Influence of hTERT Expression on Pathways Underlying Invasion and Metastasis

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CERTIFICATE

This is to certify that this thesis entitled **"Influence of hTERT expression on the pathways underlying invasion and metastasis"** submitted to the Jawaharlal Nehru University, New Delhi, by **Mr. Ramraj Prasad** is based on the studies carried out by him in School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been submitted so far, in part or in full, for any degree or diploma in this or any other university or institute.

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for **Prof. S. K. Goswami**
(Dean)

Dedicated
to
My Parents

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SYMBOLS AND ABBREVIATIONS

ALT	Alternative lengthening of telomeres
APS	Ammonium per sulphate
ATM	Ataxia Telangiectasia Mutated
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
Conc.	Concentration
DAPI	4’-6-Diamidino-2-pheyliodide
DEPC	Diethylpyrocarbonate
DKC 1	Dyskerin
DMEM	Dulbecco’s Modified Eagle medium
ºC	Degree centigrade
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
dNTP	triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetertra acetic acid
EMT	Epithelial Mesenchymal Transition
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocynate
GAPDH	Glyceraldehyde phosphate dehydrogenase
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	Hypoxia inducible factor
HGF	hepatocyte growth factor
HGFR	hepatocyte growth receptor
hTERT	human telomerase reverse transcriptase
hTR	human telomerase RNA
kb	Kilobase pair
kDa	Kilo Dalton
KLF4	Krupple like factor 4
LB	Luria broth
MET	Mesenchymal Epithelial Transition
µg	Micro-gram

µl	Microliter
MgCl2	Magnesium chloride
mRNA	messenger ribonucleic acid
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pmol	Pico mole
PMSF	Phenyl Methyl sulphonyl fluoride
POT1	Protection of telomeres 1
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic Acid
rpm	Revolutions per minute
rRNA	ribosomal RNA
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SDS-	sodium dodecyl sulphate-polyacrylamide
PAGE	gel electrophoresis
TAE	Tris-Acetic acid{glacial)-EDTA
TBE	Tris-Borate EDTA
TEMED	N, N, N', N', Tetramethylethylenediamine
TRF1	Telomeric repeat binding factor 1
TRF2	Telomeric repeat binding factor 2
Tris	Tris (hyroxymethyl) amino ethane
v/v	volume/volume
w/v	weight/volume
α	Alpha
β	Beta
γ	Gamma
λ	Lambda
µ	Micro

INTRODUCTION

1 INTRODUCTION

Cancer is a leading cause of mortality and disability worldwide. Primary tumors can be surgically removed and eradicated by radiation and chemotherapeutic. But when primary tumor cells start metastasizing then none of the therapies are effective. Chemotherapy creates some possibility, but it has high side effect over the body. Prevention of invasion and metastasis process in the initial stage of cancer can be useful strategies to reduce this disease pathogenesis. So, It is important to understand the pathways underlying invasion and metastasis.

Telomerase is a ribonucleoprotein complex. It consists of an RNA component hTR and protein component human telomerase reverse transcriptase (hTERT)[1]. Telomerase uses the hTR as a template to maintain the telomere length[2]. It adds the hexanucleotide repeats at the 3' overhang of DNA telomere. Telomere length gets shorter in every cycle of division and when it reaches to critical level cells stop dividing. So, for actively dividing cells (stem cells and cancer cells) maintenance of intact chromosomal termini is essential[3]. Telomere maintenance provides the chromosomal stability that leads to cellular immortalization[4]. hTERT is overexpressed in 80 to 90 percent of cancers and can be used as a biomarker in cancer pathology as a diagnostic or therapeutic target[5]. Recent reports suggest that hTERT is not only involved in the maintenance of telomere length but also performed extracurricular activity[6,7]. hTERT regulates molecular pathways associated with cancer cell survivability, proliferation invasion, and metastasis[8,9]. However, the role of hTERT in cancer invasion and migration is not well established. Therefore, it is important to explore the hTERT regulated pathway leading to invasion and metastasis.

c-MET is disulfide link heterodimer that made up of the alpha subunit (50 kD) beta subunit (145 KD). Alpha subunit contains only one domain that presents at the extracellular surface while beta subunit has three domain, extracellular beta domain, transmembrane helix, and intracellular tyrosine kinase domain for tyrosine phosphorylation[10]. This protein synthesized as precursor protein in cells and processed into active formed by proteolytic cleavage. c-MET encoding gene present on the 7th chromosome and its transcriptional regulation play a critical role in cancer pathogenesis[11]. c-MET activation starts after binding of HGF ligand on its extracellular domain that leads to phosphorylation of intracellular tyrosine kinase domain and

activates several signaling pathways; like PI3K, Gab1, STAT and β-catenin[12,13]. c-MET promotes the cancer cell invasion and metastasis by promoting EMT, ECM degradation and invadopodia formation by reorganization of the intracellular cytoskeleton[14,15].

p53 is a tumor suppressor protein playing an important role in genomic stability, and survival of healthy cells. p53 expression in normal cells prevents transformation into tumor cells. p53 signaling is initiated in response to many stress signal related to DNA damage that promotes transcriptional activation of target genes including p21, Bax, and Bcl-2 which regulate the cell cycle arrest or apoptosis[16]. Most of the cancer cells have mutant form p53 mutated that make tumor or cancer cells more aggressive. Mutant p53 gains tumor promoting role and exerts dominant negative effect over wild-type p53[17]. Genes regulated by p53 are associated with cell cycle arrest, apoptosis, senescence, angiogenesis, metastasis and DNA repair[18,19]. Wild type p53 acts as the suppressor for invasion and metastasis process. Those cancer cells have mutant or null p53 expression shows increases invasive and metastatic properties[20].

Both hTERT and c-MET proteins are well known for their role in cancer progression. Both proteins show the positive impact on cancer cell proliferation, survivability, invasion, and metastasis. These two proteins seem to share common pathways that are not yet established. Role of hTERT in cancer progression and its association with c-MET need to be investigated.

Therefore, we made the following objectives for our study.

1. To verify the effect of expression of hTERT in the process of cell invasion and migration.
2. To find out cross-talk between hTERT and HGFR/MET.
3. Investigation of hTERT-modulated HGFR/c-MET signaling in cancer cell invasion and migration.

REVIEW

OF

LITERATURE

2 REVIEW OF LITERATURE

2.1 Biological Relevance of Telomeres

2.1.1 Telomere: Discovery and Structure

Telomere is the specially organized terminal part of the eukaryotic chromosome. The concept of telomere was given in 1930 by H.J. Muller and Barbara Mc.clintock. They had observed the type and frequency of chromosomal rearrangement after x-ray irradiation[21,22] and suggested that end broken chromosomes are highly unstable and join end to end and form dicentric, ring and other kinds of unstable chromosome[21]. This observation on chromosomal instability due to loss of chromosomal end led to the origin of telomere concept.

Telomere is a specialized complex structure at the end of eukaryotic chromosome without which chromosomes are unstable[23]. In 1975-77 Blackburn discovered the structure of chromosomal termini (telomere) which is a repeat of hexanucleotides (TTGGGG) at the chromosomal end of *Tetrahymena thermophila*[24].

Telomere is a complex structure of DNA and protein. Telomere consists of G-rich DNA repeat sequence of hexa- nucleotide (TTAGGG in Human) which is present on 5' to 3' strands and runs towards chromosomal termini[23]. The uncopied portion at the 3' end of DNA template protrudes as a single-stranded G-rich tail. This G- rich tail invades and inserts in double-stranded DNA of telomere to form the telomere loop or T-loop[25]. Invasion by the single stranded 3' terminal DNA also causes displacement of the invaded region forming a D-loop (Fig. 1). Telomere length and the sequence of oligonucleotide repeat vary from species to species in eukaryotes, and human telomere consists of around 15 kb[26].

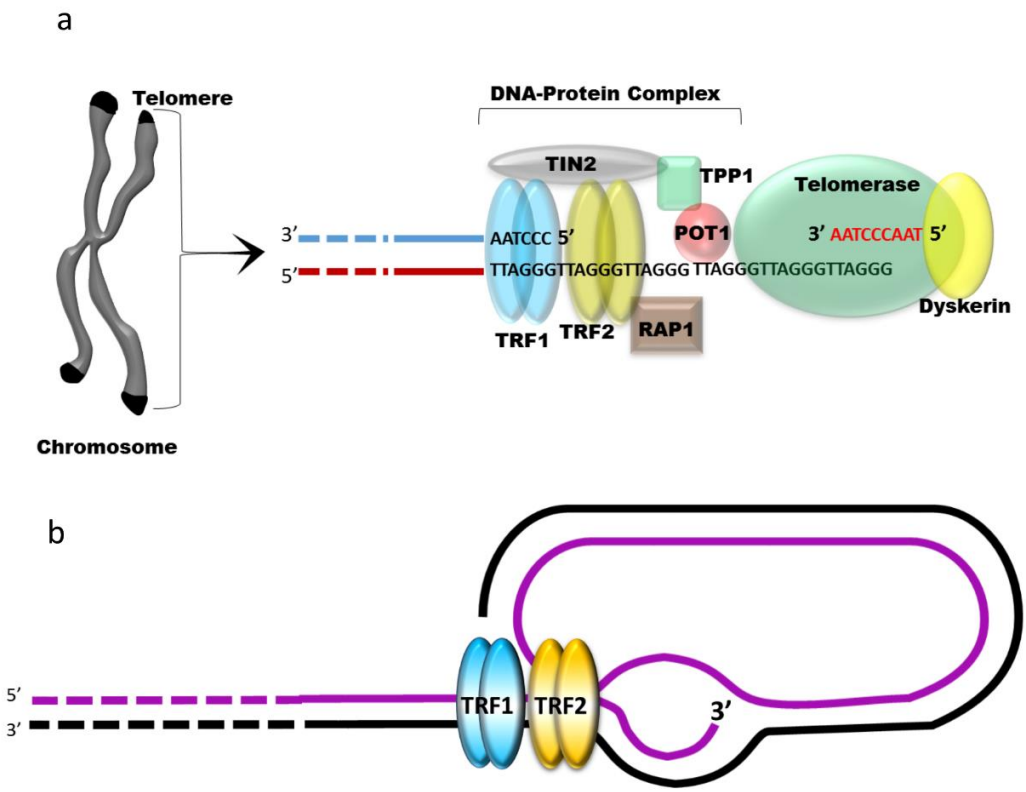

Figure 1: Structure and position of telomere in the chromosome.

a) A schematic view of chromosome and position of telomere and its associated protein. b) Single stranded 3' end of DNA template protrudes to form G-rich tail. This G- rich tail invades and inserts in double-stranded DNA of telomere to form the telomere loop or T-loop. Invasion by the single stranded 3' terminal DNA also causes displacement of the invaded region forming a D-loop.

2.1.2 Telomere: Replication and Maintenance

Telomere is replicated by DNA polymerase in each cell cycle. In each cycle, some part of the 3' terminal region remains un-replicated; thus, the copy strand is shorter than template due to end replication problem. This problem of shortening is solved by telomerase an RNA-dependent DNA polymerase employing an external RNA template. Telomerase similar to DNA polymerase adds nucleotide at 3' hydroxyl group[27,28]. Ciliates telomerase, TRC has template recognition element (TRE), located immediately 3' end of the template. TRE element is required for correct positioning of the template to the active site of telomerase[29]. Vertebrate telomerase requires only primer-template pairing for elongation rather than a template recognition element in flanking region of RNA [25].

Telomerase binds to 3' overhang of DNA in such way that some nucleotides of DNA overhang are paired with RNA template of telomerase RNA component. Telomerase adds nucleotides from 5' to 3' direction; further telomerase dissociates and translocates without losing the synthesized products to synthesized new repeated sequence[30]. When telomere reached to maximum length (~ 15 kb) telomerase dissociate from the overhanging strand, then primase adds a small primer over the end of synthesized DNA strand, and DNA polymerase fills the gap[31] (Fig. 2).

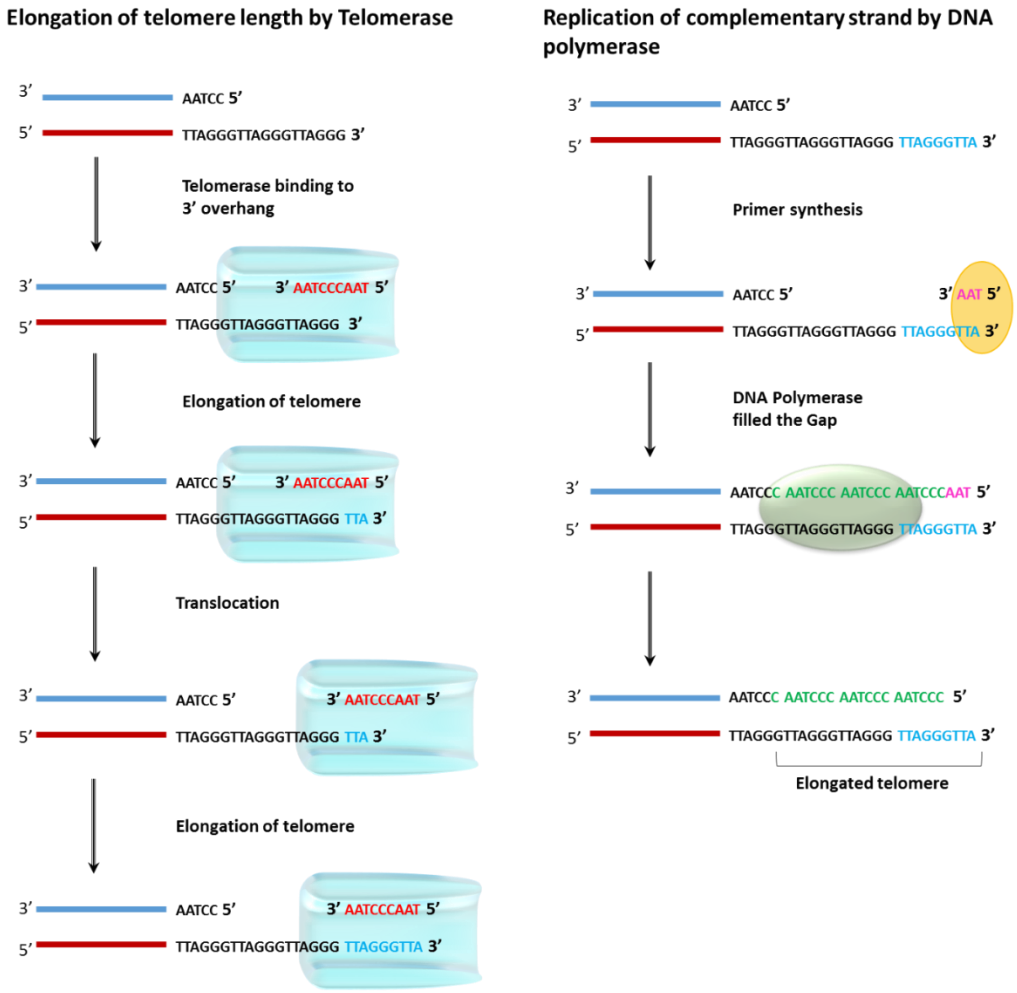

Figure 2: A schematic view of telomere elongation by telomerase and DNA polymerase.

Telomerase binds to 3' overhang of DNA and adds nucleotides from 5' to 3' direction. It dissociates and translocates without losing the synthesized products to integrated new repeated sequence. When telomere reached to maximum length (~ 15 kb) telomerase dissociate from the overhanging strand, then primase adds a small primer over the end of synthesized DNA strand, and DNA polymerase fills the gap.

Alternative lengthening of telomeres (ALT) is telomerase-independent telomere lengthening process found in telomerase negative tumor and immortalized cells[32].

ALT positive cells maintained the telomere length by:

Resolution of T-loop by NBS1 and XRCC3 leads to the formation of t- circles which spread and anneal with another chromosomal telomere and replicate the end by rolling circle replication[33] (Fig. 3a).

Homologous recombination-dependent telomere replication is completed by the invasion of 3' overhang of one telomere to duplex telomere of another chromosome followed by replication by DNA polymerase[34] (Fig 3c).

Another mechanism of telomere lengthening could involve Telomere-sister chromatid exchange followed by replication and homologous recombination between sisters chromatids telomere[35] (Fig. 3b).

ALT positive cells have heterogeneous length of the telomere and protein- DNA complex is referred to as ALT-associated promyelocytic leukemia protein nuclear bodies (APBs)[36].

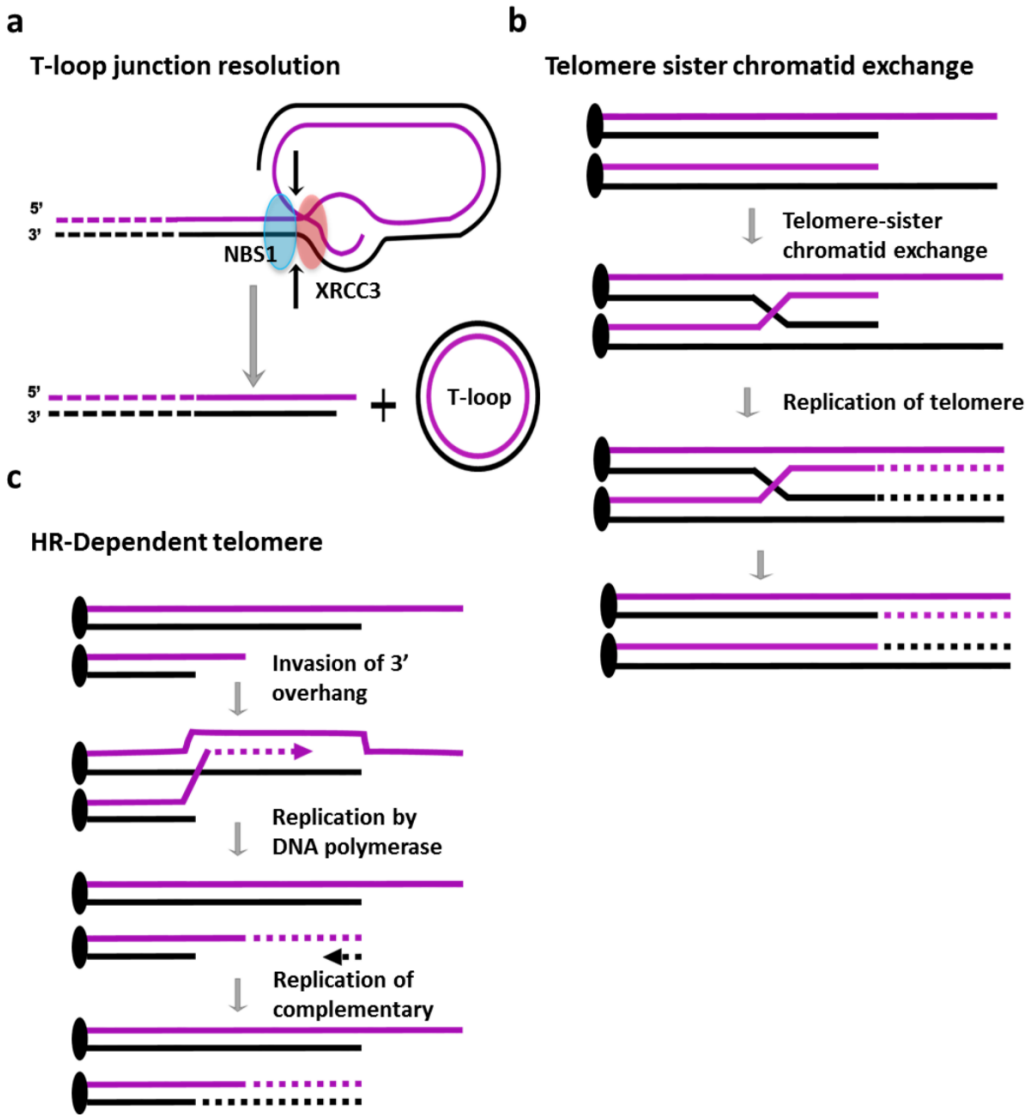

Figure 3: Models of different type of ALT mechanism involved in the maintenance of telomere length in telomerase negative cells.

a) NBS1 and XRCC3 resolved the T-loop of telomere which spread and anneal with another chromosomal telomere and replicates the end by rolling circle replication. b) Homologous recombination-dependent telomere replication is completed by the invasion of 3' overhang of one telomere to duplex telomere of another chromosome followed by replication by DNA polymerase. c) Telomere of telomerase negative also maintained by exchange sister chromatid telomere followed by replication and homologous recombination between sisters' chromatid strands telomere.

2.1.3 Telomere-associated protein

Telomere is a complex structure of DNA and protein referred as shelterin complex, and proteins associated with this complex are shelterin proteins[37].

Human telomere is protected by six shelterin proteins.

I. **TRF1 (telomerase repeat factor 1):** TRF1 is a homodimer, bound to hexanucleotide repeat (TTAGGG) of the telomere. TRF1 prevents the telomerase-mediated addition of nucleotides on telomere after achieving maximum length[38]. During telomere replication, TRF1 recruits helicase to facilitate the unwinding of telomeric DNA[26] (Fig 4a &b).

II. **2-TRF2 (telomerase repeat factor 2):** this is a homodimeric protein also binding to the double-stranded telomeric repeat[39]. TRF1 and TRF2 share common structural domains. These two proteins interact with other shelterin proteins and recruit them on telomere[40] (Fig 4a &b).

III. **3-POT1 (Protection of Telomeres 1):** POT1 contains oligonucleotide and oligosaccharide-binding domain (OB-fold) at the N-terminus. POT1 recognizes and binds to the single stranded 3' overhang of the telomere and prevents it from nuclease digestion[41] (Fig 4a &b).

IV. **4-TPP1:** TPP1 is a multi-domain protein, each domain defined to interact with specified proteins. This protein does not directly bind to DNA but interacts with TIN2 and recruits other proteins over the telomere. TPP1 directly interacts with POT1 and acts as a processivity factor for telomerase during telomere replication. TPP1 maintains the telomere length by recruiting the telomerase at the end of the chromosome[42] (Fig 4a &b).

V. **5-TIN2 (TRF1-interacting nuclear protein 2):** TIN2 is a major factor in shelterin complex that stabilizes the whole complex. TIN2 works as bridging protein between double stranded and single stranded DNA-binding proteins. TIN2 directly interacts with TRF1, TRF2, and TPP1[43] (Fig 4a &b).

