transcriptional regulation [67]**.**

Expression of *c-Myc* in the normal cell is exquisitely regulated by external cues, such as growth factors and extracellular matrix contacts, as well as by internal clocks, such as the cell cycle. The resting cell normally expresses little to no *c-Myc*, whereas cells stimulated by growth factors to proliferate robustly increase *c-Myc* expression as an immediate early gene. The *c-Myc* gene then returns to

its basal quiescent state in resultant resting daughter cells. Ectopic overexpression of *c-Myc* in primary cells, however, appears to activate a protective pathway through the induction of p19/p14ARF that results in a p53-dependent cell death pathway. Through this means, normal cells that overexpress *c-Myc* and deviate toward neoplasia are eliminated from the host organism through apoptosis.

1.2.9.2 MYC Structure in Cancer [66]

In the 1970s, Weinberg and co-workers showed that rat embryo cells could be transformed when *c-Myc* and *ras* were co-transfected. Since then a variety of models have clearly indicated that *c-Myc* is oncogenic. When immortalized fibroblasts are engineered to overexpress *c-Myc*, they acquire the ability to grow in soft agar, signifying loss of contact inhibition commonly found in tumor cells. Transgenic animals overexpressing *c-Myc* form tumors in a variety of tissues. More recently, it has been shown that the conditional induction of *c-Myc in vivo* in keratinocytes or hematopoeitic cells can lead to proliferation and clonal expansion, a hallmark of neoplasia.

Although a link between *c-Myc* and cancer is quite established both *in vivo* and *in vitro*, the mechanism of *c-Myc*-mediated transformation is not fully known. Recent work has established a role for *c-Myc* in cell cycle progression, metabolism, apoptosis, and genomic instability. One model proposes that *c-Myc* promotes cell proliferation and genomic instability by accelerating cells through G1 and S phases of the cell cycle, abrogating cell cycle checkpoints, and increasing cell metabolism. In many settings, these alterations lead to apoptosis, or cell death.

However, in the background of additional mutations that activate antiapoptotic signals, *c-Myc* can lead to full-blown neoplasm.

To better understand *c*-Myc function and its role in neoplasia, recent efforts have concentrated on identifying genes that are induced or repressed by *c*-Myc. Direct targets of *c*-Myc are defined as those target genes whose expression is directly altered due to *c*-Myc binding at their promoter or intronic sequences. Indirect targets are those activated two or more steps downstream of Myc (i.e., induced by transcription factors, which have been induced by a c-Myc-mediated event) chosen in Table 1.3.

Table 1.3 Putative c-Myc target genes [66]

Gene product	Regulation	Technique	Relevance to c-Myc
ARF or p19	Up	Guess	Apoptosis
CAD	Up	Promoter	Growth and metabolism
Cdc2	Up	Guess	Growth related
Cdc25A	Up	Guess	Growth related
Cyclin A	Up	Guess	Growth related
Cyclin D1	Up or down	Guess/diff	Growth related
Cyclin E	Up	Guess/diff	Growth related
DHFR	Up	Promoter	Growth and metabolism
ECA39	Up	Diff	Amino acid transport
eIF-2α	Up	Guess	Growth-related metabolism
eIF4E	Up	Guess	Growth-related metabolism
ISGF3γ	Up	Guess	Stress response
LDH-A	Up	Diff	Growth and metabolism
MrDb	Up	Binding	Metabolism

Table 1.3 Putative c-Myc target genes(Continued)

Gene product	Regulation	Technique	Relevance to c-Myc
ODC	Up	Diff/guess	Growth related
α-Prothymosin	Up	Diff	Growth related
p53	Up	Diff/promoter	Growth related
RCC1	Up	Guess	Growth related
Rcl	Up	Diff	Growth related
Telomerase	Up	Guess	Immortality
TK	Up	Guess	DNA metabolism
Collagens a1(I), a2(I), a3(VI), a1(III)	Down	Diff	Adhesion
C/EBPα	Down	Promoter	Differentiation
Gadd 45	Down	Diff	Growth
LFA-1	Down	Guess	Adhesion
MHC class I	Down	Guess	Immune surveillance
Thrombospondin	Down	Diff	Metastasis

As mentioned above, Myc has been noted to activate telomerase, an enzyme that sustains telomere length and leads to immortalization of cells (i.e., an ability to be passaged indefinitely). The relevant clinical correlation is the intriguing observation that both N-myc amplification and telomerase levels are parallel predictors of poor outcome in neuroblastoma. This observation might be construed to suggest that N-myc is responsible for the elevation of telomerase in neuroblastoma; however, hard evidence for this and for the connection between Myc and telomerase in human cancers remains sparse.