VI. **6-RAP1 (Repressor Activator Protein 1):** RAP1 interacts with TRF2 and helps the proteins recruitment associated with TRF2. RAP1 prevents the chromosomal end fragility and telomere recombination, but this does not significantly prevent the chromosomal end to end fusion[44] (Fig 4a &b).

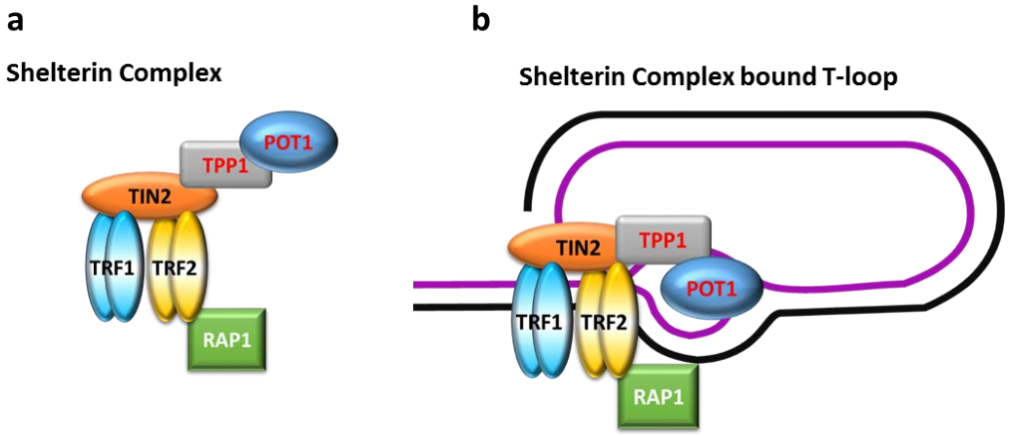

Figure 4: Theoretical model is representing the structural component of shelterin complex and its association with the telomeric DNA.

2.1.4 Organization and function of telomere

Telomere protects the chromosome end to end fusion, recombination of terminal region and degradation from nuclease[45]. Normal cells divide up to 50 cycles due to shortening of the telomere, so telomere length is an important determinant for cell division and lifespan of cells[46]. In cancer and stem cells, telomere length remains constant due to active telomerase enzyme[47]. Cells transfected with telomerase expressing constructs gain immortality[48]. Telomere loss and maintenance both are associated with disease, e.g., loss of telomere leads to dyskeratosis congenital syndrome which is a rare syndrome of premature aging while telomere lengthening causes cancer[49].

2.2 Telomerase

2.2.1 Telomerase: discovery

E. Blackburn and C. Greider discovered the telomerase in 1985 as a catalytic unit that extends telomeric region in *Tetrahymena thermophila*. Later they were found out that a ribonucleoprotein complex consisting of two components is involved in end replication[50]. RNA component of ribonucleoprotein provides a template for synthesis of telomeric DNA repeat[2]. For the discovery of telomerase C. W. Greider, E. Blackburn and J. W. Szostak were awarded Nobel Prize in Physiology or Medicine for the year 2009.

2.2.2 Telomerase: structure

Telomerase is a ribonucleoprotein complex. Its core complex consists of a protein component, i.e., telomerase reverse transcriptase (TERT) and an RNA component telomerase RNA (TR) or telomerase RNA component (TERC)[23]. Other than this two principal components telomerase has several other proteins that interact with it, but these molecules are not considered as the integral component of telomerase since they are not required for reconstitution of telomerase activity.

2.2.3 Telomerase reverse transcriptase (TERT)

TERT protein was first identified as homologous genes from *Schizosaccharomyces pombe* and human by Nakamura et al. in 1997[51]. Human TERT gene was cloned in 1997 by Meyerson et al. and shows significant similarity with TERT of lower eukaryotes, and they had referred this gene as EST2[52]. Independently Kilian et al. in 1997 also cloned the human reverse transcriptase and designated as TCS1[53].

2.2.4 Telomerase reverse transcriptase (TERT) Gene and transcripts

Gene encoding for hTERT is located on chromosome region 5p15.33 mapped by Meyerson et al. in 1997 and length of the gene is around 40 kd[52]. In 1999 Cong et al. find out that hTERT gene consists of 16 exons and 15 introns which give seven transcript variants after alternative splicing[54]. Mature full-length hTERT mRNA is 4.0 kb long. Around seven selective splicing sites are present on hTERT pre-mRNA. Out of seven splicing site, three are (α, β, and γ) deletion site and four insertions site[55]. α deletion transcript variant is generated by deletion of 36 nucleotides from exon six which is translated in a protein that lacks telomerase activity and shows a dominant negative effect for normal telomerase[56]. β variant is produced by 182 nucleotides deletion from exon seven and eight along with insertion of 38 bp in exon 4. β variant undergoes premature termination during translation and gives a nonfunctional protein[51,57]. γ variant is generated by deletion of exon 11, and this variant protein does not exhibit any function[58]. Remaining variants are produced by insertion in 2, 4, 11 and 14 exon region. These insertions lead to premature termination during translation and generate nonfunctional proteins[59] (Fig. 5).

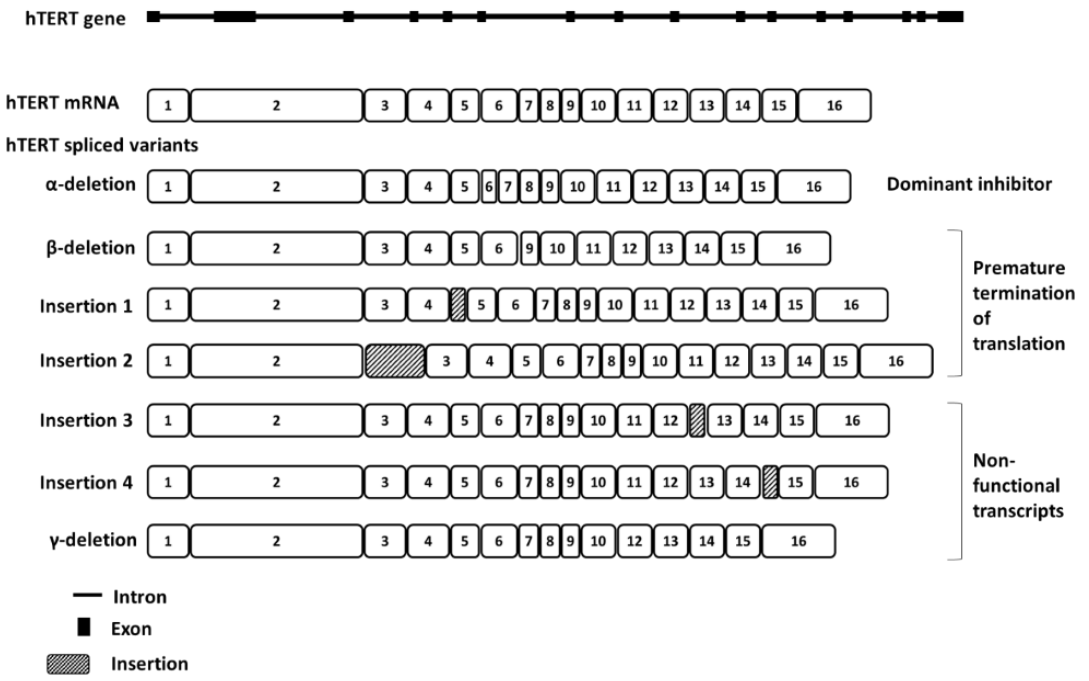

Figure 5: The hTERT mRNA and its splice variants resulting in dominant negative form, premature termination of translation or non-functional transcripts

2.2.5 Regulation of hTERT expression

hTERT core promoter was identified within the region of 300 bp upstream of transcription start site and 37 bp of exon 2 of hTERT gene. hTERT promoter is GC rich and contains binding sites for several transcription factors like E-box and GC-box[54].

hTERT is an essential subunit of telomerase which is limiting factor for this enzyme[60]. hTERT expression determines the telomerase activity in different cancer[61]. hTERT expression regulation is a complex process that involved genetic and epigenetic modifications. Several transcription factors are known till date that associated with regulation of hTERT. These transcription factors could be categorized as positive and negative regulator[62].

2.2.5.1 Positive regulator of hTERT

c-Myc is an essential oncogene associated with the cell growth and survivability[63]. c-Myc forms heterodimer with Max and binds on E-box of hTERT core promoter region[64]. c-Myc promotes the transcription activity by recruitment of histone acetyltransferase complex (HAT) on hTERT promoter[65]. c-Myc interacts with other

transcription factors like human foreskin keratinocytes (HFKs), Specificity Protein 1 (SP1) and Forkhead Box O3A (FOXO3a) and regulates the hTERT expression[66]. Nf-kB is transcription factor activated in various responses like inflammation, tumorigenesis, apoptosis and cellular differentiation[67]. It activates the hTERT expression by directly binding to the hTERT promoter or by modulating the expression of another transcription factor that regulates hTERT expression[68]. SP1 is another transcription factor that universally expresses in all cancer and normal cells. SP1 binds to the GC-box (5´-GGGCGG-3´) of hTERT promoter. SP1 increases the hTERT expression and telomerase activity by interacting with MCAF1, HMGA2 and NFAT in cancer and stem cells[69–71]. STAT3 expression promotes cell growth and survivability. STAT 3 interacts with the CD44 and Nf-kB to activate the hTERT expression[72]. Hypoxia-inducible factor-1(HIF-1) in the tumor is known to promote angiogenesis. HIF-1 induces the expression of hTERT by activation of enhancer-binding protein-2 (AP-2). Activated AP-2 binds to the hTERT promoter and promotes its expression[73].

2.2.5.2 Negative regulators of hTERT expression

Mad1 is a transcription repressor that antagonizes the effect of c-Myc. Mad1 competed with c-Myc to bind and forms a heterodimer complex with Max[74]. Mad1/Max complex compete with c-Myc/Max complex to bind on E-box of hTERT promoter[75]. AP1 belongs to Jun and Fos family and has a binding site on hTERT promoter, upstream to transcription start site. Binding of AP1 to hTERT promoter represses the hTERT expression[76]. SP3 binds to the GC-box of hTERT promoter. SP3 antagonized the SP1 by recruiting the histone deacetylase (HDAC) on hTERT promoter[77]. p53 is a universal suppressor protein that regulates the expression of many genes associated with proliferation, differentiation, senescence and apoptosis. p53 expression is suppressed or mutated in most of the cancer. As a master controller, p53 suppresses the expression of hTERT by preventing the binding of SP1 protein on hTERT promoter[78]. Retinoblastoma protein (pRb), Wilms´ tumor 1 suppressor (WT1) and PITX1 are some other transcription factors associated with repression of hTERT expression[79–81].

2.2.5.3 hTERT gene copy number and mutations in promoter in regulation of hTERT expression

The copy number of hTERT promoter increased in cancer cells due to polyploidy or specifically multiplication in copy number of hTERT locus gene[82,83]. This is one aspect

of increase in hTERT expression and telomerase activity in cancer cells. Other than copy number point mutation in hTERT promoter is another mechanism of increase in hTERT expression. First point mutation in hTERT promoter was reported in 2013[84]. Two important point mutations are found in hTERT, cytidine to thymidine (C > T) at position 124 and 146 from translation start site ATG. These mutations in hTERT promoter create new E26 transformation-specific (ETS) binding site that promotes the hTERT expression in cancer cells[84,85]. Mutation in hTERT promoter increased the telomerase-associated cancer progression.

2.2.5.4 Epigenetic modification on hTERT promoter in hTERT expression

Epigenetic changes in the hTERT promoter is another mechanism by which hTERT expression could be regulated in cancer cells. Promoter hypermethylation is related to gene silencing and hypomethylated promoters being associated with gene activation. hTERT promoter lacks TATA or CAAT boxes but is rich in GC nucleotides that show the possibility of methylation. hTERT promoter shows discrete pattern of methylation. Some tumor cells have methylated hTERT promoter that increased the hTERT expression and in other finding methylation associated with silencing[86,87]. In most of the cancer cells hTERT CpG sites are hypermethylated at the distal position (-600 bp) and hypomethylated in proximal region (-150 bp to +150 bp) of hTERT promoter[88]. Hypermethylation of hTERT promoter prevents the binding of CTCF repressor[89]. Some CpG sites remain non-methylated in hTERT promoter which allows the binding of activator protein like SP1 and c-Myc. Histone modification mediated chromatin remodeling is also involved in hTERT expression[90].

2.2.5.5 miRANA mediated regulation of hTERT expression

Some micro-RNAs, like miR-346 and miR-138, are reported to interfere with hTERT transcript and regulate the hTERT protein synthesis. miR-346 promotes the hTERT translation while miR-138 inhibits the translation[91].

2.2.6 Telomerase reverse transcriptase (TERT): structure

Telomerase core component is composed of telomerase reverse transcriptase (TERT) and telomerase RNA (TR)[23]. These two components interact with other accessory proteins to form a holoenzyme. First TERT protein was identified in

Schizosaccharomyces through genetic screening[92]. Later active telomerase was biochemically purified from the ciliates[93]. TERT from *E. aediculatus* was identified as homologous protein of yeast. Yeast TERT, RT domain has a catalytic triad of amino acids present at the active site of TERT. Mutation or removal of this catalytic triad from TERT leads to shortening of the telomere. Later similar homologous proteins were identified in ciliates, rodents, and human[51,52,94]. Human TERT is an 1132 amino acid long protein. The molecular weight of this protein is 126.997 kd.

Mutational and bioinformatics work shows TERT has three distinct domains.

I. N-terminal extension domain (NTE)
II. Catalytic reverse transcriptase domain (RT)
III. C-terminal extension domain (CTE)

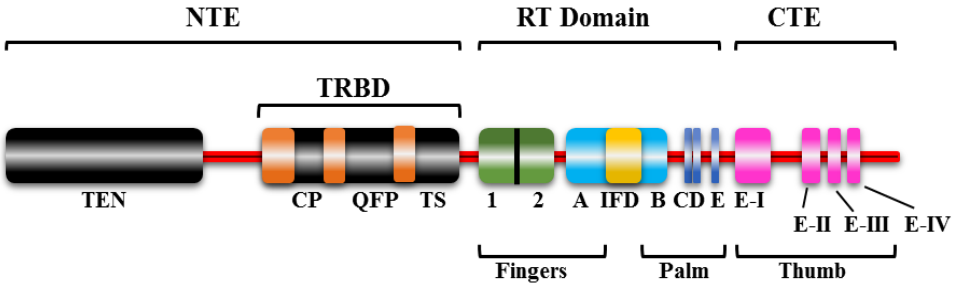

Figure 6: Structural representation of different domain and subdomain of the TERT.

It contains N-terminal extension, catalytic RT domain and C-terminal extension (CTE). N-terminal (TEN) domain made-up of TEN domain and TRBD. TRBD has three distanced motif CP, QFP, and TS. RT domain has seven conserved motifs, four (1, 2, A and B) in fingers subdomain and three (C, D and E) in palm subdomain. C-terminal extension domain has a single thumb domain that has three motifs EII, EIII, and EIV.

I. N-terminal extension domain (NTE)

N-terminal extension domain of TERT contained two main sub-domain first TEN domain and the second one are telomerase RNA binding domain (TRBD). These two domains have specific protein folding for binding with the single-stranded RNA. TEN and TRBD domains are linked with a flexible linker part that provides the flexibility to enzyme[95,96] (Fig. 6). TEN domain of N-terminal extension shows the binding capacity to single-stranded DNA of telomere, and it contains some important sequences that are required for telomerase activity[97].

Crystal structure of TRBD domain indicates that it has two asymmetric structure. TRBD contains 12 alpha helices linked by several loops and 2 beta sheets. Helices are organized in such a way that they create two distinct asymmetric halves that were connected by three loops. Larger half contained nine alpha helices while smaller one has three alpha helices[95]. TRBD has three distanced motif CP, QFP, and TS[95]. CP and TS motifs are involved in the interaction with telomerase RNA while QFP is thought to be essential for ribonucleoprotein assembly[1]. TEN and TRBD domain co-operatively ensure the sequence-specific binding of TERT-TR complex during telomerase activity.

II. Catalytic reverse transcriptase domain (CRT)

RT is one of the best characterized domains of TERT protein. It contains seven evolutionary conserved RT motifs that are required for reverse transcriptase activity[98–100]. TERT-RT domain has two subdomain, fingers, and palm that resembles with typical reverse transcriptase enzyme subdomain[101,102]. These two domains are linked with a loop that contained conserved primer grip[103]. This loop directly interacts with RNA-DNA hybrid of 3' end of single-stranded DNA. Primer grip of loop help in the positioning of 3' end of single-stranded DNA at the active site of enzyme[103]. RT domain of TERT shows a unique feature that differs from other reverse transcriptases in that there is the insertion of a large motif between A and B subdomains known as insertion in fingers domain (IFD). IFD domain has two antiparallel alpha helices present between finger and palm subdomain of RT. IFD domain helps in organization, stabilization and interaction with telomeric DNA and TERT-RT domain[51,101]. RT domain has seven conserved motifs, four (1, 2, A and B) in fingers subdomain and three (C, D and E) in palm subdomain (Fig. 6). TERT-RT catalytic domain contained three aspartic acid in motif A. these residues are conserved in TERT-RT domain and form a catalytic triad that is directly involved in nucleotide addition during telomere elongation[101].

III. C-terminal extension domain (CTE)

C-terminal extension domain has less conserved sequence as compare to NTE and RT domain. This may have species-specific amino acid sequence but this sequence fold and form a similar structural domain. C-terminal extension domain has a single thumb domain that has three motifs EII, EIII, and EIV[103] (Fig. 6). CTE is a bundle of alpha helices interconnected with several loops that may be involved in the formation and stabilization of DNA-RNA heteroduplex complex on the active site of the enzyme[104].

This domain shows species-specific function in human and *Tetrahymena*, removal of this region causes inactivation of telomerase enzyme while in yeast it reduces the only processivity of the enzyme[105,106].

2.3 Telomerase RNA component

Telomere cap protects eukaryotic chromosomal end that is synthesized by a ribonucleoprotein complex enzyme telomerase. Telomerase has two components viz., the catalytic domain TERT and another, the telomerase RNA component (TR). Telomere consists of a repeat sequence complementary to TR template[107]. Telomerase RNA component discovered in 1995 by Feng et al. they cloned the 451 long RNA and represented as TRC3. TRC3 is highly expressed in germ line cells and tumor cells which have high telomerase activity while normal cells like kidney, liver and prostate cells have very low TR level[107].

2.3.1 Telomerase RNA component (TRC): Gene and transcript

Gene encoding for telomerase RNA component (TR) is located on chromosome 3 at q arm. The exact location of TR gene on the chromosome is 3q26.2. TR gene has one transcript and size of the transcript is 541 bp[108].

2.3.2 Telomerase RNA component (TERC/TR): structure

Structure and size of RNA component of telomerase differ from species to species. Telomerase RNA component length in Ciliates 150 nucleotides, vertebrate 450nucleotides and yeast 1500 nucleotide long[109–111]. Vertebrate and yeast telomerase RNA are synthesized by RNA polymerase II while ciliates TR component is synthesized by RNA polymerase III. Different species acquired different types of RNA domains in telomerase RNA during evolution that helps in the binding of the telomerase-associated protein.

Vertebrate Telomerase RNA component contains four conserved domains, pseudoknot, CR4-CR5, BoxH/ACA and CR7[109]. Telomerase RNA domain is divided into two half, one half includes pseudoknot and CR4-CR5 that are essential for telomerase activity while second half includes BoxH/ACA and CR7 required for RNA stability[112,113] (Fig.7).

I. **Pseudoknot:** Pseudoknot is the less conserved domain of telomerase RNA component than other domains. Pseudoknot contained a TERT binding site and template sequence that is utilized by TERT to synthesise telomere[114].

II. **CR4-CR5:** This is a highly conserved region. CR4-CR5 independent of pseudoknot interact with TERT, and this interaction is crucial for telomerase activity. CR4-CR5 contained P5, P6 helix and a P6.1 stem-loop structure. Stem region of P6.1 is required for binding of telomerase RNA component to TERT and loop of P6.1 is essential for catalytic activity of telomerase enzyme[115].

III. **BoxH/ACA: T**his domain is required for assembly of a functional telomerase enzyme. BoxH/ACA contains a sequence and hairpin-hinge-hairpin-tail structure. BoxH/ACA interacts with cytoplasmic protein dyskerin. BoxH/ACA-dyskerin interaction is essential for processing of telomerase RNA component from large RNA precursor and this complex is also vital for stability of telomerase RNA component and its localization within the cells[116].

IV. **CR7:** This is a highly conserved region in telomerase RNA component. CR7 domain contained two helices P8a and P8b and a loop P8[109]. CR7 contains a signal sequence that helps in the localization of telomerase RNA component in a specialized nuclear compartment where telomerase assembly occurs[117].

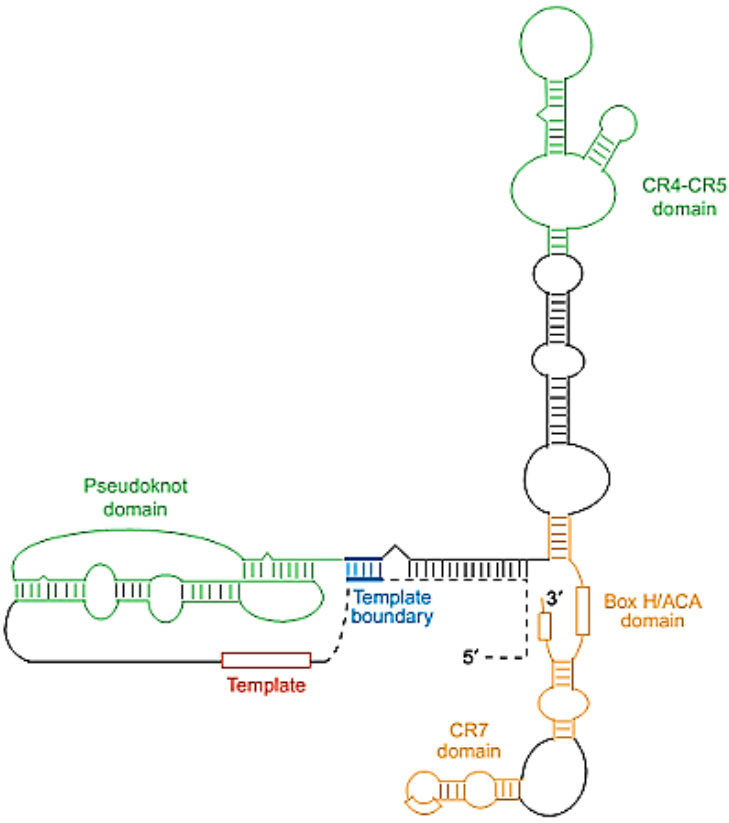

Figure 7: Structure of telomerase RNA showing the pseudoknot region in the vicinity of the template region and other CRs[118].

2.3.3 Function of telomerase

2.3.3.1 Canonical function of telomerase

Hanahan and Weinberg have described the hallmarks of cancer for successful transformation of the normal cell to cancerous cells[119]. They have proposed that the normal cells must take ten important steps to transform into cancer cells. Out of ten one essential step is enabling replicative immortality. Normal cells divide up to 50 cycles due to shortening of telomere[120]. Cancer cells overcome this problem by maintaining the telomere length. Telomerase is a specialized ribonucleoprotein complex involved in the lengthening of telomere in the cancer cell. Telomerase lengthens the telomere to check replicative senescence[121]. Telomerase has a catalytic protein component TERT and RNA component that acts as a template for synthesis of hexanucleotide repeat (TTAGGG) at telomere ends. Telomerase activation and repression both are associated with the disease.

2.3.3.2 Disease related to loss of telomerase activity

2.3.3.2.1 Dyskeratosis Congenita

Dyskeratosis Congenita is a rare genetic autosomal disorder. This disease is characterized by nail dystrophy, anomalous skin pigmentation, and leukoplakia of the oral mucosa. This disease was first identified as a heterologous mutation in TERT gene. Dyskeratosis Congenita is dominant and recessive autosomal disease. Mutation in TERT gene leads to the synthesis of the non-functional catalytic component. Telomerase with mutant TERT shows impaired activity and leads to shortening of telomeres and causes Dyskeratosis Congenita[122]. Many mutations in TERT gene are associated with Dyskeratosis Congenita listed below (Table 1).

Table 1: Transversion and transition mutation in TERT associated with dyskeratosis congenita

S.N.	Mutation in TERT gene	Type of mutation	Position	Change in amino acid
1	G to C	Transversion	Exon 11	lys902 to asn
2	G to A	Transversion	1892	arg631 to gln
3	C to T	Transition	Exon 8	arg811-to-cys
4	C to T	Transition	Exon 11	arg901-to-trp
5	C to T	Transition	Exon 5	pro704-to-ser

2.3.3.2.2 Pulmonary Fibrosis

Pulmonary Fibrosis is interstitial lung disease in which scars are made inside the lungs. Scars were formed by the accumulation of fibrous connective tissue. This accumulation leads to thinking of lungs wall and reduces the oxygen supply to the blood. Pulmonary Fibrosis occurs due to mutation in TERT gene and results in shortening of telomeres. Many mutations in TERT gene are associated with pulmonary fibrosis[123]. These mutations and change in amino acid are mentioned below in the Table 2.

Table 2: Transition, transversion and frame shift mutations associated with the Pulmonary Fibrosis.

S.N.	Mutation in TERT gene	Mutation	Position	Change in amino acid
1	G to A	Transition	2594	Arg to his
2	Deletion of T	Frame shift	2240	Truncated protein
3	G to A	Transition	Intron 1	-
4	T to A	Transversion	-	leu55-to-gln
5	A to C	Transversion	Intron 9	-
6	G to A	transition	2371 exon 7	val791-to-ile

2.3.3.2.3 Bone marrow failure

Bone marrow failure disease is characterised by production of insufficient RBC, WBC, and platelets. TERT mutation is one of the reasons for development of this disease. Telomerase with mutant TERT incapable of maintaining the telomere length resulted in reduced growth of bone marrow stem cells and produced an insufficient amount of blood cells. Missense mutation in TERT gene associated with Bone marrow failure[124]. These mutations are listed below (Table 3).

Table 3: Missense mutations in hTERT gene are associated with Bone marrow failure.

S.N.	Type of mutation	Position	Change in amino acid
1	Missense	Exon 2	his412-to-tyr
2	Missense	Exon 5	val694-to-met
3	Missense	Exon 7	tyr772-to-cys
4	Missense	Exon 15	val1090-to-met

2.3.3.3 Disease associated with gain of telomerase activity

Telomerase has high activity in actively dividing cells like stem cells and cancer cells[125]. Telomerase maintains the telomere length to check the cell from entering into replicative senescence[126]. Now, this is well established that around 90 % of human cancers have high telomerase activity. Ectopic expression of telomerase in normal and telomerase negative cells increase the life span of cells while inhibition of its activity leads to decrease the growth of cancer cells. During tumorigenesis telomerase

expression is reactivated. TERT expression in cells is regulated by many transcription factors and coregulators like c-myc, nf-kb, and beta-catenin. Increased telomerase expression and activity enables proliferatinig cancer cells to bypass checkpoint that is activated due to shortening of telomere which can induce cellular senescence and cell death.

2.3.4 Non-canonical function of telomerase

The first evidence of telomere-independent function of telomerase was observed in mice. Mice have very long telomere (20 to 50 kb) compared to human (5 to 15 kb). So murine tumor does not require telomerase to maintain the telomere length and prevent replicative senescence, but even in mice tumor has high expression of telomerase[127,128]. This suggests that TERT might be may be playing a role other than maintenance of telomere length. Ectopic expression of TERT in mouse mammary carcinomas and epidermal tumors increase cell proliferation[128]. Some human cells maintained the telomere length by ALT pathway. H-Ras is a GTPase protein involved in the regulation of cell growth. Constitutive expression of H-Ras in ALT positive cells does not transform the cell. However, co-expression of H-Ras and TERT effectively transformed the ALT positive cells[129,130]. So, if the only function of telomerase is telomere-length maintenance, then ALT mechanism would replace for telomerase. Further siRNA-mediated knock down of hTERT shows reduced cancer growth and proliferation while wild-type or catalytically mutant TERT promotes the cancer growth and cell proliferation[131]. Catalytic subunit (hTERT) of telomerase performed other multiple functions; it is found to be associated with RNA component of mitochondrial RNA processing endoribonuclease (RMRP) and works as RNA-dependent RNA polymerase[132]. The molecular mechanism behind the non-canonical function of telomerase is widely unknown. Now some reports are suggesting the extracurricular activity of hTERT. Recent advancement in non-canonical function of telomerase shows that it might work as a transcription factor or co-modulator and regulate the expression of another gene associated with cancer progression. Some important non-canonical functions of telomerase are discussed in the following section.

2.3.4.1 hTERT promotes the cancer cell growth and proliferation

Transgenic induction of telomerase in mouse skin epithelial cells induces the cells from resting phase to active dividing phase[133]. Telomerase activation is required for the immortalization of cells. Immortalized cells have high expression level of DNA methyltransferase I (DNMT1). hTERT regulates the transcription of growth factor by modulating the expression and activity of DNA methyltransferase I (DNMT1)[134]. Ectopically expressed hTERT in human fibroblast cells leads to high level of epiregulin that is a potent growth promoting factor[135]. Over-expression of hTERT in human mammary epithelial cells (HMECs) shows increase in growth of cells without exogenous mitogen. hTERT promotes the HMECs growth by enhancing the expression of EGFR gene. Inhibition of EGFR expression reverses the hTERT mediated proliferation of HMECs[136]. Catalytically inactive hTERT expression in mammalian skin cells promotes the keratinocyte proliferation and activates the dormant hair follicle stem cells. hTERT promotes the proliferation and stemness in skin cells by promoting the expression of c-Myc and Wnt pathway genes[137]. Alternative splicing in hTERT genes gives eight different variants. One variant Δ4-13 lacks catalytic domain required for telomerase activity. Inhibition of Δ4-13 variant in cells reduces the cell proliferation, while ectopic expression of Δ4-13 variant increased the cell proliferation. Δ4-13 variant regulates the cellular activity by modulating the Wnt signaling pathway[138]. All these studies show that hTERT have growth-promoting role in actively dividing cells independent of its role in maintenance of telomere length.

2.3.4.2 hTERT promotes the cell survivability and inhibits apoptosis

Cancer cells are resistant to apoptosis and senescence. Telomerase inhibits apoptosis and senescence by the maintenance of the telomere. However, some data shows that telomerase catalytic domain makes cancer cells resistant to apoptosis and senescence independent to telomerase function. hTERT expression in cancer cells antagonizes p53 mediated apoptosis[139]. hTERT expression reduces the activity of caspases 3, 8 and 9 and promotes the expression of anti-apoptotic protein Bcl-2 and reduced the expression of pro-apoptotic protein BAD, and BAX [140]. hTERT also interacts with PARP and p53 and reduces their expression in cancer cells to inhibit the apoptosis and senescence[141]. hTERT overexpression induces the production of intracellular ROS and enhances the mitochondrial function to inhibit the ROS-mediated apoptosis[142]. hTERT promotes the expression of Nf-kB target survival genes by interacting with Nf-kB

subset and recruitment to the Nf-kB target gene like IL-6 and TNF-α and help the cancer cells to escape from apoptosis[143].

2.3.4.3 hTERT promotes invasion and metastasis

Invasion and metastasis is a major step in cancer development. hTERT is found to be upregulated in these steps and promotes the epithelial to mesenchymal transition that is a most initial step in cancer progression[144]. hTERT directly interacts with ZAB1 and binds to the E-cadherin promoter and inhibits the E-cadherin expression and promotes the mesenchymal character[145]. hTERT interacts with beta-catenin and regulates the expression of Wnt pathway associated genes to induce the EMT and invasion in cancer cells [146]. hTERT interacts with c-Myc and binds to the heparinase promoter and promotes the expression of heparanase in cancer cells. Heparanase hydrolysed the heparan sulfate, extracellular matrices and helped in cancer cell invasion and migration. hTERT also is known to elevate the expression of other extracellular proteases independent of telomerase function. hTERT interacts with Nf-kB and these complex binds to the MMP 2 and 9 promoter and promotes its expression. MMPs degrade the extracellular matrices and facilitate cancer cells invasion and migration[147]. These results show that hTERT is involved in the cancer cells invasion and migration independent of telomerase function but more studies are needed to understand the hTERT mediated cancer invasion and metastasis.

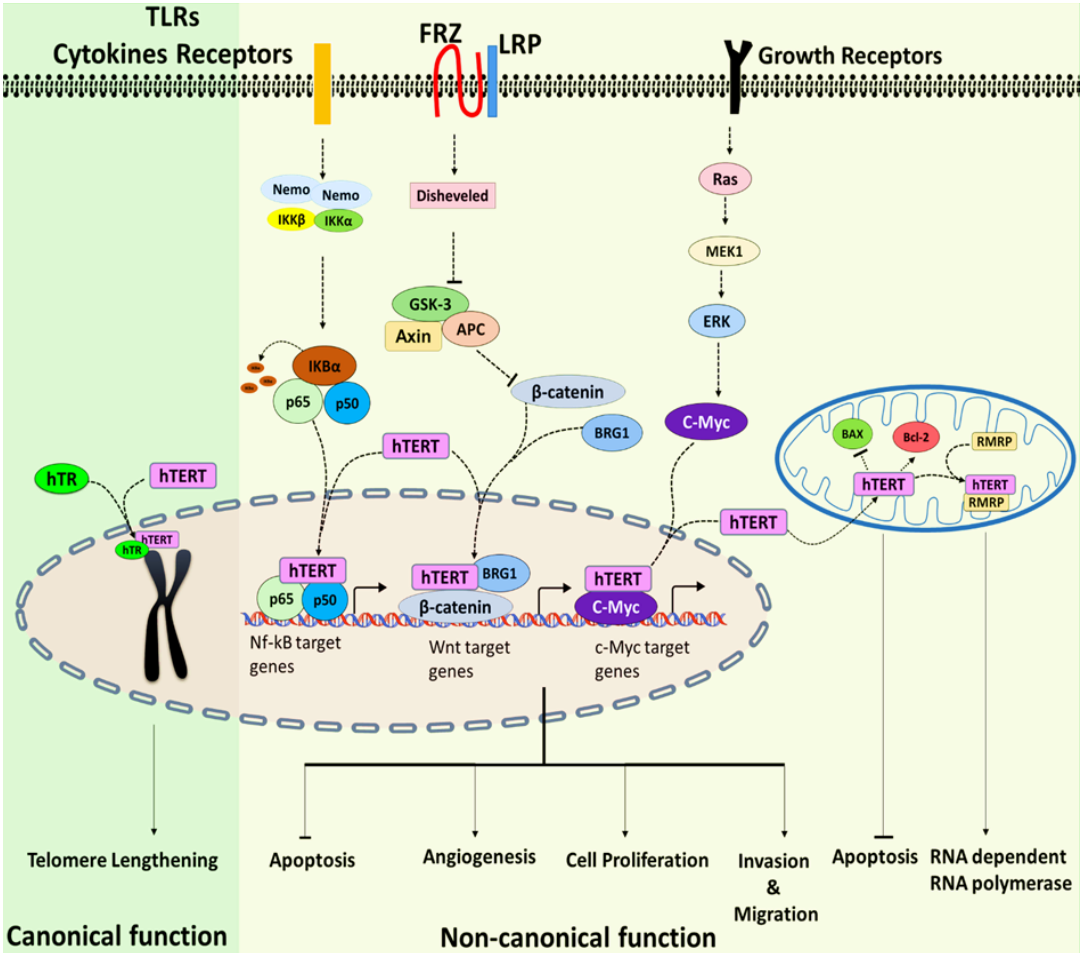

Figure 8: The proposed model represents the canonical and non-canonical function of telomerase in actively dividing and cancer cells.

2.4 c-MET, the hepatocyte growth factor receptor

2.4.1 Discovery of c-MET

Cooper et al. (1984) identified the transforming fusion protein in osteosarcoma cells as a potent oncogenic factor[148]. In 1985 Dean et al. demonstrated that MET is tyrosine kinase protein belonging to the tyrosine kinase family. c-MET shows the sequence similarity with human insulin growth receptor and ABL proto-oncogene[149]. Park et al. in 1987 established that MET is cell surface receptor protein and consists of the disulfide linked alpha subunit (50 kd) and beta subunit (145 kd). A fully processed MET protein has extracellular alpha and beta subunits, trans-membrane helices and intracellular tyrosine kinase domain[10].

2.4.2 c-MET Gene and transcripts

Dean et al. in 1985 mapped the c-MET gene on chromosome 7 by in situ hybridization. They had shown that gene encoding for c-MET was located at q31.2 of on chromosome 7 and size of the gene was around 145 kb[149]. c-MET gene has 21 exons and 20 introns, and after alternative splicing, it produces seven transcript variants (MET 201 to MET 207) (Fig.9 a)[150]. c-MET gene exon 1 to 12 code for the extracellular domain, exon 13 codes for transmembrane helix and exon 14 to 21 codes for intracellular domain region[151]. Transcript variant MET 201 has complete 21 exons and length of the transcript is 4632 nucleotides long (Fig. 9b). It codes for longest c-MET isoform 1408 aa[152]. MET 202 transcript variant consists of 6635 nucleotides and codes for 1390 aa long functional peptide (Fig. 9c)[151]. Other transcript variants (MET 203 to MET 206) lack many coding exons and translated into small peptides (Fig.9 d-g). Seventh transcript variant has five exon regions and two introns between second and third exon, and this transcript does not code for any protein(Fig.9 h)[151].

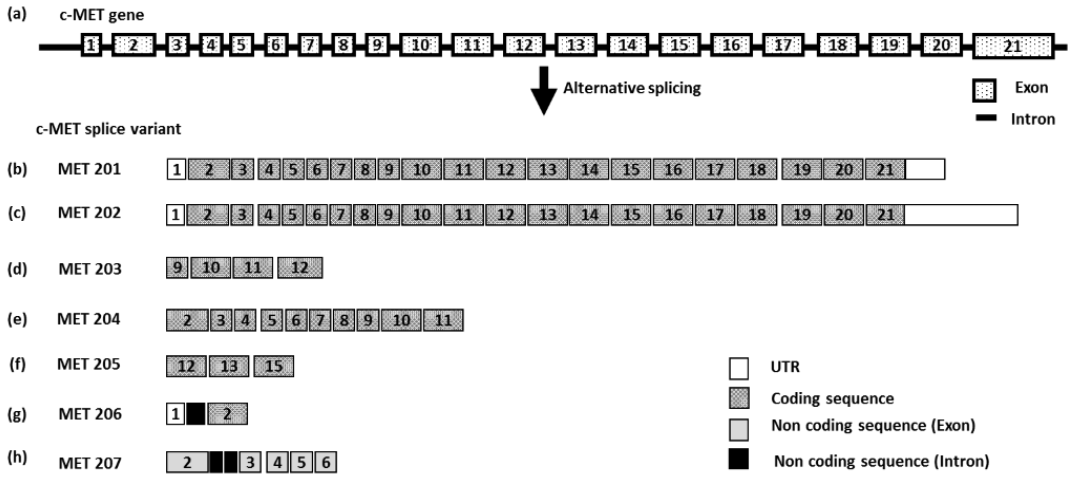

Figure 9: Different c-MET mRNA transcripts generated by alternative splicing.

2.4.3 Regulation of c-MET expression

Youhua Liu in 1998 characterized the c-MET promoter. The 2.6 kb of upstream DNA sequence contained most of the regulatory elements[150]. The c-MET promoter does not have TATA or CAAT elements, but it contains highly GC-rich sequence within a 600 bp upstream region of transcription start site (positions −400 ~+200)[153].

2.4.3.1 Positive transcription regulator of c-MET

Sequence analysis of c-MET promoter shows strong positive factor- binding site present at position -233 to -68 bp. This region contains multiple Sp1 binding sites. Deletion of DNA fragment corresponding to -223 to -68 bp significantly reduced promoter activity. These multiple Sp1 sites in the proximal promoter region are responsible for c-MET constitutive expression of c-Met[150]. c-MET core promoter region has activator protein-1 (AP-1) binding site (TGAGTCA) at position -158 to -152. The c-MET promoter has binding site for SP family of transcription factors (Sp-1 and Sp-3) adjacent to the AP-1 site. SP binding depends on the binding of AP-1 to its associated site in the c-MET promoter region. The increase in the level of AP-1 protein enhances the c-MET promoter activity while inhibition of AP1 binding reduced the c-MET promoter activity[154]. Promoter analysis of c-Met gene shows Nf-kB binding site at −745 to −697 from transcription start site. C/EBP transcription factor also has binding site within this region upstream of the Nf-kB binding site[155]. Nf-kB and C/EBP physically interact and synergistically promote the expression of several genes. Recent findings show that C/EBP and Nf-kB form a complex and bind to the c-MET promoter to promote c-MET expression[155]**.** c-MET expression is also regulated by Wnt signaling pathway. β-catenin/ T-cell factor (TCF) bin d to the c-MET promoter and activate transcription [156]. Some other molecules like Ets (E-twenty six), and Pax3 (paired box 3) also promote the expression of cMET during the development and cancer progression[157,158].

2.4.3.2 Negative regulator of c-MET expression

p53 is tumor suppressor protein, expressed in the cell to protect the cell from uncontrolled growth. But in cancer cells, p53 is reduced or mutated. Reduced p53 expression or mutant p53 help the cancer progression by enhancing the cell proliferation, survivability, invasion, and migration. Some recent reports show that p53 regulates the cancer cell invasion and motility by regulating the expression of c-MET. Wild-type p53 regulates the expression of c-MET by two ways: (i) transactivation of miR-34 gene whose transcript targets the c-MET, (ii) inhibition of the binding of SP1 at the c-MET promoter causing down regulation of c-MET expression. Some cancer cells express mutant p53 lacking the suppressor properties[159]. Esophageal squamous cell carcinoma is an aggressive human cancer expressing mutant p53(R175H) that lacks binding capability on the c-MET promoter, so the c-MET expression remains high[160].

2.4.4 Structure of c-MET

c-MET is synthesized as a 170kDa single chain large precursor protein (pro-c-MET) which undergoes proteolytic processing in the post-Golgi compartment by serine proteases[161]. After processing c-MET forms a heterodimer which consists of alpha and beta subunits. c-MET is single-pass, disulphide-linked α/β heterodimer which consists of extracellular, transmembrane and cytoplasmic region[10] (Fig. 10).

2.4.4.1 Extracelluar region

The extracellular region of fully processed c-MET is composed of three domains; SEMA domain, PSI domain, and IPT domain. The extracellular region comprises of 25 to 932 aa residues of the c-MET protein. The extracellular region includes binding sites for c-MET with its natural ligand HGF[162].

I. SEMA domain: This is the N-terminal region of the c-MET protein and consists of 500 aa residues which fold to form a large semaphorin (SEMA) domain, which includes the entire α chain and part of the β chain (Fig. 10). c-MET SEMA domain consists of the seven-bladed β-propeller structure. SEMA domain helps in Met receptor dimerization. It is also involved in interaction with the β chain of HGF. SEMA-HGF interaction is responsible for activation of c-MET signaling cascade[163].

II. PSI domain: PSI domain is a cysteine-rich domain found in the extracellular region of c-MET and spans over approximately 50 aa residues[162]. PSI domain is receptor important for c-MET function. PSI consists of cysteine knot with short regions of secondary structure including a three-stranded antiparallel beta-sheet and two alpha-helices. c-MET, PSI domain has eight cysteine residues involved in disulfide bonds. PSI domain links SEMA and immunoglobulin domains and is responsible for the correct positioning of the ligand-binding site of the receptor[164] (Fig. 10).

III. IPT domains (immunoglobulin-like regions in plexins and transcription factors): IPT has four immunoglobulin-like domains (IPT1-IPT4), which comprise four disulfide-linked loop structures (Fig. 10). IPT domain of Met cooperates with the SEMA domain in binding to HGF. IPT-HGF-alpha interaction provides binding strength, and the SEMA-HGF-beta interaction involves the activation of c-MET protein[162,165].

2.4.4.2 Transmembrane region

Transmembrane region spans 933 to 955 aa residues of the c-MET protein. These residues form helices and connect the extracellular region to cytoplasmic region[166] (Fig. 10).

2.4.4.3 Cytoplasmic region

This region includes 956 – 1390 aa residue of c-MET protein[162]. The cytoplasmic domain consists of three domains:

I. **Juxtamembrane domain (JM):** Juxtamembrane domain (JM) contains Serine at 975 position which undergoes phosphorylation and negatively regulates the c-MET activity. This domain also has a tyrosine residue at 1003 position which is involved in the endocytosis of c-MET after phosphorylation (Y1003) (fig. 10).