1.2.10 *Zingiber officinale Roscoe*

- **Botanical description of *Zingiber officinale Roscoe*** [68]

Zingiber officinale Roscoe or known as ginger (Figure 1.12) is the underground stem of the ginger plant. Its actual name is rhizomes of ginger. However, it is commonly referred to as ginger, as the meaning is well known. The taxonomic position is as follow: Kingdom, Plantae; Division, Angiosperma; Class, Monocotyledoneae; Order, Scitaminaea; Family, Zingiberaceae; Genus, *Zingiber*; Species, *officinale*.

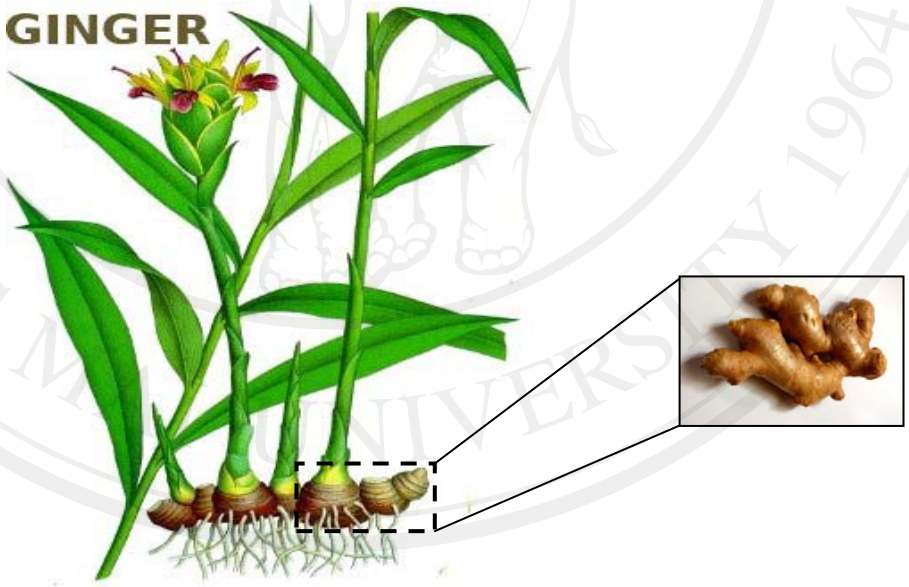

Figure 1.12 Ginger (*Zingiber officinale Roscoe*)

1.2.10.1 Chemistry of Ginger [69]

The sensory perception of ginger in the mouth and the nose arises from two distinct groups of chemicals: Volatile oils and Non-volatile pungent compounds. The volatile oil components in ginger consist mainly of sesquiterpene hydrocarbons, predominantly zingeberene (35%), curcumene (18%) and farnesene (10%). The non-volatile pungent compounds consists of gingerols, shogaols, paradols and zingerone (Figure 1.13) that produce a ‘‘hot’’ sensation in the mouth.

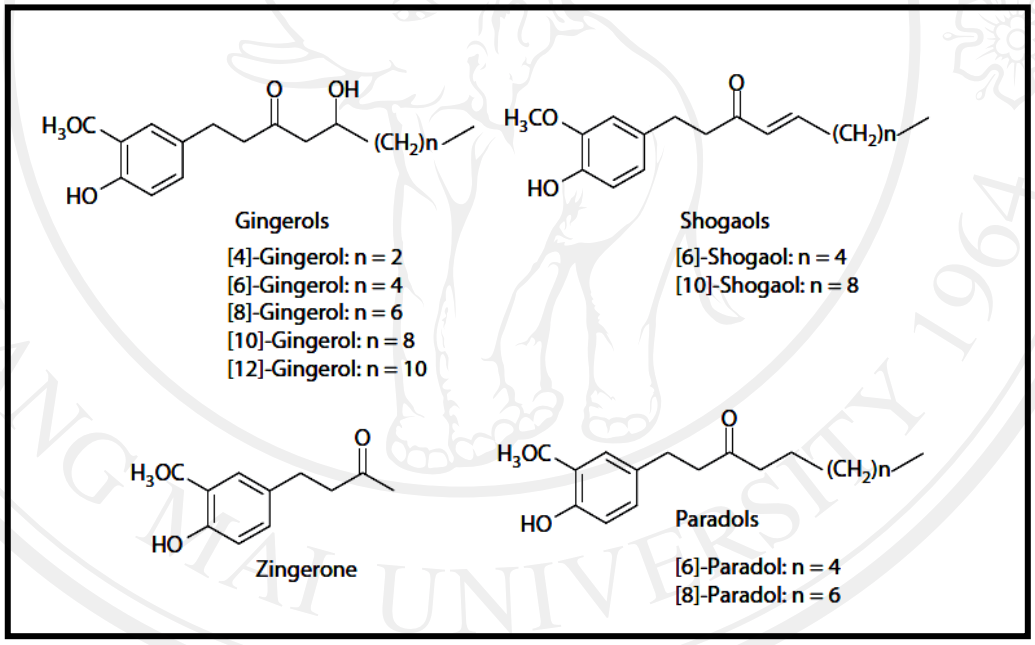

Figure 1.13 Chemical structures of some phenolics compounds from ginger.