II. **Tyrosine kinase (TK) domain**: Tyrosine kinase (TK) domain contains two important tyrosine residues at 1234 and 1235 position. Tyrosine kinase (TK) domain kinase activity is up-regulated by phosphorylation at these two tyrosine residues[167] (Fig. 10).

III. **C-terminal multifunctional docking site (MFDS):** C-terminal multi-functional docking site (MFDS) mediates recruitment of cytoplasmic signaling molecules and adaptor proteins via Y1349 and Y1356[168] (Fig. 10).

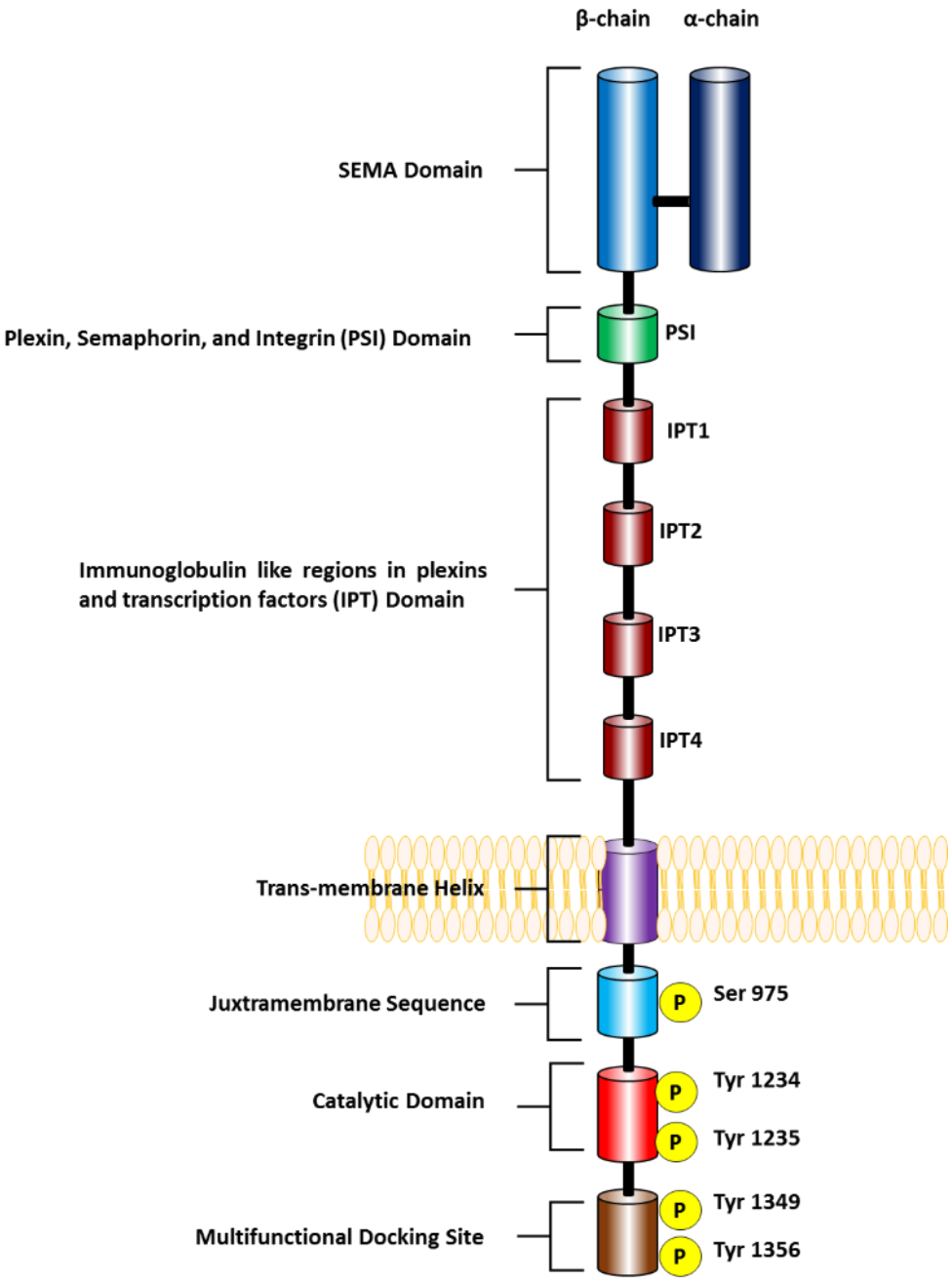

Figure 10: Model structure represent the multidomain component of c-MET protein.

2.5 Multiple forms of HGF

Gohda et al. (1988) isolated and purified HGF from hepatic failure patient's plasma. They had shown that HGF exists in various forms with the molecular mass between 76 and 92 kD. HGF contains two chains, heavy (54 to 65 kD) and light (31.5 and 34.5 kD and these chains are associated with each other by disulfide bonds[169]. Miyazawa et al. (1989) and Nakamura et al. (1989) sequenced cDNAs encoding HGF. Cloned sequence codes for pre-pro-protein that comprises of 728 aa residues. Single mRNA encodes for

heavy and light chains of HGF and translated as a common peptide which is processed by the proteases to give rise to two subunits[170,171]. Gherardi and Stoker (1990) found a structurally similar factor to HGF that stimulates the scattering of epithelial cells. They had termed this factor as scattered factor(SF)[172]. Later in 1991 Weidner et al. confirmed that HGF and SF are same proteins encoded by a single gene and also these two proteins have similar structure and function[173].

2.5.1 HGF gene and transcripts

Fukuyama et al. (1991) had shown that gene encoding for HGF present on chromosome 7 by spot-blot hybridization[174]. Further Weidner et al. (1991) mapped the HGF gene on q11.2-q21 region of chromosome 7[173]. Later Saccone et al. (1992) by using non-isotopic *in-situ* hybridization method mapped the HGF encoding gene on chromosome 7q21.1[175]. HGF gene encodes for five transcript variants. The HGF gene consists of 18 exons and 17 introns spanning 91.5 kb. HGF transcript undergoes alternative splicing and produces five variants. Transcript variant-1 has 5989 nucleotides that encode the longest isoform that consists of 728 aa residues. Transcript variant-2 has deficiencies of multiple 3' exons but contains an alternate 3' exon comparative to variant 1. Variant-2 is 1184 long nucleotide transcript and encoded truncated protein containing 290 amino acids. Transcript variant-3 consists of 2249 nucleotides and encodes 723 amino acids long peptide. Variant-3 lacks FLPSS sequence at positions 162-166 compared to variant-1. Transcript variant-4 contains 1111 nucleotides and encodes for 285 amino acids long peptide. This variant is a combined form of variant-2 and 3. Transcript variant-5 consists of 1990 nucleotides and lacks multiple 3' exons but has an alternate 3' segment that is only present in this variant. Variant-5 encodes for 210 amino acids long peptide[176,177].

2.5.2 Structure of HGF

HGF/SF is a multidomain protein. It is synthesized as pre-pro-HGF (728 aa) which is cleaved at or near 37 position of the peptide by proteinases into pro-HGF before secretion in ECM. Single chain pro-HGF is further cleaved between residue 94 and 95 to produce biologically active heterodimeric HGF[171]. Final activation of HGF/SF is achieved by three serine proteinases: HGF activator (HGFA), type II transmembrane enzymes matriptase and hepsin[178]. Matriptase and hepsin proteinases are expressed on

MET-expressing target cells ECM[178]. HGFA is a soluble proteinase, activated by thrombin that is associated with tissue-regenerative responses[179]. These proteinases are associated with the modification of two HGF inhibitors, which are HGF activator inhibitor 1(HAI1) and HGF activator inhibitor 2 (HAI2)[180,181]. Cancer has high level of matriptase and hepsin expression which regulate the expression level of HAI1 and HAI2 to promote the activation of HGF[11].

Mature HGF consists of a 69 kDa α chain (32 to 494 aa residues) and a 34 kDa β chain (495 to 728 aa residue). The α chain contains N-terminal domain and four kringle domains (K1–K4). The β chain contains serine proteinase homology (SPH) domain[182] (Fig. 11).

I. **N-terminal domain:** The N-terminal (N) domain of HGF contains a hairpin-loop that is essential for binding with the receptor. The N-terminal domain has heparin or heparan sulfate binding site, which promotes receptor-ligand interaction and modulates c-MET-dependent signaling[183].

II. **Kringle domain:** HGF has four kringle domains K1 to K4. Kringle domain 3 and 4 have structural similarity to c-MET, IPT3, and IPT4 (Fig. 11). HGF N-terminal domain and first kringle domain are necessary for tight binding with c-MET receptor[184].

III. **Serine proteinase homology (SPH) domain:** HGF at c-terminal region has a serine proteinase homology (SPH) domain that is structurally similar to the catalytic domain of serine proteinases, but enzymatically inactive (Fig. 11). This domain has low-affinity interaction with SEMA domain of c-MET[184,185].

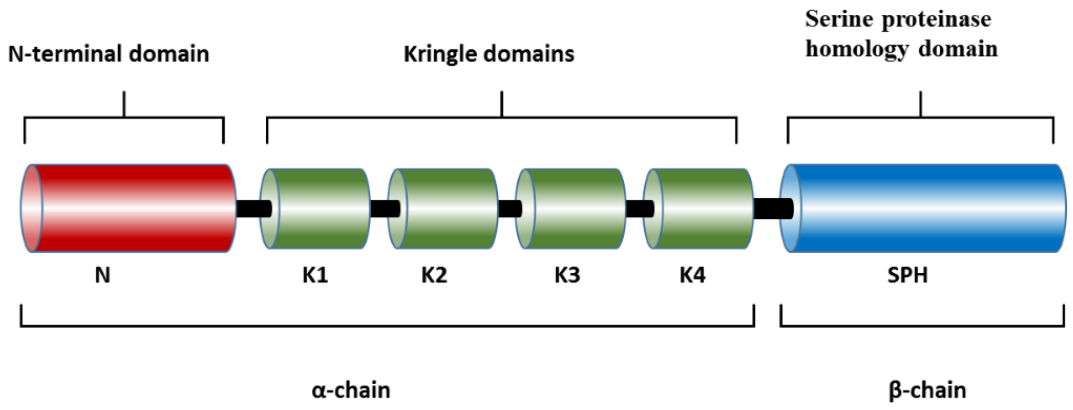

Figure 11: Multidomain structure of HGF protein.

2.5.3 HGF/c-MET signaling

Stromal cells secrete HGF which binds receptors of the adjacent cells and activates the signaling cascade in a paracrine fashion (Fig 12). HGF binds to the c-MET and induces dimerization of receptor and trans-autophosphorylation of the tyrosine amino acid (Y1003) in the juxtamembrane domain, and tyrosine 1234 and 1235 residues (Y1234 and Y1235) of the catalytic domain. Active catalytic domain induces the phosphorylation at tyrosine residues Y1349 and Y1356 of the serine proteinase homology (SPH) domain [185] (Fig. 12). Phosphorylation at tyrosine Y1349 and 1356 promotes the recruitment of intracellular adaptor proteins like; GAB1, GRB2, phospholipase C (PLC), PI3K and SRC[186,187] (Fig. 12). These proteins are phosphorylated on tyrosine residue after interaction with the c-MET receptor and start downstream signaling. These adaptor proteins activate several signaling pathways and cellular responses[188–190]. PI3K activates the AKT that activates downstream mTOR, GSK3β, and MDM2/p53 pathways to promote cell survivability and cell proliferation (Fig. 12). c-MET also promotes the cell survivability by activating the STAT3 pathway[167,187]. c-MET-mediated activation of PI3K and Src promotes the nuclear localization of NF-kB by activating the IKK complex which increased phosphorylation of IkB-α that undergoes proteasomal degradation and released the NF-kB complex that moves to the nucleus and promotes expression of genes associated with survivability and proliferation[191] (Fig. 12). GAB1, GRB2, SOS, and SRC complex is activated in response to c-MET which activates the Ras/MAPK cascade[192]. Ras small GTPase which is activated by son of sevenless (SOS) and inactivated by GTPase activating proteins (GAPs), like; p120 Ras-GAP (p120). c-MET activates the SOS and inhibits p120[167]. Mitogen-activated protein kinase (MAPK) cascades have three subfamilies, and each subfamily comprises of three protein kinases which activate one another sequentially. The terminal transcription molecules like; (ERKs), (JNKs) and p38s are translocated to the nucleus and regulate the genes responsible for survivability and proliferation[167,193–195] (Fig. 12). SRC, Ras/MAPK, and PI3K also activate focal adhesion kinase and cytoskeleton remodeling complex that is responsible loss of cell adhesion and change in orientation of cytoskeleton protein to promote the cell migration and invasion[196].

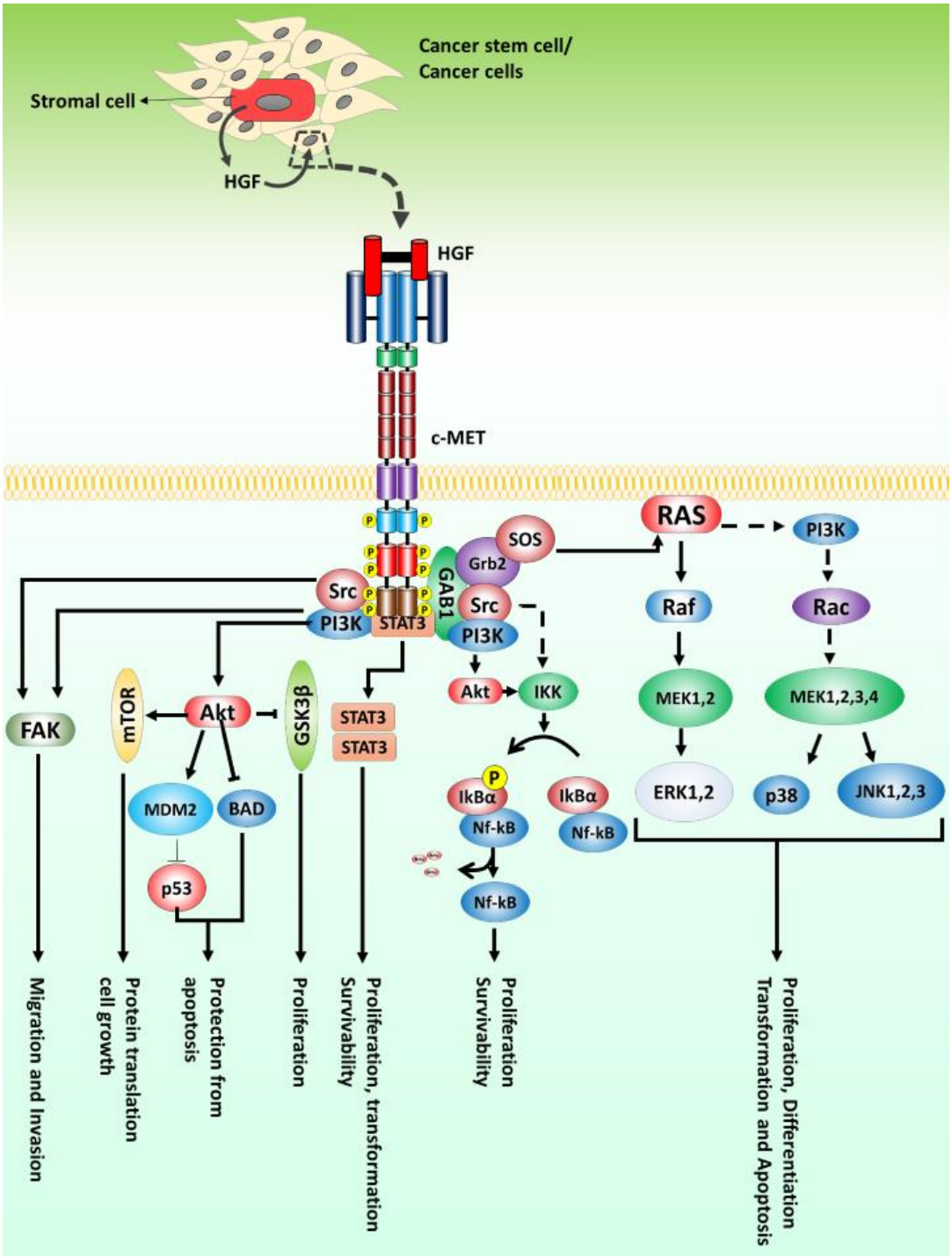

Figure 12: HGF/c MET signaling pathway components, interaction network, and mechanisms of regulation of the different cellular process.

2.5.3.1 Role of c-MET in cancer progression

HGF/c-MET signaling is essential for growth and survival of hepatocytes and placental trophoblast cells during development. Deletion of c-MET or HGF in developing embryos leads to reduced growth of liver and the placenta[197,198]. The HGF/c-MET

signaling is also involved in the proliferation and motility of muscle progenitors cells. These muscle progenitors cells split into thin layers from the dermomyotome and move to limbs, tongue, and diaphragm, where these cells differentiate to form hypaxial muscles. Deletion of HGF or c-MET genes leads to loss of migration of muscle progenitors cells[199]. HGF/c-MET signaling promotes the liver regeneration of chemically or surgically removed liver. HGF from partially hepatectomized subject activates the c-MET of hepatocytes in a paracrine manner that promotes the hepatocytes growth and liver regeneration[200]. c-MET signaling also plays an important role in wound healing. HGF along with FGF and EGF promotes the re-epithelialization[201,202]. Depletion of HGF or c-MET led to reduced cell proliferation and impaired liver regeneration and reduced the wound healing process[202,203]. HGF/c-MET signaling is tightly regulated during development, regeneration and wound healing. This signaling process promotes the regeneration of terminally differentiated cells. Cancer seems like a persistent regeneration process that is unable to define its end point. Tumors have been represented as wounds that do not heal[204].

2.5.3.2 Genetic abnormalities that cause aberrant HGF-MET signaling

c-MET mutation led to sporadic and inheritable renal, osteofibrous, hepatocellular and several other cancer types[205]. Most of these mutations occur in the catalytic domain of c-MET. Replacement of endogenous c-Met with a mutant form in the mouse germ line causes development of tumors in mouse[206]. The ectopic expression of c-MET in cells promotes mesenchymal feature[207]. Thus, increased expression or expression of mutant c-MET can initiate cancer progression. Some of the mutations in the c-MET gene and associated abnormality are listed below (Table 4).

Table 4: Different type of mutation in the c-MET gene and change in protein composition and associated disease.

S.N.	Type of mutation	Position	Change in amino acid	Cancer type
1	T-C transition	Exon 17	Met1149-to-Thr	Renal cell carcinoma[205]
2	G-T transversion	Exon 18	Val1238-to-Ile	
3	G-A transition	Exon 19	Val1238-to-Ile	
4	G-A transition	Exon 19	Asp1246-to-Asn	
5	G-A transition	Exon 19	Tyr1248-to-Cys	
6	C-G transversion	Exon 18	Leu1213-to-Val	
7	A-G transition	Exon 16	His1112-to-Arg	
8	C-T transition	Exon 17	Thr to Ile	Hepatocellular carcinomas[208]
9	G-A transition	Exon 19	Met to Ile	
10	A-G transition	Exon 19	Lys to Arg	
11	G-C transversion	-20 bp in Promoter	_____	Unclassified[209]
12	26-bp deletion	Exon 14	Deletion of Leu964 to Asp1010	Osteofibrous dysplasia[210–213]
13	Deletion of exon 14	Exon 14	_____	

2.5.3.3 Crosstalk between MET and other signaling pathways

c-MET interaction with EGFR TGF-β, ERBB2 and IGF1R are reported in disease associated condition[214,215]. Interaction of c-MET with other receptor proteins is an important mechanism for cancer development and resistance to the therapy[216]. c-MET also shows the interaction with developmental signaling pathways like; TGF-β and Wnt-β catenin[217]. c-MET and integrin α3β1activates the transcription of WNT7B and HGF/c-MET signaling also induces the nuclear localization of β-catenin-TCF complex in the nucleus to promote the transcription of Wnt target genes[218,219]. c-Met interacts with plexin B1 receptor in epithelial cells and binds with the Sema4D ligand to activate signaling pathways associated with invasion and migration[220,221]. c-Met and CD44

interaction are required for HGF mediated migration and invasion in cancer cell lines[222]. Pancreatic carcinoma cells have high expression of the IGF-I receptor (IGF-IR) which along with c-Met is known to promote migration and invasion in cancer cells. Treatment of pancreatic carcinoma cell with HGF or IGF-I or both inhibits the cell migration and invasion. c-MET alone enhances the cancer progression, but with interaction with other receptor molecules, c-MET increases the cancer malignant properties and resistance to therapy[214].

2.5.3.4 HGF/SF and MET in angiogenesis

Angiogenesis is an essential step in the growth of tumor and metastasis. The vascular endothelial growth factor and its receptor (VEGFR) have principal role in tumor growth and metastasis[223]. HGF/SF–MET are also known to induce the endothelial cell growth[224]. c-MET induces VEGFR expression in cancer cells to promote angiogenesis, and it also activates the VEGFR signaling molecules SHCs[225]. HGF/cMET signaling suppresses the expression and activity of Thrombospondin 1 (TSP1) that is a negative regulator of angiogenesis[226]. c-MET and VEGFR interaction is not known till date but these two proteins synergistically regulate the common signaling intermediates like; ERK– MAPK, AKT and focal adhesion kinase (FAK)[225]. A recent finding shows that hypoxia induces the expression and activity of c-MET in cancer cells[227]. Hypoxia also promotes the expression of the hypoxia-inducible factor 1α (HIF1α) which activates the VEGFR to increase the angiogenesis in the tumor. Inhibition of HIF1α reduces the vascularization and increases the hypoxia in tumor thus targeting the vascularization in tumor may leads to c-MET induces tumor cells spreading[228,229].

2.5.3.5 HGF/SF and MET signaling in metastasis

Epithelial-mesenchymal transition (EMT) is an initial essential step of metastasis that involves the loss of cellular polarity and adhesion and gain of fibroblast-like properties. EMT is a cellular process that is also required during embryonic development. Several developmental processes like; gastrulation, neural crest formation, and heart morphogenesis require the epithelial to mesenchymal transition[230]. A similar mechanism is utilized by tumor cells to escape from the tumor mass. HGF treatment to the kidney epithelial cells changes cells into migratory fibroblast-like cells[231]. HGF promotes the expression of SNAIL by activating the ERK MAPK pathway[232]. HGF /c-MET signaling promotes expression of the mesenchymal markers such as vimentin, N-

cadherin, and fibronectin while it reduces the epithelial marker E-cadherin and cytokeratin[233,234]. Cells are attached to each other by the adhesion proteins. Adhesion proteins can be categorized into three group; adherents' junctions, tight junctions, and gap junctions. These proteins maintain the intercellular connection and membrane integrity. Loss of cell-cell adhesion is important for the cancer metastasis which is achieved by regulation of adhesion proteins[235]. HGF regulates the cancer cell metastasis through altering the cell junctions by regulating the stability and the expression of adhesion proteins. HGF regulates the expression of tight junction molecules like; occludin and claudin in cancer cells[236]. HGF/c-MET signaling also regulates the expression of other cell adhesion molecules like E-cadherin and ZO1 which are involved in cancer cell migration and invasion. Expression analysis of c-MET and E-cadherin in gastric cancer shows that these two proteins have a inverse correlation. Gastric cancer tissue has high expression of c-MET and low expression of E-cadherin. Further, overexpression of c-Met in gastric cancer cell reduces the expression of E-cadherin[237]. ZO1 is an important molecule involved in cell-cell adhesion. HGF treatment in H1299 cells reduces the expression of ZO1 to promote the cancer cell scattering[238]. Cells are attached to the extracellular matrices and substratum through the transmembrane protein known as focal adhesins (FAs). Intracellular part of focal adhesin provides the binding sites for various signaling molecules by which the cells monitor their environment. Formation and disassembly of focal adhesins are regulated during cancer cell migration. Integrins are cell surface receptor required for the cell to the extracellular matrix (ECM) adhesion[239,240]. HGF treatment in cancer cells promotes adhesion to some ECM proteins like laminins 1 and 5, fibronectin, and vitronectin[241]. HGF also promotes the expression and clustering of integrins at the site of invadopodia formation[242]. Cytoskeleton reorganization is another step essential for the cell movement and invasive behavior. HGF/c-MET signaling is involved the cytoskeleton remodeling, and its importance in cytoskeleton dynamics is still expanding. Cytoskeletal remodeling is of central importance in the development of cancer cell phenotype and invasive behavior. The reorganization of the cytoskeleton is regulated by the Rho family of small GTPases. Three kinds of GTPases are well characterized in the cytoskeleton remodeling that is RhoA, Rac1, and Cdc42. RhoA regulates reorganization of stress fibers and assembly of focal adhesins[243]. Rac1 is involved in the formation of lamellipodia[243] and Cdc42 is associated with the formation of filopodia which help in the directional movement of cancer cells during migration[244].

HGF/c-MET signaling regulates cytoskeleton reorganization through these small GTPase activity[245–247]. HGF/c-MET signaling promotes the activity of effector molecules like Arp2/3, WASP/WAVE, cortactin, and coflin[248–250]. Extracellular matrix (ECM) degradation is one of an essential steps in metastasis which provides the path for migratory cells. ECM degradation is achieved by activation of extracellular proteases that are present outside the cells in latent form. HGF is involved in the expression and activation of matrix metalloproteinase (MMP-2, MMP-3 and MMP-9) which degraded the ECM to promote the cell migration and invasion[251,252].

MATERIALS AND METHODS

3 MATERIALS AND METHODS

3.1 Cell culture and transfection

Cells used for present study were obtained from NCCS, Pune are listed in the Table 5. A549, H1299 and HepG2, Cells were grown in DMEM media (Gibco) and HCT116 and HCT116 p53-/- cells were cultured in RPMI media (Himedia) supplemented with 10% FBS (Gibco) and 100 Units/ml penicillin, and 100 µg/ml streptomycin (Himedia) at 37°C in a humidified chamber and 5% CO2. Puromycin (Sigma) was used for selection of stable cell line. Both A549 and H1299 cells were selected at 2.0 µg/ml concentration of puromycin. Lipofectamine 3000 (Invitrogen) and Metafectene (Biontex) were used to transfect the cell.

Table 5: List of cell line

Cell line	Characteristic	Morphology
A549	Lung Carcinoma Hypo-triploid	Epithelial
H1299	Non-small cell lung Carcinoma The cells have a homozygous partial deletion of the p53	Epithelial
HEK 293T	human embryonic kidney cells	Epithelial
HCT116	colon cancer Diploid	Epithelial
HCT116 p53-/-	Colon cancer Diploid Homozygous deletion of p53	Epithelial
HepG2	Liver carcinoma	Epithelial

3.2 Cloning

3.2.1 hTERT and p53 were cloned in lentiviral pLVX-puro expression

hTERT is 127 kDa protein expressed mostly in dividing cells. hTERT has seven transcripts, of which one transcript contains all functional components. For exogenous expression of hTERT, we used the pBABE-hTERT construct from Addgene (Fig. 13a & b). We sub-cloned the hTERT in pLVX-puro vector between EcoRI and XbaI sites. hTERT cDNA was PCR amplified by Pfu DNA polymerase and purified by Qiagen kit.

PCR product and pLVX-puro vector were digested with EcoRI and XbaI and ligated with T4 DNA ligase (Promega). A549 cDNA was used to clone the p53 coding region in the pLVX-puro expression vector. CDS region of p53 was PCR amplified by Pfu DNA polymerase and cloned in between BamHI and EcoRI. Ligated product was transformed into the DH5α and plated on Ampicillin (100µg/ml) containing agar plate. Positive colonies were screened by colony PCR and confirmed by sequencing.

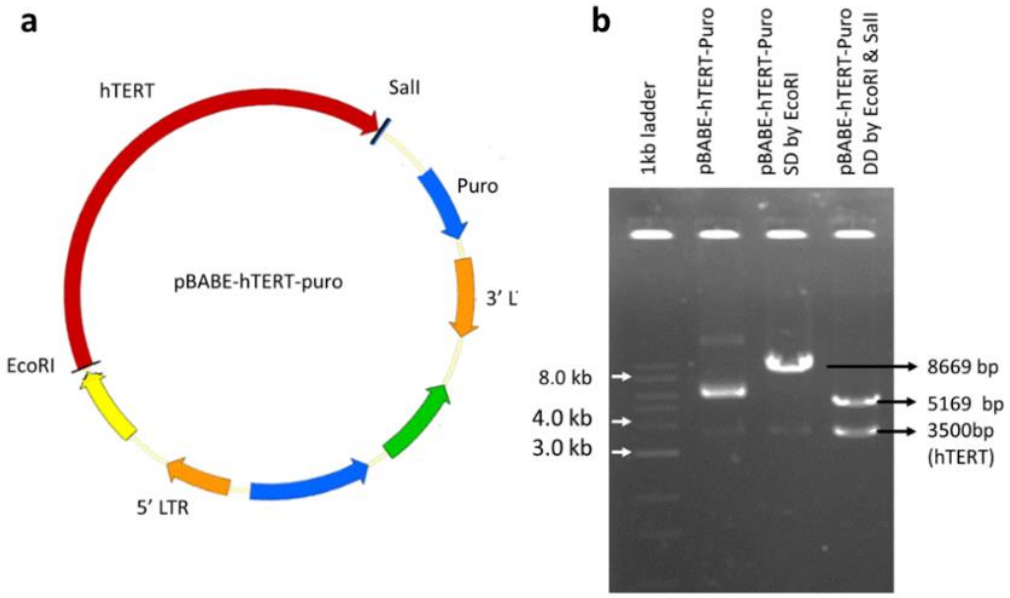

Figure 13: hTERT gene cloned in pBABE-puro vector.

a) Vector map of pBABE-HTERT-puro. b) Size of insert (hTERT) was confirmed by double digestion by EcoRI and SalI.

Table 6: Expression construct and cloned genes.

Constructs	Gene	Selection marker
pBABE hTERT	hTERT	Puro
pLVX-hTERT-HA	hTERT tag with HA	Puro
pLVX-p53	p53	Puro

3.2.2 Cloning of shRNAs in pLVX-shRNA, pLKO1-shRNA vector

shRNAs were used to knock down the expression of the genes by targeting the gene's transcript. shRNAs were designed using WI siRNA Selection Program. Sense and antisense both strands were synthesized from Sigma. Both strands were dissolved in nuclease-free water, and equal moles of both strands were added in PCR tube and

incubated at 95 ^{0}C for 5 minutes. The mixture of oligos was allowed to cool to room temperature for 10 minutes. Annealed Double stranded DNA was ligated in pLKO1-shRNA vector between AgeI and EcoRI and between BamHI and EcoRI in the pLVX-shRNA1 vector. Ligated products were transformed into DH5α and plated on ampicillin (100 µg/ml) containing agar plates. shRNA positive colonies were screened by colony PCR and confirmed by sequencing. shRNAs used in this study are listed in Table 7.

Table 7: Lentivirus shRNA constructs and target genes

Constructs	Gene	Selection marker
pLKO1-hTERT-shRNA	hTERT	Puro
pLKO1-p65-shRNA	RELA	Puro
pLKO1-c-Myc-shRNA	c-Myc	Puro
pLKO1-Scramble shRNA	Scramble	Puro
pLVX-c-MET-shRNA	c-MET	Puro
pLVX-p50-shRNA	NF-kB1	Puro
pLVX-p53-shRNA	p53	Puro

3.2.3 Cloning of HGFR/c-MET promoter in pGL3 vector

Genomic DNA was isolated from the A549 cells by Qiagen kit and used to amplify HGFR/c-MET promoter (1850 bp) by Pfu DNA polymerase at 68 ^{0}C annealing temperature. HGFR promoter was cloned into pGL3 vector between XhoI and HindIII restriction site. The ligation product was transformed into DH5α and insert positive colonies were screened by colony PCR. Cloning was confirmed by double digestion with XhoI and HindIII and sequencing (Fig. 14a & b).

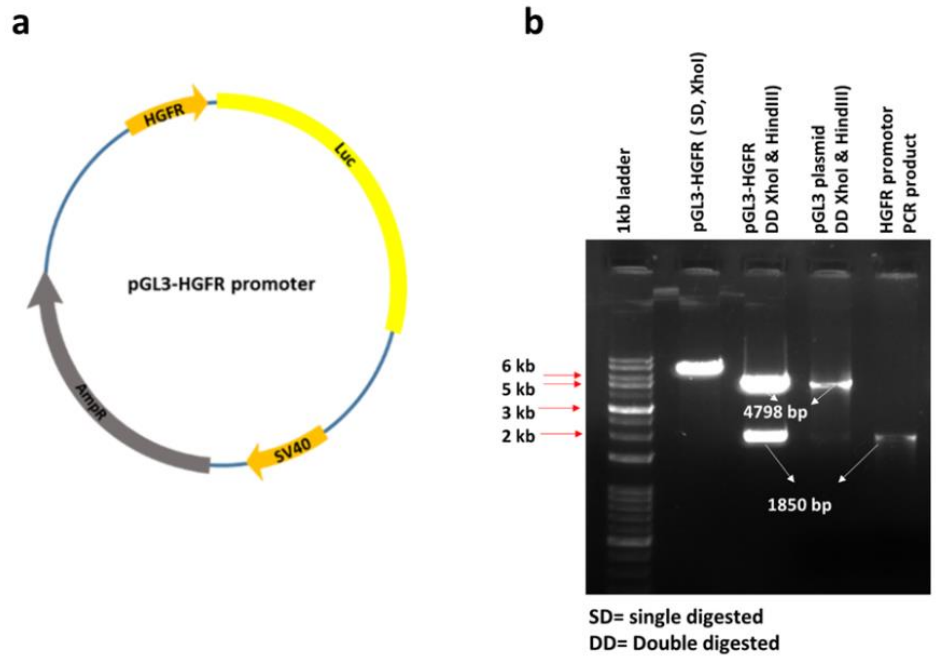

Figure 14: Cloning of HGFR/c-MET promoter in pGL3 vector.

a) Vector map of cloned c-MET 1.85 kb promoter in pGL3 luciferase vector. b) Agarose gel picture of double and single digested pGL3-c-MET promoter construct that confirms the right length of insert in pGL3 vector.

3.3 Lentiviral particle synthesis

1. 0.8 x 10^6 293T cells were seeded in 60 mm dish. Cells were grown in DMEM complete media at 37°C and 5% CO_2 for 24 hours.
2. Next day, old media was replaced with fresh 2 ml complete DMEM media.
3. Lentiviral vectors mix were prepared as described below for 60 mm dish. We used second-generation lentiviral vectors that have two packaging vectors and one transfer vector. Transfer vector, psPAX2, and pMD2.G were used in 4:3:1 ratio for lentivirus production (Table 8).

Table 8: Plasmids used for lentivirus production form 60 mm dish

Plasmids	Amount of plasmids (µg)
psPAX2	1.5 µg
pMD2.G	0.5 µg
Transfer vector	2.0 µg

4. Lentiviral vectors were added in 250 µl optiMEM, and in a separate tube lipofectamine (2000 or 3000) was added in 250 µl optiMEM as per manufacturer protocol. After 5-minutes lentiviral vector solution was added in lipofectamine-containing tube. Plasmids and lipofectamine mixture was incubated for 15 to 20 minutes at room temperature.

5. After 15 to 20 minutes the plasmids-lipofectamine mixture was added drop wise to 293T cells.

6. Transfected cells were incubated for 6 to 8 hours at 37 ^{O}C and 5% CO2.

7. Transfection media was removed after 6 to 8 hours and replaced with 3 ml fresh complete media. Cells were incubated for next 12 hours.

8. After 12 hours used media was discarded in sodium hypochlorite solution. Fresh 4 ml complete media was added to the plate and incubated in cell culture incubator for 24 hours.

9. After 24 hours, the first batch of viral particles in media were harvested and stored at -80 ^{0}C as aliquots. Cells were supplemented with fresh complete media and incubated for another 24 hours for next round of viral particle production.

10. Next day second viral media was harvested and stored at -80°C. Virus-producing Cells were incubated with 100% sodium hypochlorite for 15 to 30 minutes.

3.4 Transduction and cell selection

1- Cells were plated in 60 mm dish, and at 70 to 80 % confluency polybrene was added in the plate at 10 µg/ml concentration before transduction. Virus containing media was added to the plate and incubated for 12-18 hours.

2- After 12 to 18 hours media was replaced with fresh complete media and cells incubated for another 12 hours.

3- Virus transfected cells were split and cultured in selection media containing puromycin. In parallel, healthy cells also were grown in selection media as a control for cell selection.

4- Virus transfected cells were cultured in selection media until all the cells in control plate died. Then transfected cells were grown in maintenance media that has lower concentration (1 µg/ml) of selection antibiotics.

Table 9: Concentration of puromycin antibiotics for selection different cancer cells

Cell lines	Puromycin concentration
A549	2.0 µg
H1299	1.8 to 2.0 µg
HepG2	2.0 µg
HCT116, HCT116 p53-/-	1.0 µg
293T	2.0 µg

3.5 Cell proliferation assay

Cells were transduced with lentivirus for target genes or 2.0 x 10^4 cells per well seeded in 12 well plate in triplicate were transiently selected. Cells were allowed to grow for next 1, 2, 3 and 4 days. Each day cells were trypsinized and diluted with appropriate amount of media and counted with haemocytometer. Graph Pad software was used for making graph and statistical analysis.

3.6 Wound healing assay

Wound healing assay was used to study the *in-vitro* cell migration. To assay, the cell migration, around 8 x 10^5 cells were seeded per well in 6 well plates and allowed to adhere to the surface for 12 hours. After 12 hours cells were treated with mitomycin c (5 µl/ml for A549, 2.5 µg/ml for H1299) and incubated for 1 hour. Wounds were created in confluent cells artificially with 200 µl tip. Cells were washed with media to remove the cell debris. Cell migration was monitored at 0, 12, 24 and 36 hours. Cell movement was calculated at different time intervals. In wound healing, assay cells migrate and fill the artificially created gap. This movement mimics the in-vivo cell movements. Statistical analysis and the graphical representation is done by Prism Graph Pad software.

3.7 Colony formation assay

Colony formation assay was performed to observe the cancer cell survivability and proliferation capacity in the isolated condition. Cells were trypsinized and counted with haemocytometer. About 1000 cells were seeded per well in six-well plates and supplemented with 2 ml complete media. Cells were incubated in 37 ^{0}C humidified chamber containing 5% CO2 for 10 days. After 10-days media was removed and the plate was washed thrice with PBS and cells were fixed with 4% formaldehyde for 30 minutes. After 30 minutes formaldehyde was removed and the plate was rinsed thrice with PBS. Cells were stained with crystal violet for 15 to 20 minutes and excess stain was washed with PBS. Number of colonies in each well were counted by using the microscope. Groups of cells containing more than 50 cells were considered as the colony. Graph pad was used for making graph and statistical analysis.

3.8 Protein isolation and Western blotting

Cell lysis buffer (CLB) was used to isolate the whole cell protein from different cancer cell line. Media of cells were removed and washed with PBS to remove remaining residual media from plates. Plates (60 mm) containing cells were incubated with 200-300 µl cell lysis buffer for 15 to 20 minutes on ice. Cells were Scraped and stored in Eppendorf. The cell lysate was centrifuged at 13000 rpm for 30 minutes. After 30 minutes supernatant was transferred to a new tube and stored at -80 ^{0}C. Protein concentration was estimated by Bradford method in triplicates for each cell lysate. 50µg of isolated proteins was loaded into each well of SDS-PAGE gel for electrophoresis at 120 volts. Proteins were transferred to PVDF membrane by wet transfer system at 70 volts for 3 hours. Transfer efficiency visualized by Ponceau stain. The membrane was incubated with skimmed milk blocking solution for 2 hours at room temperature. After blocking, the membrane was rinsed with TBST (0.05% tween 20). The membrane was incubated with primary antibodies for overnight on gentle sacking platform. After overnight incubation, the membrane was washed with TBST three times for 10 minutes. The membrane was further incubated with HRP conjugated secondary antibody at room temperature for 1 hour and the membrane was washed with TBST three times. Protein bands were visualized by HRP substrate ECL (Millipore) and chemiluminescent image was captured on X-ray film.

Table 10: List of Antibodies used for western blotting

Antibody	Isotype	provider	Working dilution
hTERT	IgM (Mouse)	Novus	1:1000
c-MET	IgG (Rabbit)	Santa Cruz	1:2000
Nf-kB (p65)	IgG (Rabbit)	Santa Cruz	1:2000
p53	IgG (Mouse)	Santa Cruz	1:10000
E-cadherin	IgG (Rabbit)	CST	1:1000
Vimentin	IgG (Rabbit)	CST	1:1000
N-cadherin	IgG (Rabbit)	CST	1:1000
Phosphor p65	IgG (Rabbit)	CST	1:1000
IkBα	IgG (Mouse)	Santa Cruz	1:1000
Phosphor IkBα	IgG (Rabbit)	CST	1:1000
β-Actin	IgG (Rabbit)	Thermo Scientific	1:5000
IKKα/β	IgG (Rabbit)	CST	1:1000
c-Myc	IgG (Rabbit)	e-Biosciences	1:1000
Phosphor c-MET	IgG (Rabbit)	CST	1:1000
SMAD4	IgG (Rabbit)	CST	1:1000

3.9 Immunofluorescence imaging of cells

For immunofluorescence, equal number of cells were seeded on tissue culture treated coverslips and incubated overnight. Next day media was removed, and coverslips were washed thrice with PBS. Cells were fixed in 4% formaldehyde for 15 minutes at room temperature. After fixation cells were washed with PBS and permeabilized with 100% methanol for 10 minutes at -20 ^{0}C or 0.2% triton-X100 for 10 minutes at room temperature. After permeabilization cells were incubated in blocking buffer for 1 hour at 4 ^{0}C. Coverslips were incubated with primary antibodies on Parafilm in humidified chamber overnight. Primary antibodies were removed, and coverslips were washed with PBST for 5 minutes three times and then incubated with FITC labelled secondary antibodies for 1hour at room temperature. After 1-hour coverslips were removed and washed with PBST and mounted in 50% glycerol. Cells were observed, and the image was captured under Nikon TiE florescence microscope. The image was analyzed by Nikon NIS software.

Table 11: List of antibodies used for immunofluorescence

Antibody	Isotype	provider	Working dilution
c-MET	IgG (Rabbit)	Santa Cruz	1:200
Nf-kB (p65)	IgG (Rabbit)	Santa Cruz	1:200
p53	IgG (Mouse)	Santa Cruz	1:500
E-cadherin	IgG (Rabbit)	CST	1:100
Vimentin	IgG (Rabbit)	CST	1:100
SMAD4	IgG (Rabbit)	CST	1:300
Cytokeratin	IgG (rabbit)	CST	1:300
Anti-mouse FITC	IgG (Goat)	Santa Cruz	1:250
Anti-rabbit FITC	IgG (Goat)	G-Biosciences	1:250

3.10 Quantitative Real-Time PCR

Cells were trypsinized and counted with haemocytometer and nearly 2 x 10^6 cells used to isolate the RNA. The plates were washed with PBS to remove residual media and cells were centrifuged. 1ml of TRIZOL (Sigma) reagent was used to lyse the cells and RNA was isolated as per manufacturer protocol. 1 µg of RNA was used to make cDNA by Verso cDNA synthesis kit. For quantitative real-time PCR, sample was prepared by adding 5 µl of SYBR green (Applied Biosystems), 1µl of prepared cDNA (100 ng) and 0.2 µl of each of 10 picomole concertation primers in each vial. Each sample was taken in triplicates for qRT-PCR. Primers used for real-time PCR listed are listed in Table A14 with their sequence. Actin was used as an endogenous control for qRT-PCR reaction. Applied Biosystems 7500 fast was used to detect the expression level of genes. Relative expression level of the gene was represented as ΔΔCT value. Statistical analysis was done and graph made by Prism graph pad software.

3.11 Luciferase assay

For luciferase assay, equal number of control and treated cell were seeded in 6 well plate and incubated for overnight to allow the cells to attach to the surface. Next day 1 µg pGL3-c-MET promoter were co-transfected with different amounts of hTERT-shRNA (0.25 µg, 0.5, µg and 1.0 µg) in A549 cells by using Lipofectamine 3000. Transfection media was replaced with complete media after 8 to 10 hours, and cells were incubated at 37 ^{0}C in a humidified chamber under 5% CO_2 for 36 hours. After 36

hours cells were trypsinized and collected in the tube and centrifuged at 2000 rpm for two minutes. The cell pellet was washed with PBS to remove remaining media. In each tube 150 µl cell lysis buffer (provided in Biovision luciferase assay kit) was added and after 5 minutes the cell lysate was centrifuged at 13000 rpm for 2 minutes. The supernatant was transferred to a new tube and kept on ice. 50 µl per well supernatant was added in triplicate to 96 well plate, and luciferase substrate was added to each well as per manufactured protocol of Biovision. Luminescence was recorded by thermo varioskan lux multimode microplate reader in scanning mode. Luminescence value was normalized with reference to protein concentration of the sample.