Ginger rhizome has been used in traditional oriental herbal medicine for the treatment of a variety of human ailments including common colds, fever, rheumatic disorders, gastrointestinal complications, motion sickness, diabetes, cancer, etc. [70]. Ginger contains active phenolic compounds such as gingerol, paradol, shogoal and zingerone which is a pungent ingredient that have been shown to possess

antioxidant activities in vitro and also has many interesting pharmacological and physiological activities in vivo such as anti-inflammatory, anti-cancer, analgesic, anti-carcinogenic and anti-atherosclerotic properties [71,72]. The biochemical basis of cancer chemopreventive and therapeutic potential of ginger-derived pungent phenolic substances is illustrated in Figure 1.14.

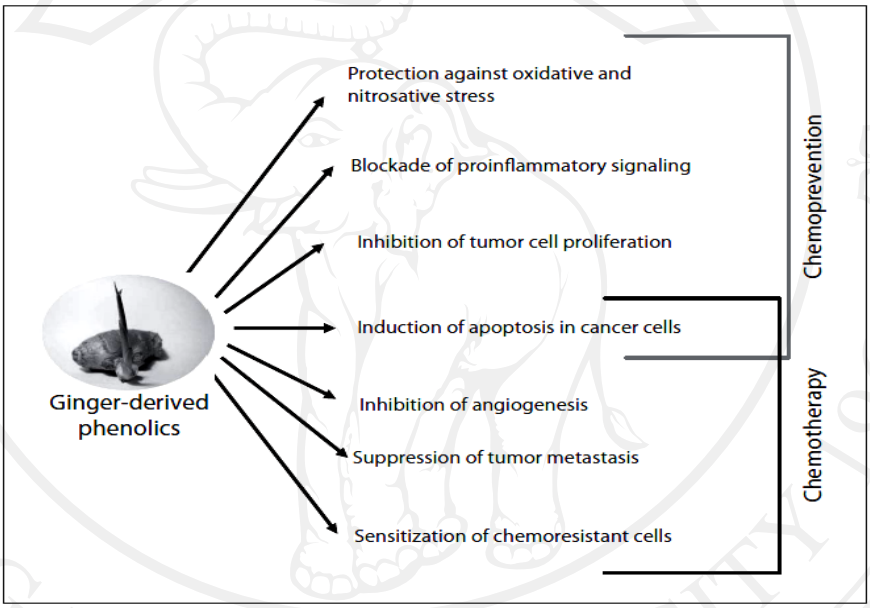

Figure 1.14 Biochemical mechanisms underlying anticarcinogenic effects of ginger-derived phenolic substances.

1.2.10.2 Antitumor-Promoting Effects [70]

6-Gingerol and 6-paradol inhibited epidermal growth factor (EGF)-induced transformation of cultured mouse epidermal JB6 cells following apparently different mechanisms. While 6-paradol inhibited transformation of JB6 cells stimulated with EGF through induction of apoptosis, 6-gingerol inhibited cell transformation by blocking EGF-induced transactivation of activator protein-1 (AP-1), a ubiquitous transcription factor involved in tumor promotion. 6-Gingerol

blocked TGF-β1-induced epithelial-mesenchymal transition in c-Ha-ras-transfected immortalized human keratinocyte (HaCaT) cells. 6-gingerol significantly reduced the formation of HCl-induced gastric lesions, a predisposing factor for gastric carcinogenesis. Both 6-gingerol and 6-paradol have been found to protects TPA-induced ear edema, epidermal ornithine decarboxylase (ODC) activity, and skin tumor promotion in female ICR mice.

1.2.10.3 Inhibition of the Growth and Proliferation of Tumor Cells [70]

Ginger phenolics have been reported to suppress the growth of tumor xenografts. While 6-shogaol suppressed the growth of human gastric cancer xenografts in athymic nude mice, 6-gingerol alone merely displayed a growth inhibitory effect. However, when co-treated with TNF-related apoptosis-inducing ligand (TRAIL), 6-gingerol significantly potentiated the growth-inhibitory effect of TRAIL. 6-Shogaol exerted antiproliferative effects in two transgenic mouse ovarian cancer cell lines, C1 (genotype: $p53^{-/-}$, c-*myc*, K-*ras*) and C2 (genotype: $p53^{+/+}$, c-*myc*, *Akt*).