3.12 Analysis and Statistics

Prism GraphPad software was used to plot the graphs. Data obtained from real-time PCR, growth assay, colony forming assay, migration assay, and luciferase assay were analyzed by Prism GraphPad software. The statistical parameters were selected to fit with the experimental design. The results of statistical analysis are represented by the asterisk mark to indicate significance at the given p value.

RESULTS
AND
DISCUSSION

Objective 1

Effect of expression of hTERT on the process of cell invasion and migration

4 RESULTS AND DISCUSSION

4.1 Objective 1: Effect of expression of hTERT on the process of cell invasion and migration

4.1.1 hTERT knockdown causes change in cellular morphology of A549

A549 cells were transduced with viral vector for expression of hTERT-shRNAs to downregulate the expression of hTERT, and knockdown efficiency was estimated by western blotting. Two hTERT shRNAs that targeting two different regions in hTERT transcripts have been used. Both hTERT-shRNAs reduce the hTERT expression in A549. hTERT shRNA1 shows ~ 40% knockdown efficiency while hTERT-shRNA2 ~ 70 -80% (Fig. 15a). So for further studies, hTERT shRNA2 was used (Fig. 15b). hTERT knockdown in A549 cells leads to change in the morphology of cells that appear flattened and larger in size (Fig. 15c).

4.1.2 Cell proliferation and survivability are associated with hTERT in cancer cells

To view the effect of hTERT knockdown on cell proliferation, viruses delivering hTERT-shRNA2 and scramble-shRNA were transduced in A549 and selected for puromycin resistance and knock down was verified by western blotting (Fig. 16a). 2.0 $X10^4$ cells were seeded in 12 well plate in triplicate, and cell growth was observed for four days. hTERT downregulation reduces the cell proliferation rate around 30 percent on the fourth day (Fig. 16b). We also overexpressed hTERT in H1299 and checked its effect on H1299 cell proliferation. pBABE hTERT transfected H1299 cells show increased cell proliferation (Fig. 16d & e). Further, to find out role of hTERT in cancer cell survivability, hTERT-shRNA 2 and scramble-shRNA virus were transduced in A459 cells. 1000 cells of hTERT shRNA and scrambled shRNA were grown in 6 well plate for ten days. After ten days clonogenic survivability was calculated and result shows that hTERT downregulation reduces the survivability by up to 60 percent in A549 cell (Fig. 16c).

4.1.3 hTERT expression helps in migration

Results presented in preceding sections indicate that hTERT expression increased the cell proliferation and survivability. Along with proliferation and survivability cell migration is a crucial step in cancer development. To find out the effect of hTERT on migration, hTERT was downregulated in A549, and its effect was assayed by wound healing (Fig.17a). hTERT knockdown reduces the cell migration around 20 % in A549 cell (Fig. 17b). Further, in reciprocal experimental design, hTERT was overexpressed in H1299 cells and wound healing assay was performed (Fig. 17c). hTERT overexpression in H1299 increases the migration potential as assayed by wound healing (Fig. 17d).

4.1.4 hTERT downregulation reduces EMT in A549

hTERT expression is positively associated with the cell migration as described above. Migratory and invasive properties in cells increase after the loss of epithelial character and gain of mesenchymal feature, this change being referred to as epithelial to mesenchymal transition (EMT). So, to find out the correlation between hTERT and EMT, expression of EMT markers was examined in hTERT downregulated A549 cells (Fig. 18a). Vimentin and N-cadherin are mesenchymal markers and expression of these proteins were decreased in hTERT knockdown A549 (Fig. 18c & d). E-cadherin is an epithelial marker protein which is found upregulated in hTERT downregulated A549 cells (Fig.18b). Further, results of western blots validated by immunofluorescence analysis of vimentin are in consonance with the assumption that hTERT indeed promotes EMT in A549 cells (Fig. 18e).

4.1.5 hTERT expression promotes mesenchymal feature in H1299 cells

hTERT down-regulation has a negative association with EMT markers shown in previous result. Here we overexpressed the hTERT in H1299 cells by using pBABE-hTERT expression vector and checked the effect on vimentin expression in H1299 (Fig. 19a). hTERT expression promotes the vimentin level in H1299 cells (Fig. 19a, b).

4.1.6 SMAD4 expression positively associated with expression of hTERT

SAMD 4 is an EMT-associated transcription factor, and this is also a TGFβ responsive protein. SMAD4 interacts with other members of SMAD family proteins like; SMAD2/3 and binds to promoters of EMT-associated genes and modulates their expression. SMAD4 is known to regulate the expression of E-cadherin and vimentin in cancer cells, and our previous results show that hTERT expression is associated with expression of EMT marker proteins. So we checked the effect of hTERT on SMAD4 expression and result indicates that hTERT knockdown in A549 cells reduces the expression of SMAD4 at protein level (Fig. 20a & b).

4.1.7 hTERT knockdown promotes cytokeratin-18 level in A549 cells

Cytokeratin 18 is epithelial cell specific intermediate filament protein. Expression of this protein in cells is associated with cell differentiation[253]. In initial results, we have shown that hTERT knockdown in A549 causes changes in cellular morphology as if cells are acquiring the epithelial morphology. So, we checked the cytokeratin level in hTERT downregulated A549 cells, and we found an increase in cytokeratin level in cells (Fig. 21a & b).

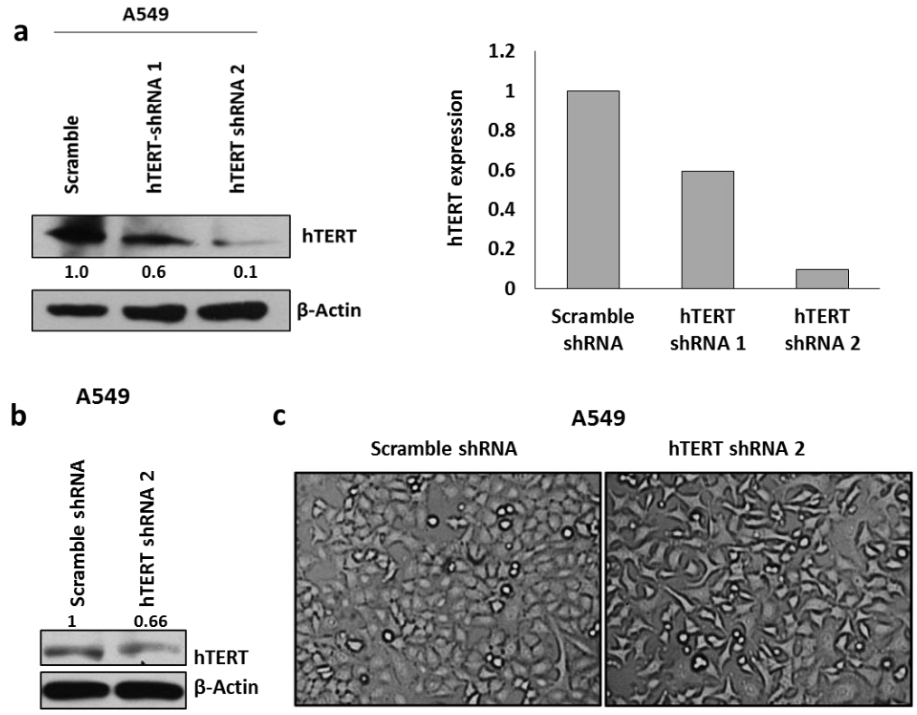

Figure 15: hTERT knockdown causes change in cellular morphology of A549.

a & b) Immunoblotting of hTERT protein in hTERT downregulated A549 cells. Two shRNAs were used against hTERT. hTERT shRNA 1 reduced the hTERT expression by ~ 40% while hTERT shRNA 2 shows more conspicuous reduction by ~ 80%. c) hTERT knockdown in A549 cells causes change in cellular morphology, hTERT shRNA transfected cells become more flattened and larger in size.

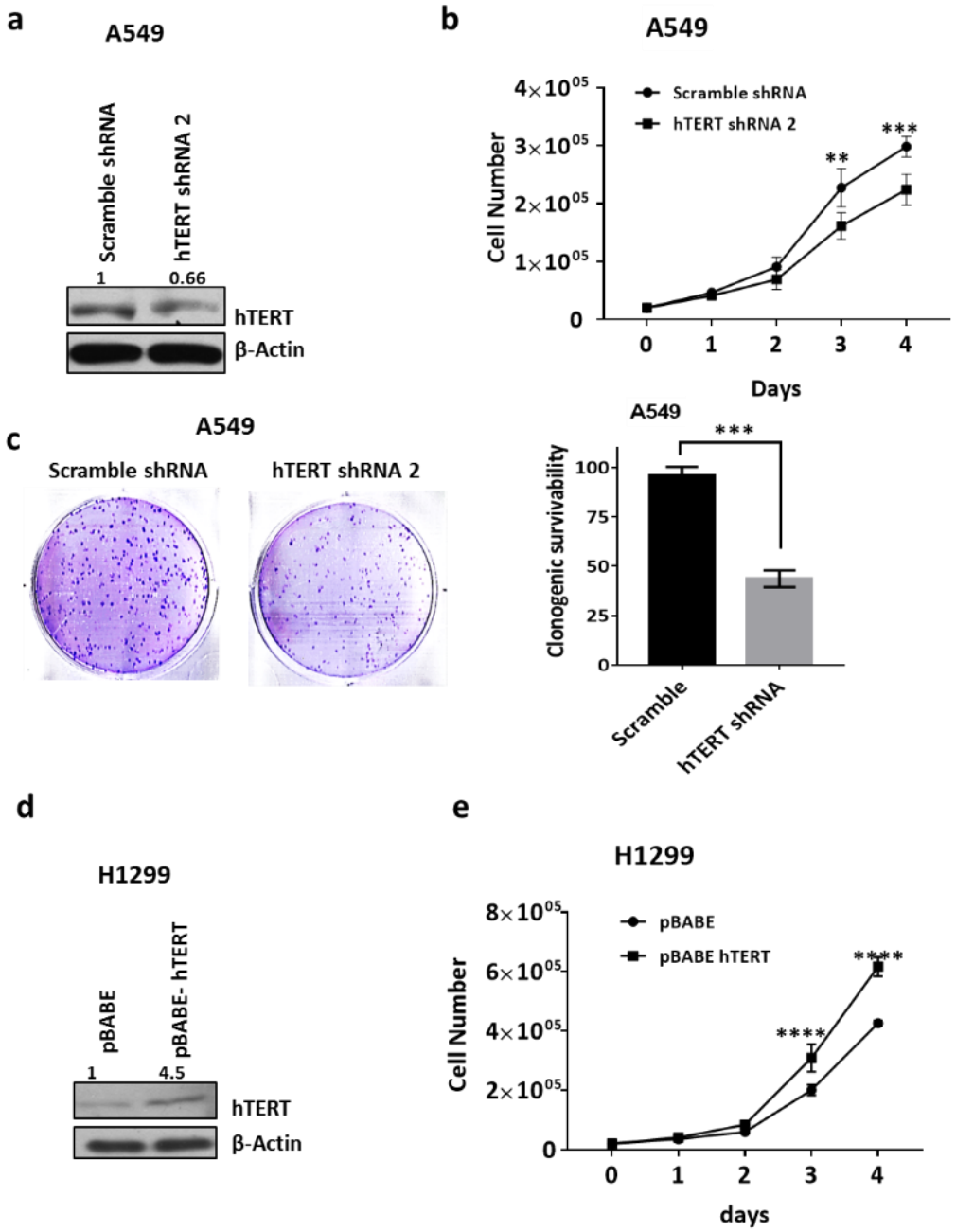

Figure 16: Cell proliferation and survivability are associated with hTERT in cancer cells.

a) Western blot analysis of hTERT in hTERT downregulated A549 cells. b) Cell growth assay of hTERT knockdown A549 cells that show reduced cell proliferation. c) Clonogenic survivability was performed in hTERT downregulated A549 cells and result shows that hTERT knockdown in A549 cells reduces their survivability. d) H1299 is small lung cell carcinoma in which hTERT was overexpressed by using pBABE hTERT expression vector and expression was assayed by immunoblotting. e) Role of hTERT expreesion in cell proliferation was assayed, and result indicates that hTERT expression in H1299, promotes the cell proliferation. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

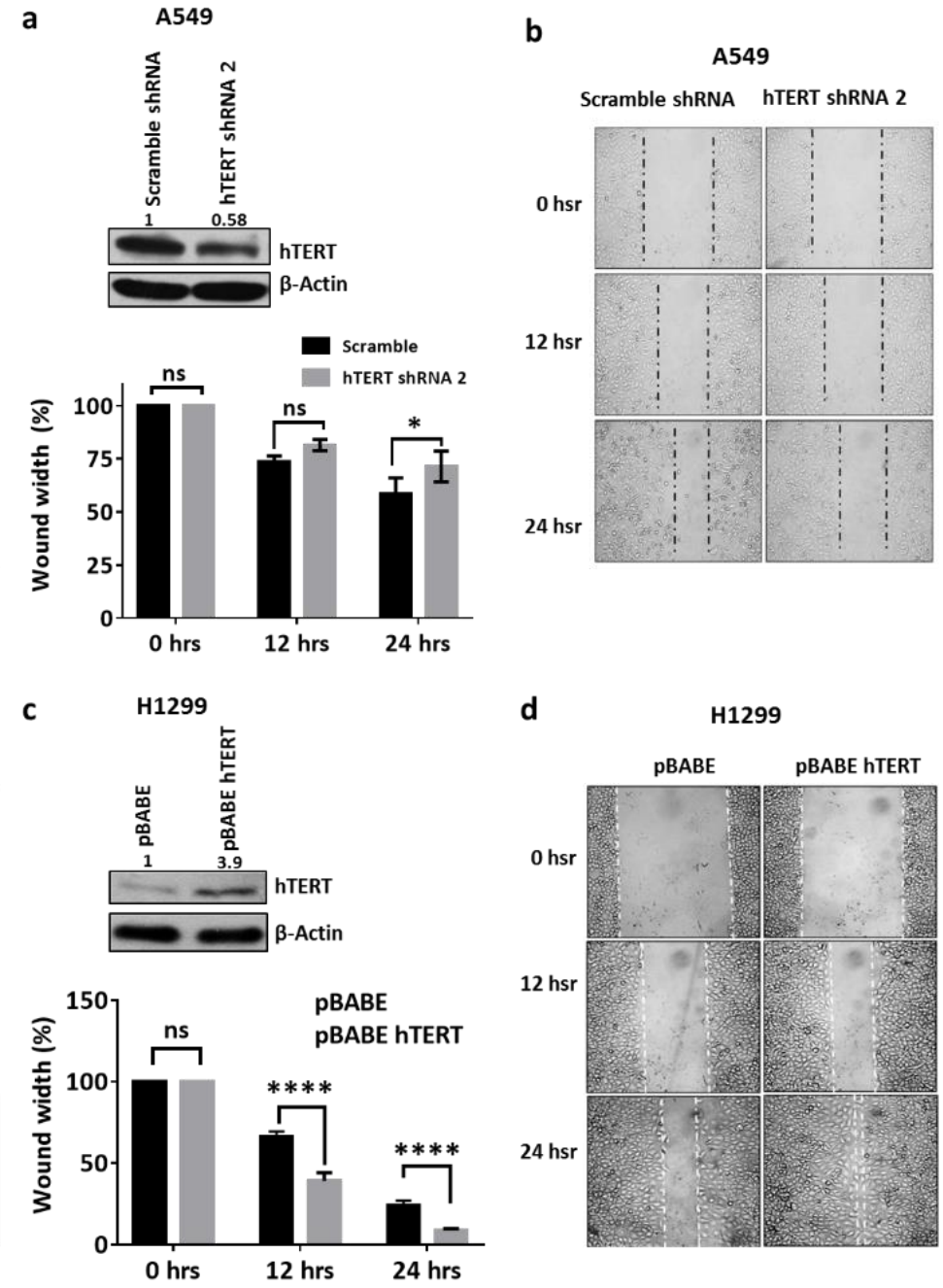

Figure 17: hTERT expression helps in migration.

a) hTERT expression in hTERT downregulated A549 cells assessed by immunoblotting. b) hTERT knockdown in A549 cells reduces the cell migration. c) Overexpression of hTERT in H1299 cell is confirmed by western blotting. d) hTERT overexpression in H1299 promotes the cell migration. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

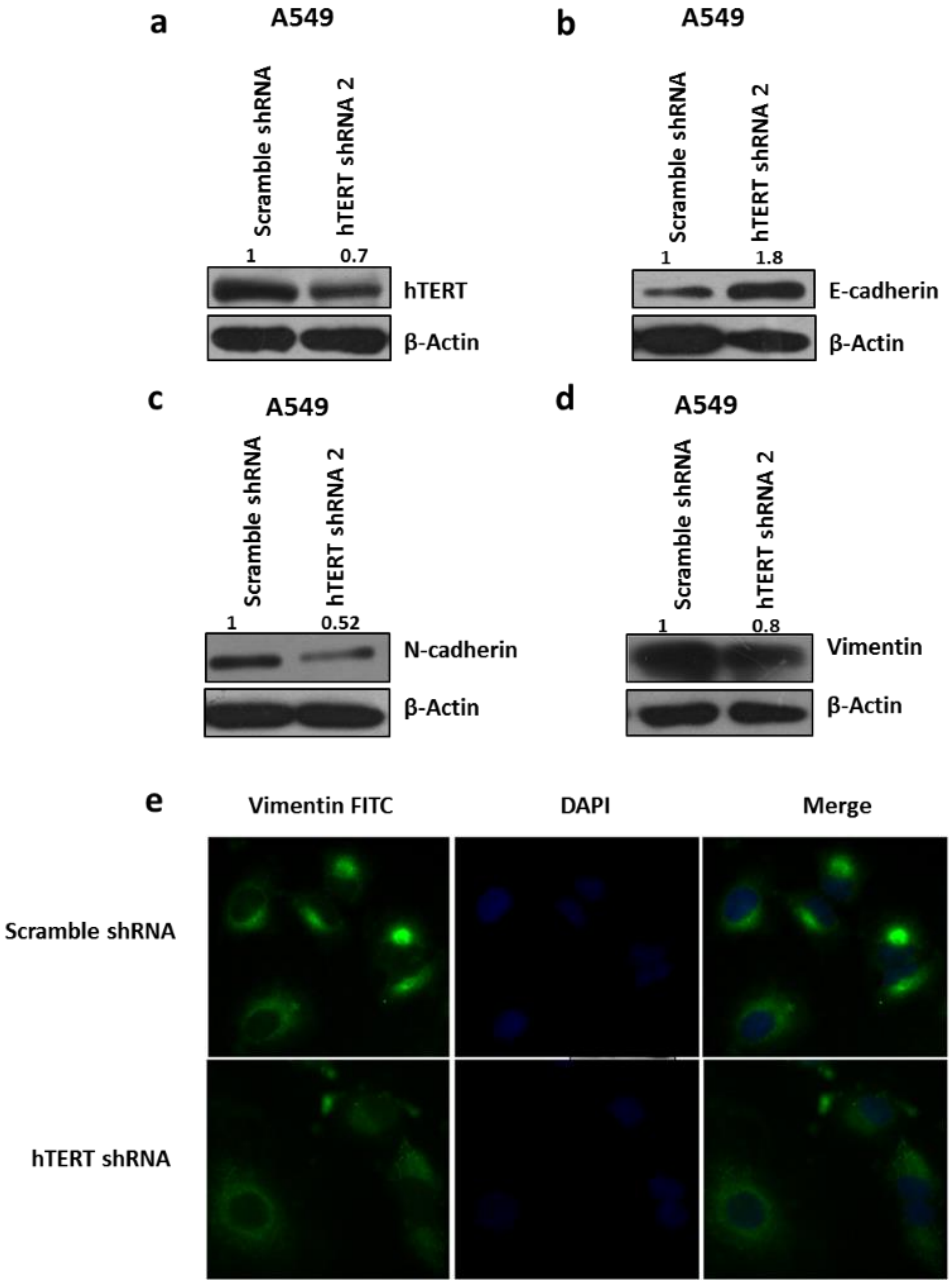

Figure 18: hTERT downregulation reduces EMT in A549.

a) Western blot analysis of hTERT in hTERT shRNA transfected A549 cells. b) E-cadherin is an epithelial marker and was found upregulated in hTERT knockdown A549 cells. c & d) N-cadherin and vimentin are mesenchymal marker and their expression level was reduced following hTERT knockdown.

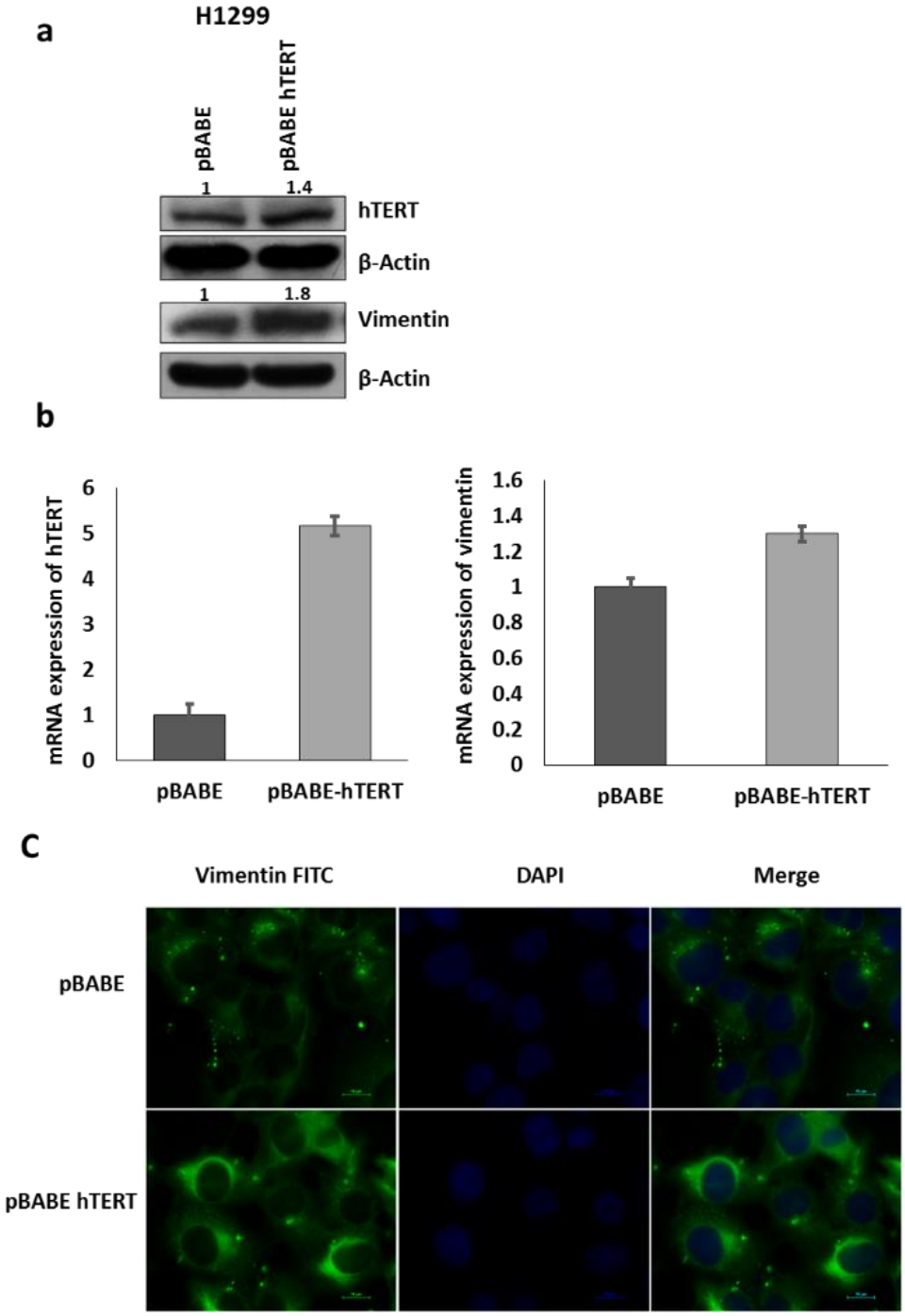

Figure 19: hTERT expression promotes mesenchymal features in H1299 cells.

a) Western blot analysis of hTERT overexpression in H1299 cells and accompanying rise in expression of vimentin. b) mRNA expression level of hTERT and vimentin in pBABE-hTERT and pBABE transfected cells. c) Immunofluorescence analysis of vimentin in hTERT overexpressing H1299 cell that shows hTERT expression promotes the vimentin expression.

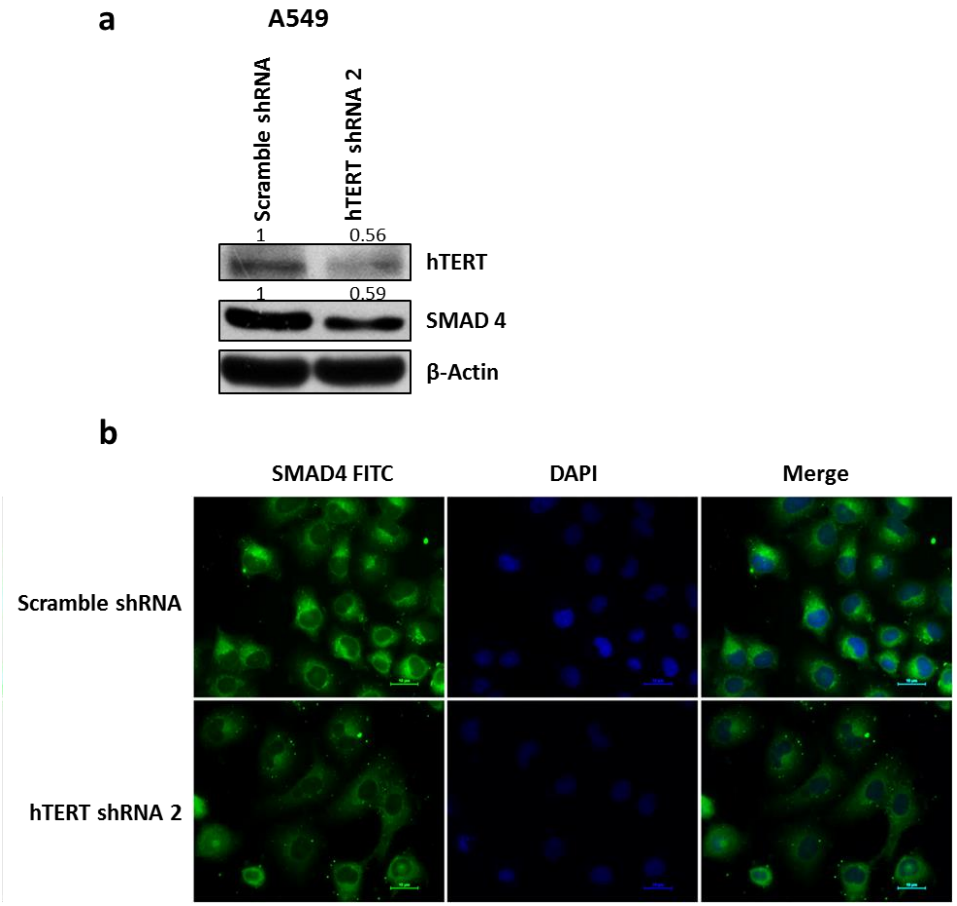

Figure 20: SMAD4 expression is positively associated with expression of hTERT.

a) Western blot analysis of hTERT knockdown A549 cells that shows the reduction in hTERT expression. hTERT knockdown also reduces the expression of SMAD4 expression in A549 cells. b) Immunofluorescence of SMAD4 protein in hTERT knockdown A549 cell. hTERT targeting in A549 reduces the SMAD4 expression.

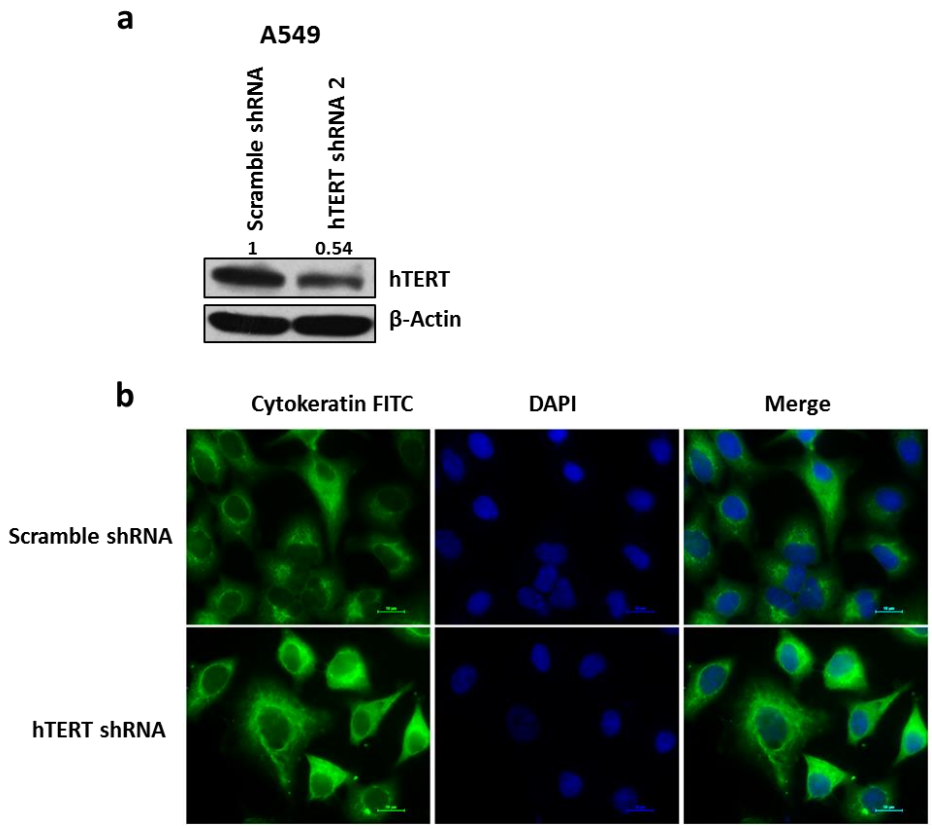

Figure 21: hTERT knockdown promotes cytokeratin level in A549 cells.

a) Western blot of hTERT in hTERT down regulated A549 cells. b) Immunofluorescence analysis of cytokeratin protein. hTERT targeting in A549 cells increased the expression level of cytokeratin protein.

4.1.8 Discussion

hTERT, protein component of telomerase, has a positive associated with telomerase-mediated cellular immortalization, cell growth, and cancer malignancy[254]. Till date, very limited information is available on the telomerase-independent functions of hTERT and its association with cell growth and survivability, migration, and EMT[255]. In the present study, our results suggest the role of hTERT in cancer growth survivability, migration, and EMT. We found that hTERT knockdown causes the morphological change in A549 cells. A549 cells knocked down for hTERT expression have larger cell size and flattened morphology. hTERT promotes the cellular immortalization by telomere maintenance[256]. Telomere length gets reduced in every cell cycle, and when the length of telomere reaches a critical low, cells stop dividing[257]. Telomere length reaches critical threshold level after about 50 cell cycles. But when we knock down or overexpressed the hTERT in cancer cells, we got change in cell proliferation rate in only four cell cycles. So, hTERT does not regulate the cell growth only by telomere maintenance, but it has some other important roles in cancer cells. We found that hTERT knockdown increased the p53 level in the cell. p53 is a master regulator of cell growth-related cell cycle proteins. p53 induces the transcriptional activity of p21WAF1, CIP1, and Sdi1 which is an inhibitor of the cyclin-dependent kinases (CDKs)[258]. hTERT expression promotes cancer cell survivability in isolation condition as confirmed by clonogenic survival assay. hTERT knockdown cells show reduced survivability as compared to control cells. During cancer development tumor cells invade the surrounding cell and migrate to reach into blood or lymph. This process is completed by involvement of two important steps, invasion and migration. Recent finding shows that hTERT promotes the expression of MMPs which help in the degradation of extracellular matrices which paves the path for cancer cells to escape from the original mass of cells[147]. Cancer cell migration is also a critical step for cancer development. hTERT expression is associated with the cancer cell migration. hTERT knockdown inhibits wound healing efficiency of A549 cells while overexpression of hTERT in H1299 promotes it. Epithelial to mesenchymal transition (EMT) is another important process required for cancer development. EMT occurred in primary and metastasised tumor sites. During EMT cancer cells lose the epithelial character and gain mesenchymal character. Mesenchymal cells are motile in nature so EMT facilitates the migration of cancer cells. Recent studies show that EMT also helps in the gain of chemo-resistance in cancer cells[259]. We have also investigated the role of hTERT in

EMT process. E-cadherin is an epithelial marker which is increased when hTERT is targeted with shRNA. Mesenchymal markers vimentin and N-cadherin are reduced on hTERT knockdown in A549 cells. While increased hTERT expression in H1299 cells induces the expression of vimentin. So, hTERT has an important role in epithelial to mesenchymal transition. To find out the mechanism behind the hTERT mediated EMT regulation. We investigated the effect of hTERT on EMT regulator. It has been previously reported that hTERT regulated the EMT via snail[144]. We found that hTERT knockdown reduces the SMAD 4 level in A549 cells. SMAD4 is a positive regulator of EMT markers. It promotes the expression of mesenchymal protein vimentin and N-cadherin and suppresses the E-cadherin expression[260]. As we have discussed above, hTERT knockdown leads to change in cell morphology. Cells become more flattened and larger in size. hTERT knock down may promote the cellular differentiation into epithelial cells. We checked the expression status of cytokeratin 18 in hTERT knock down A549 cells and the results show that hTERT inhibition increased the expression of cytokeratin 18 in cells. So, hTERT may have some function in cell differentiation. hTERT regulates molecular pathways associated with cancer cell survivability, proliferation invasion, and metastasis[8,261]. However, molecular mechanism of hTERT-mediated cancer invasion and migration is not well established. Therefore, it is important to explore the hTERT regulated pathway leading to invasion and metastasis. Here we found that hTERT has important role in regulation of c-MET oncoprotein which has established roles in cancer development.

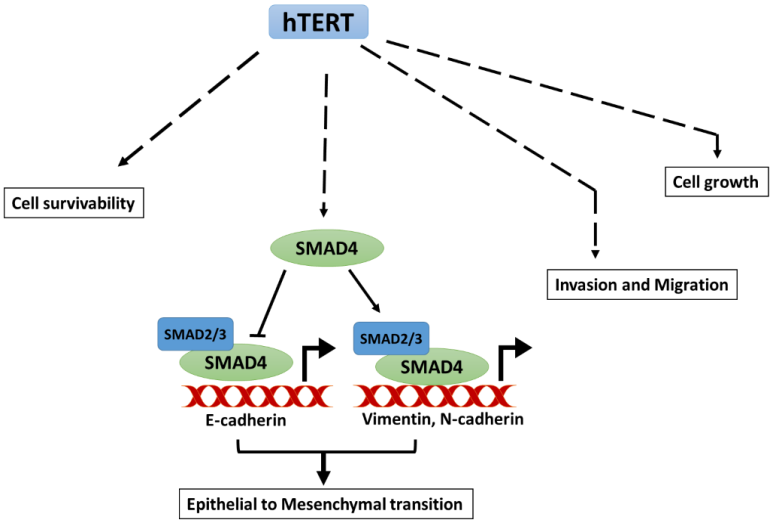

Figure 22: A schematic representation of different extracurricular roles of hTERT in cancer cells.

Objective 2

Cross-talk between hTERT and c-MET

4.2 Objective 2: Cross-talk between hTERT and c-MET

4.2.1 c-MET expression is positively associated with hTERT in cancer cells

Telomerase was initially characterized for maintenance of the telomere length of chromosomes. However, extracurricular activities of telomerase in regulating important molecules have since been documented [255]. c-MET is an important oncogenic factor found unregulated in most of the cancer cells. In this study, we have tried to investigate the role of hTERT in the regulation of c-MET oncogene. We knocked down hTERT in A549 lung carcinoma cells and found that protein level of c-MET markedly decreased when hTERT was down-regulated by shRNA (Fig. 23a). Further, we confirmed the effect of hTERT down-regulation on c-MET expression by immunocytochemistry (Fig. 23b). We also knocked down hTERT in HCT116, and HCT116 p53-/- cells and found downregulation of hTERT in both cells. We observed that hTERT knock down in HCT116 causes quantitatively greater reduction of c-MET expression as compared to HCT116 p53-/- (Fig. 23c). H1299 is derived from non-small cell lung cancer and has a homozygous partial deletion in p53 gene. We transfected the hTERT expression vector (pBABE hTERT) in H1299 and estimated expression by qRT-PCR and western blotting. Interestingly, we found that c-MET markedly increased on overexpression of hTERT in H1299 at protein as well as mRNA level (Fig. 23d-f). Our results reflect on positive effect of hTERT on c-MET expression in cancer cells.

4.2.2 hTERT is associated with c-MET expression at promoter level

Results presented in earlier sections demonstrate that hTERT has a regulatory effect on c-MET expression. hTERT is modulating the expression of c-MET at both protein and RNA levels. So, to find out the mechanism behind the hTERT mediated c-MET regulation, we did the reporter assay for c-MET promoter. The promoter region of c-MET (1.85 kb long) was cloned into pGL3 vector. The c-MET-luc construct was transfected with different amount of hTERT shRNA (0.25, 0.5 and 1.0 µg) in A549 cells. hTERT down regulation exhibits the inhibitory effect on c-MET promoter activity in a dose dependent manner (Fig. 24a). Further to find out the regulatory element of different transcription factors on the c-MET promoter, we analyzed the c-MET promoter for binding of regulatory factors by MAPPER2. We got a number of putative

transcription factor-binding sites in the promoter of c-MET. Out of these we selected three sites corresponding to transcription factors p53, c-Myc, and Nf-kb for further studies because association of these transcription factors with hTERT is already reported[262,263] (Fig. 24b).

4.2.3 hTERT modulates the expression of p53 in cancer cells

Results presented in the preceding sections indicate that c-MET has p53 binding elements on its promoter. C-MET has binding site for p53 at -185 bp and -212 bp upstream of c-MET transcription start site. These two p53 binding sites are present adjacent to the SP1 binding site. A549 and HCT116 cells were transduced with hTERT shRNA virus and consequent effect on on p53 expression was assayed. We found that downregulation of hTERT expression in A549, and HCT116 cells by shRNA induces the expression of p53 (Fig. 25a & b). Further, immunofluorescence shows an increase in p53 expression and nuclear movement in hTERT downregulated cell (Fig. 25c).

4.2.4 hTERT knockdown induces the expression of p65 in A549 cells

c-MET promoter analysis by 'Mapper' suggested the putative binding site for Nf-kB. The1.85 kb long promoter has two Nf-kB binding sites, first at -1353 bp and second at -1555 bp upstream of c-MET transcription start site. So, we checked the effect of hTERT down regulation on Nf-kB (p65) expression in A549 cells. Here we got an interesting result that hTERT down regulation induces the expression level of Nf-kB (p65) in A549 cells (Fig. 26a). Further, the result has verified by targeting the hTERT with siRNA in A549 cells, and we got a similar result (Fig. 26b). Immunofluorescence analysis Nf-kB (p65) in of hTERT targeted A549 cells shows higher expression of Nf-kB (p65) compared to the scramble transfected cells (Fig. 26c).

4.2.5 Nf-kB (p65) expression is positively associated with hTERT expression in p53 null cancer cells

As shown above, hTERT has a negative association for Nf-kB (p65) expression in A549 cells. We also inhibited the hTERT expression in HCT116 and HCT116 p53-/- cells. HCT 116 p53-/- cell has homozygous deletion for p53 gene, so it lacks p53 expression. Expression status of Nf-kB (p65) was therefore assayed in HCT116 and HCT116 p53-/- cells transduced for expression of hTERT shRNA. We found that hTERT promotes

the expression of Nf-kB (p65) in HCT116 while it lowers NF-kB(p65) expression in HCT116 p53-/- cells under otherwise identical experimental conditions (Fig. 27a). Expression of hTERT also downregulated Nf-kB (p65) in another p53 null cell, H1299. Knockdown of hTERT in H1299 also reduces the expression level of Nf-kB (p65) (Fig. 27b). 293T is normal embryonic kidney cell line in which hTERT was targeted with siRNA that also leads to decreased expression of Nf-kB (p65) 293T (Fig. 27b). We overexpressed hTERT in H1299 and checked the expression of Nf-kB (p65), and we found hTERT promotes the expression of Nf-kB (p65) (Fig 27c).

4.2.6 hTERT expression promotes the expression of c-Myc in cancer cells

c-Myc is a growth associated protein. It is also one of the proteins required for the stemness. c-Myc regulates the expression of a number of growth associated genes. c-MET is also a growth associated protein which is a target of c-Myc. Promoter analysis shows that c-Myc also has a binding site on c-MET promoter at -187 bp upstream to transcription start site. c-Myc may have some regulatory role in c-MET expression. So, we checked the effect of hTERT on c-Myc expression. pBABE hTERT expression vector was transfected into H1299 cells, and c-Myc expression was estimated by immunoblotting. hTERT overexpression in H1299 induces the expression of the c-Myc protein (Fig. 28a).

4.2.7 Western blotting confirmed p53 knockdown and overexpression in different cancer cells

In our previous results, we have showed that hTERT modulates the expression of c-MET and p53. c-MET has p53 binding elements on the promoter. So, we checked weather hTERT also regulates the expression of c-MET via p53. We made p53 shRNA construct in pLVX-shRNA-1 lentiviral vector and p53 expression vector in the pLVX-puro vector. p53 shRNA transfer vector along with helper vectors was transfected in 293T cells to make the virus. p53 shRNA virus was used to inhibit the expression of p53 in different cancer cells. We have made stable p53 knock-down cells of A549, HCT116 and HepG2 and assayed for associated changes by western blotting. A decrease in p53 expression up to 60 to 80 percent in different cancer cells (Fig. 29a, c

& d). We also overexpressed the p53 in A549, and H1299 cells by transient transfection which suppresses the expression level of c-MET protein (Fig. 29 b & e).

4.2.8 p53 expression is negatively associated with c-MET expression in cancer cells

To find out the role of p53 in the process of c-MET expression, lentivirus particles with p53 shRNA were transduced in A549 cells. We found that p53 down regulation ini these cells increased the c-MET expression by 20% (Fig. 30a). Immunofluorescence for c-MET in p53 downregulated A549 showed enhanced expression of c-MET probly due to lower expression of p53 (Fig. 30b). Over-expression of p53 in A549 cells with the help of an expression vector in A549, led to reduced expression of c-MET at protein level (Fig. 30c & d). It may be noted that c-MET promoter has p53 responsive element. So we performed luciferase reporter assay for a c-MET promoter in p53 knockdown A549 cells, and the result showed that p53 downregulation results in luciferase over-expression from c-MET promoter probably due to relief from repressor effect of p53 (Fig 30e). Further, we confirmed our results in other cancer cells and found that p53 downregulation in HCT116 and HepG2 increased the expression of c-MET (Fig. 30f & g). Expression of p53 in p53 negative H1299 cell, increased expression of p53 but caused reduced expression of c-MET (Fig 30h).

4.2.9 The level of c-MET protein in different cancer cells suggests that it may be negatively regulated by p53

p53 has a regulatory effect on c-MET expression as presented in the earlier sections. Hence, we cross-checked the expression of c-MET in different cancer cell lines. These cells have a difference in p53 expression or form. We selected four different sets of cancer cells that is lung, breast, colon, and hepatic cancer cells. A549 and H1299 are lung cancer cells, in which A549 has wild type p53 while H1299 is a p53 null cell. MCF-7 and MDA-MB-321 are breast cancer cells, MCF-7 is a wild type p53 expressing cell while MDA-MB-321 is expressed a mutant form of p53. Similarly, colon cancer HCT116 has normal p53 while HT29 contains mutated p53. HepG2 and Hep3B are hepatic cancer cells, HepG2 is a p53 positive cell while Hep3B is a p53 null cell. The difference in p53 expression or form shows the difference in c-MET expression. We

found one consistent pattern in that cells having wild type p53 have lower expression of c-MET compared to those cells having mutant or no p53 (Fig. 31a & b).