1.2.10.4 Induction of Apoptosis in Cancerous or Transformed Cells [70]

6-Gingerol, 6-paradol [60] and 6-shogaol induced apoptosis in human promyelocytic leukemia (HL-60) cells. 6-Gingerol-induced apoptosis in HL-60 cells was prevented by catalase, suggesting that the compound induced cell death through generation of reactive oxygen species (ROS). In another study, 6-shogaol, but not 6-gingerol, induced cell death in human colorectal carcinoma COLO 205 cells through mitochondrial ROS generation, cytochrome c release, caspase activation, and

DNA fragmentation. Moreover, the induction of apoptosis by 6-shogaol in these cells was associated with increased expression of proapoptotic Bax and inhibition of antiapoptotic Bcl-2 and Bcl-xL. The most potent cytotoxic effect of 6-shogaol was observed against human non-small cell lung adenocarcinoma (A549), ovarian cancer (SK-OV-3), melanoma (SK-MEL-2) and colon cancer (HCT15) cells . 6-Paradol and other structurally related derivatives like 10-paradol, 3-dehydroparadol, 6-dehydroparadol, and 10-dehydroparadol, induced apoptosis in an oral squamous carcinoma cell line, through a caspase-3-dependent mechanism. 6-Gingerol induced apoptosis in human colorectal cancer cells by accumulating cells at the G1 phase of cell cycle arrest through up-regulation of NSAID-activated gene-1 (NAG-1) [73], which is a cytokine with proapoptotic and antitumorigenic properties.

1.2.10.5 Antiangiogenic and Antimetastatic Effects [70]

Angiogenesis is a process of forming new vasculature inside benign tumors to nourish**.** Major proangiogenic factors involved in tumor angiogenesis include vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IL-8, iNOS, angiopoietins, etc. Treatment of cultured epithelial ovarian cancer cells with ginger extracts induced profound growth inhibition in A2780, SKOV3, and ES-2 cells. Ginger extracts suppressed the activation of NF-κB and diminished the secretion of VEGF and IL-8 in ES-2 cells. 6-shogaol, but not gingerols, significantly inhibited the growth of A2780 cells in culture. 6-gingerol blocks VEGF-induced capillary-like tube formation in the mouse cornea. Treatment of human breast cancer (MDA-MB-231) cells with 6-gingerol led to a concentration-dependent decrease in cell migration and motility, and reduced mRNA expression and

activities of matrix metalloproteinase (MMP)-2 or MMP-9, which are well-known markers of tumor metastasis.

1.2.10.6 Chemosensitizing Effects [70]

One of the reasons for the growing trend of chemotherapy failure is the emergence of chemoresistance. P-Glycoprotein (P-gp), a product of multi-drug resistance gene-1, is considered as a key player in developing chemoresistance. P-gp is frequently overexpressed in tumor cells resistant to multiple anticancer agents. By actively refluxing drugs from cells, P-gp reduces intratumoral concentrations of chemotherapeutic drugs and hence lowers their efficacy. 6-Gingerol inhibited the efflux of P-gp substrates in human multidrug-resistant carcinoma KB-C2 cells, suggesting that the compound may facilitate intratumoral accumulation of anticancer drugs.

1.3 Objectives

Recently, our research group has found that the ethyl acetate fraction of ginger rhizome extract inhibits *hTERT* expression in A549 lung carcinoma cells. In this study, we attempted to identify the active compound from this fraction. The process included separation of crude extract by column chromatography. Each fraction was then subjected to fingerprinting by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). All fractions were tested for their inhibitory effect on *hTERT* expression. The fraction that found to be active was further purified by another column chromatography and was retested. Finally, the active fractions were analyzed for their compositions by gas chromatography / mass spectroscopy (GC/MS). The specific objectives of this research are as follows:

1.3.1 To fingerprint ginger extracts by thin-layer chromatography (TLC).

1.3.2 To quantify 6-gingerol content in ginger extracts by high performance liquid chromatography (HPLC).

1.3.3 To assess the cell toxicity by Sulforhodamine B (SRB) assay

1.3.4 To study the ability of *Z. officinale* extracts in modulating the *hTERT* and *c-Myc* expression using RT-PCR and qRT-PCR.

1.3.5 To study the ability of *Z. officinale* extracts to modulate the c-Myc expression using Western blot analysis.

1.3.6 To study the ability of *Z. officinale* extracts to modulate the telomerase activity using TRAP assay.

1.3.7 To assess the composition of compounds in ginger extracts by GC/MS.