4.2.10 p53 knockdown promotes the growth and survivability of A549 cells

p53 is tumor suppressor protein is regarded as an essential player in growth suppression. p53 performed its function by promoting the growth arrest, apoptosis, and senescence. Difference inexpression and form of p53 significant impact on cancer cell growth, survivability, and metastasis. In this study, we have investigated the role of p53 in cancer progression. Thus, we made a p53 knock down A549 cells (Fig. 32a) and used them for assaying for cell proliferation. The growth of A549 cells was significantly increased following p53 knock down (Fig. 32b). p53 knockdown also promotes cell survivability as checked by colony forming assay (Fig. 32c).

4.2.11 Migratory properties increased in p53 downregulated A549 cells

Wound healing is an in-vitro migration assay movability of cells which is important for cell migration preceding metastasis. We have investigated the effect of p53 downregulation in A549 cell migration. Control cells and p53 knockdown cells were seeded, and artificial wounds were created and the cells allowed to fill the wound (Fig. 33a). Cell movement was monitored at 0, 12 and 24 hours' time interval. We found that p53 knock down increases the migratory capacity of A549 cells (Fig. 33b).

4.2.12 p53 expression is negatively associated with markers of EMT

Epithelial to mesenchyme transition (EMT) is an essential step required for the cancer progression. Here we studied the effect of artificially altering p53 expression on EMT markers. Knocking down p53 in A549 cells by p53 shRNA resulted in increased expression of mesenchymal marker vimentin while it reduced the epithelial and differentiation marker cytokeratin (Fig. 34a & b).

4.2.13 p53 down regulation induces the expression of the SMAD4 protein in A549 cells

The previous result confirmed the role of p53 in cell migration and EMT. To find out the regulatory mechanism behind increased migration and EMT. We checked the effect of p53 down regulation of SMAD4 expression. SMAD4 is a regulator of EMT markers. We found that p53 knockdown increased the expression level of SMAD4 in A549 cells (Fig. 35).

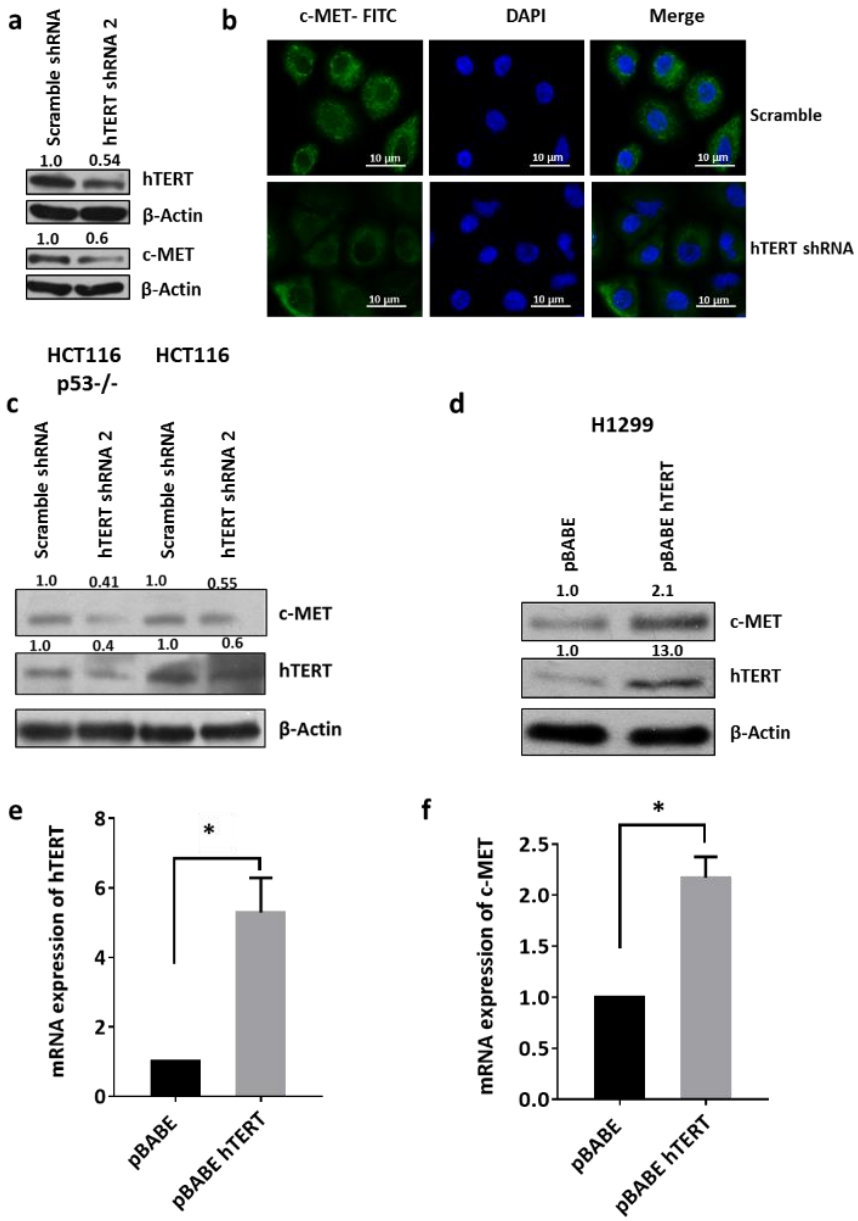

Figure 23: c-MET expression is positively associated with hTERT in cancer cells.

a) Immunoblots assaying expression of c-MET and hTERT proteins show lowering of c-Met expression in A549 cells transfected with hTERT shRNA as compared to those with scrambled RNA. b) Lower c-MET immunofluorescence in hTERT knockdown A549 cell confirms the above results. c) Immunoblot analysis of c-MET in hTERT knockdown HCT116 and HCT116 p53-/- cells shows greater response to hTERT knockdown in the latter cells. d) Western blot analysis of hTERT and c-MET expression in pBABE- hTERT and pBABE transfected H1299 cell showing elevated expression of c-Met in hTERT overexpressing cells. d-f) mRNA expression level of hTERT and c-MET in pBABE-hTERT and pBABE transfected H1299 cell showing overexpression of c-Met following that of hTERT. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant.

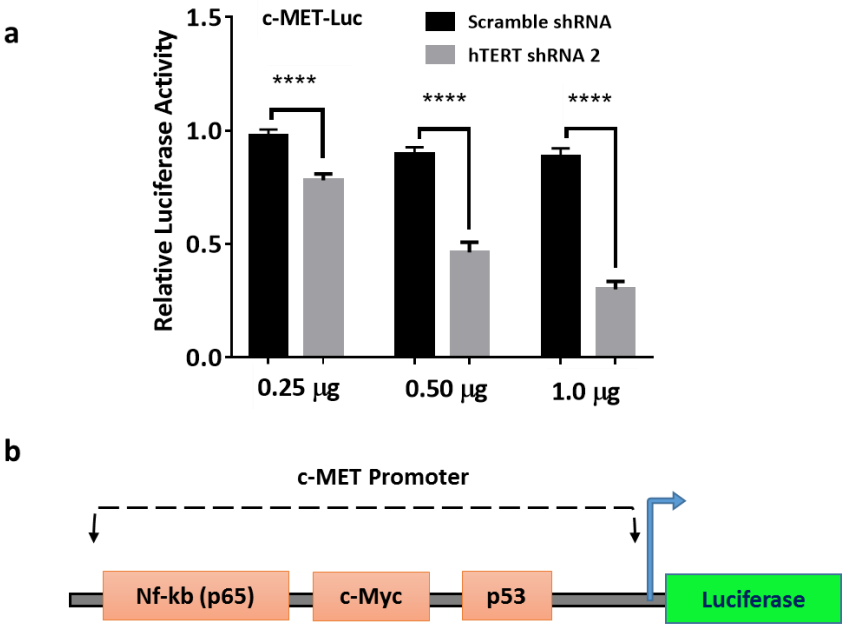

Figure 24: hTERT functionally interacts with c-MET promoter.

a) Promoter reporter assay of the c-MET promoter in hTERT knockdown A549 cells. hTERT downregulation reduces the c-MET promoter activity in dose dependent manner. b) This is a schematic representation of c-MET promoter. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

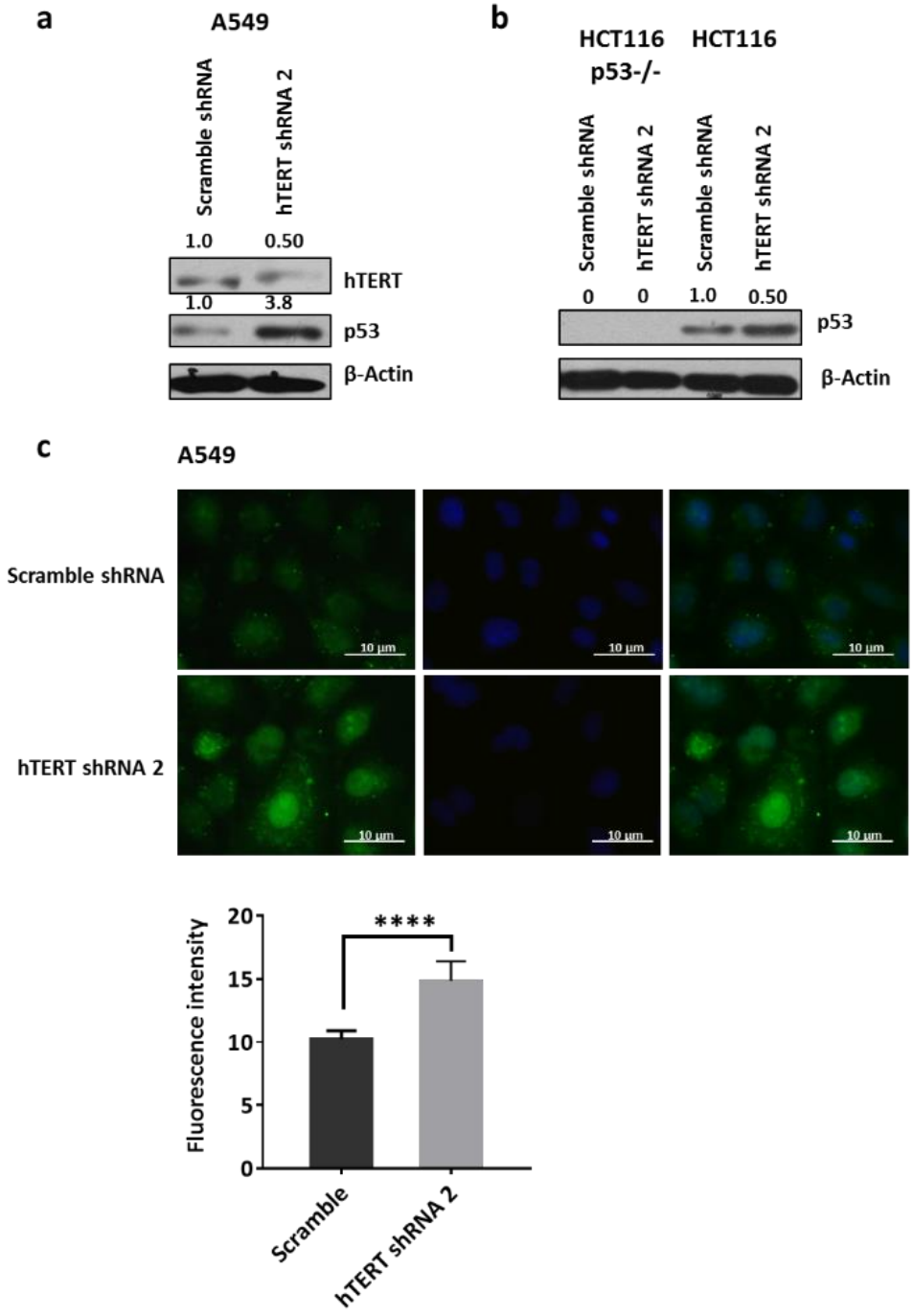

Figure 25: hTERT modulates the expression of p53 in cancer cells.

a & b) western analysis of hTERT p53 in hTERT knockdown A549 and HCT116 cells shows that the shRNA reduced the endogenous expression of p53 in these cells. c) Immunofluorescence of p53 increased in hTERT downregulated A549 cells that show increased p53 expression. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

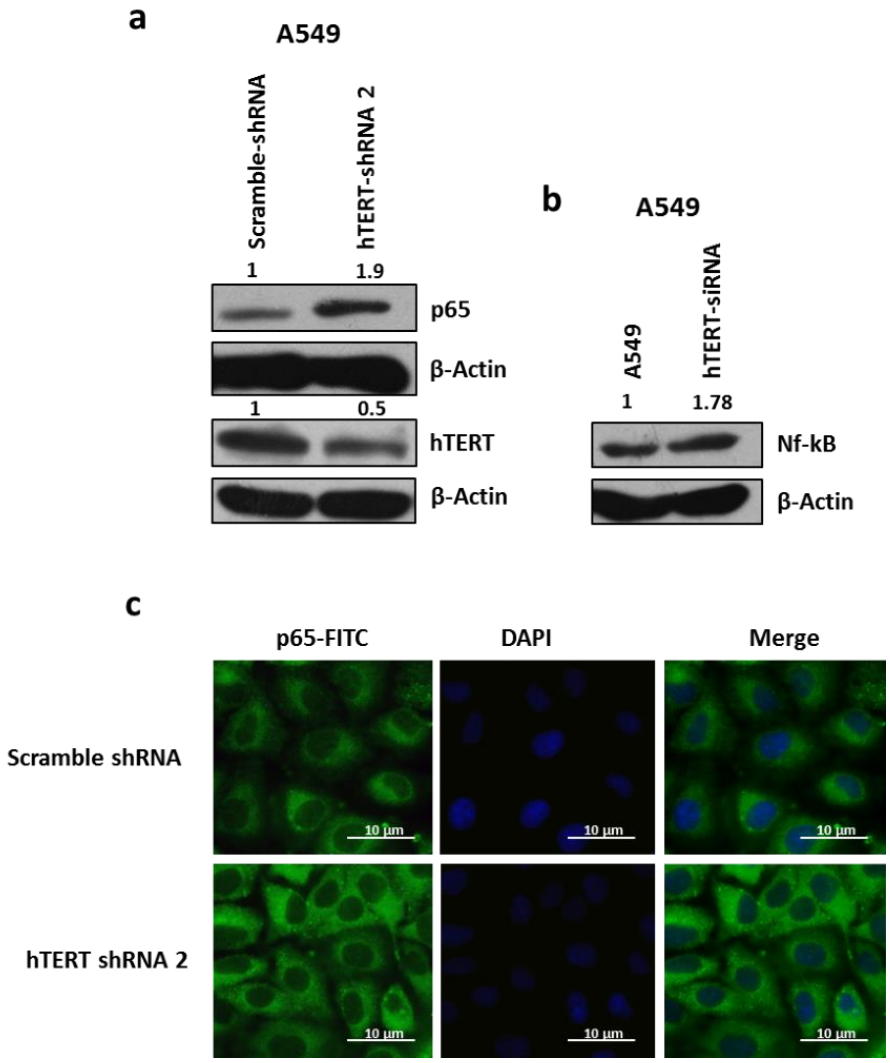

Figure 26: hTERT knockdown induces the expression of p65 in A549 cells.

a) Immunoblot analysis of hTERT and Nf-kB (p65) in hTERT downregulated A549 cells. hTERT downregulation induces the p65 protein level in A549. b) Nf-kB (p65) expression was analyzed by western blotting in hTERT siRNA transfected cells that shows similar increase in expression of NF-kB. c) Immunofluorescence of Nf-kB (p65) increases in hTERT shRNA transfected A549 cells.

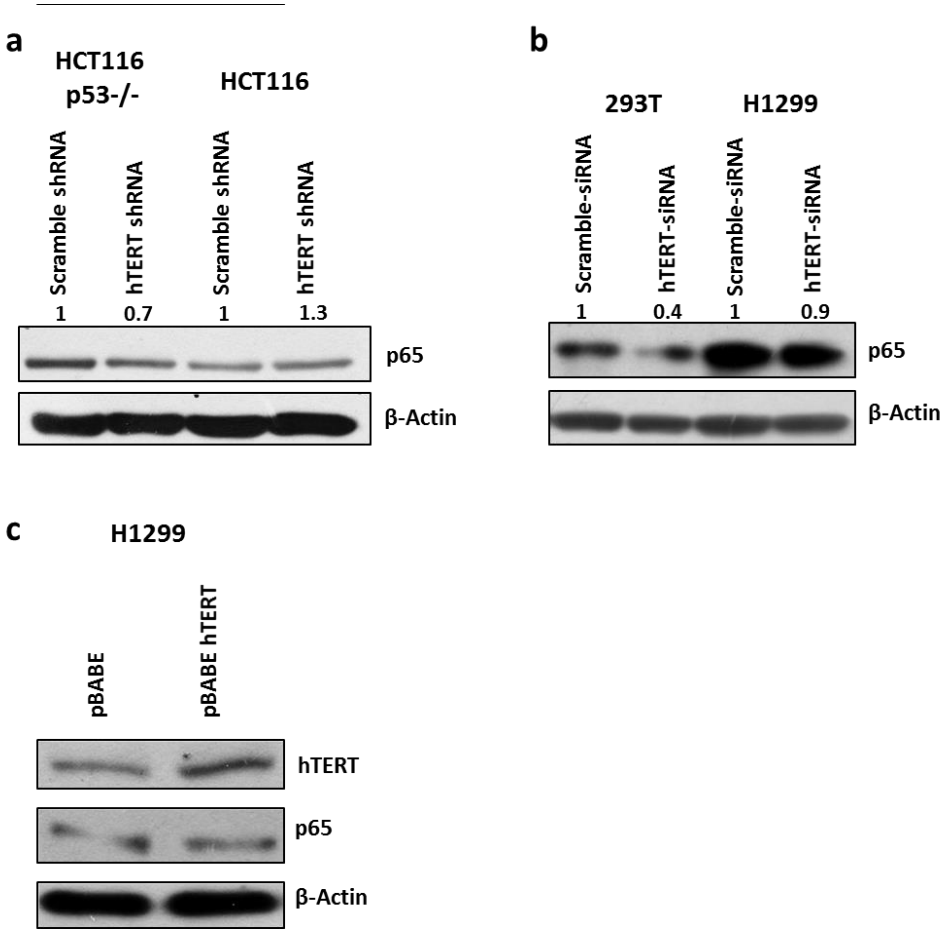

Figure 27: Nf-kB (p65) expression is positively associated with hTERT expression in p53 null cancer cells.

a & b) Western blot analysis of Nf-kB (p65) hTERT know down HCT116, HCT116 p53-/- cells, H1299, and 293T cells. hTERT down regulation in HCT116 induces the p65 expression while inhibits in HCT116 p53-/-, H1299, and 293T. c) hTERT was overexpressed in H1299 by pBABE hTERT that promotes the expression of Nf-kB (p65).

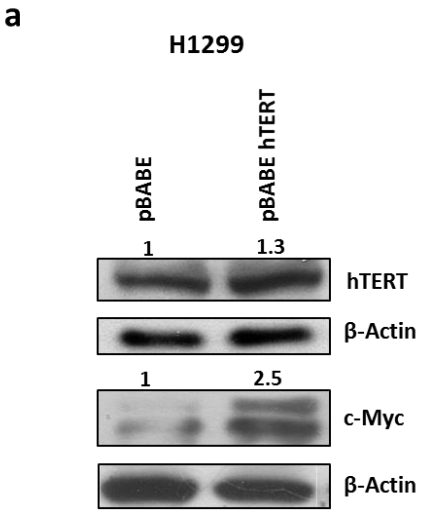

Figure 28: hTERT expression promotes the expression of c-Myc in cancer cells.

a) Western analysis of hTERT and c-Myc in pBABE hTERT transfected H1299. hTERT expression promotes the expression of c-Myc in H1299.

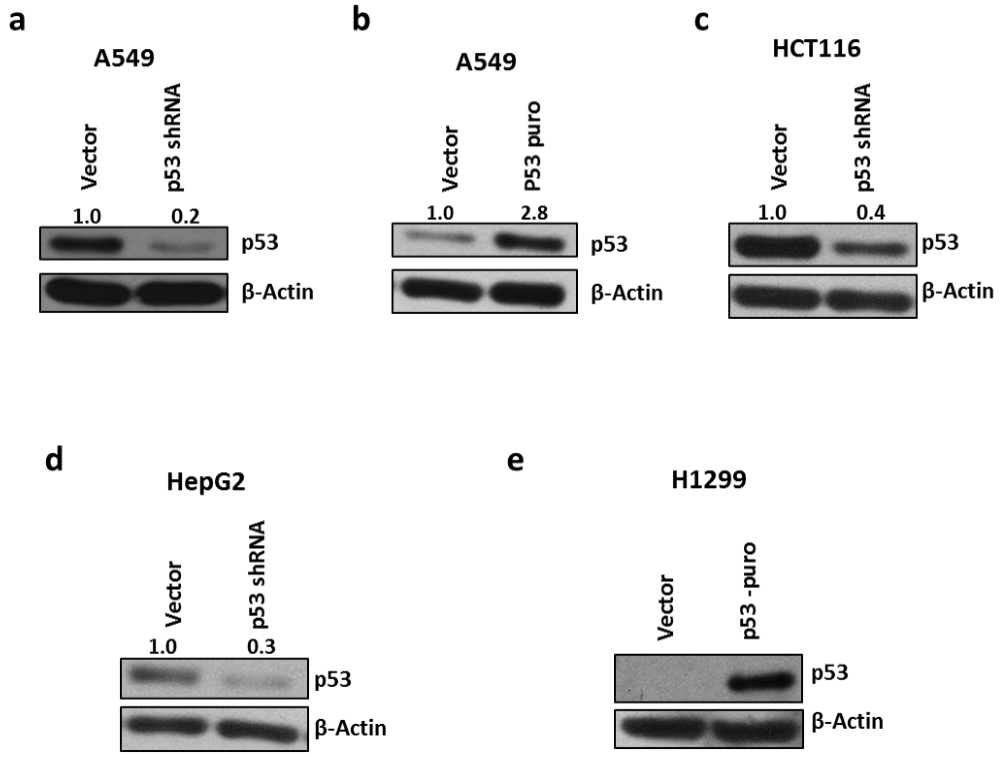

Figure 29: Western blotting confirmed p53 knockdown and overexpression in different cancer cells used for assaying associated changes.

a-e) western analysis of p53 protein in p53 knockdown A549, HCT116 and HepG2 cells and over-expression in A549 and H1299 cells.

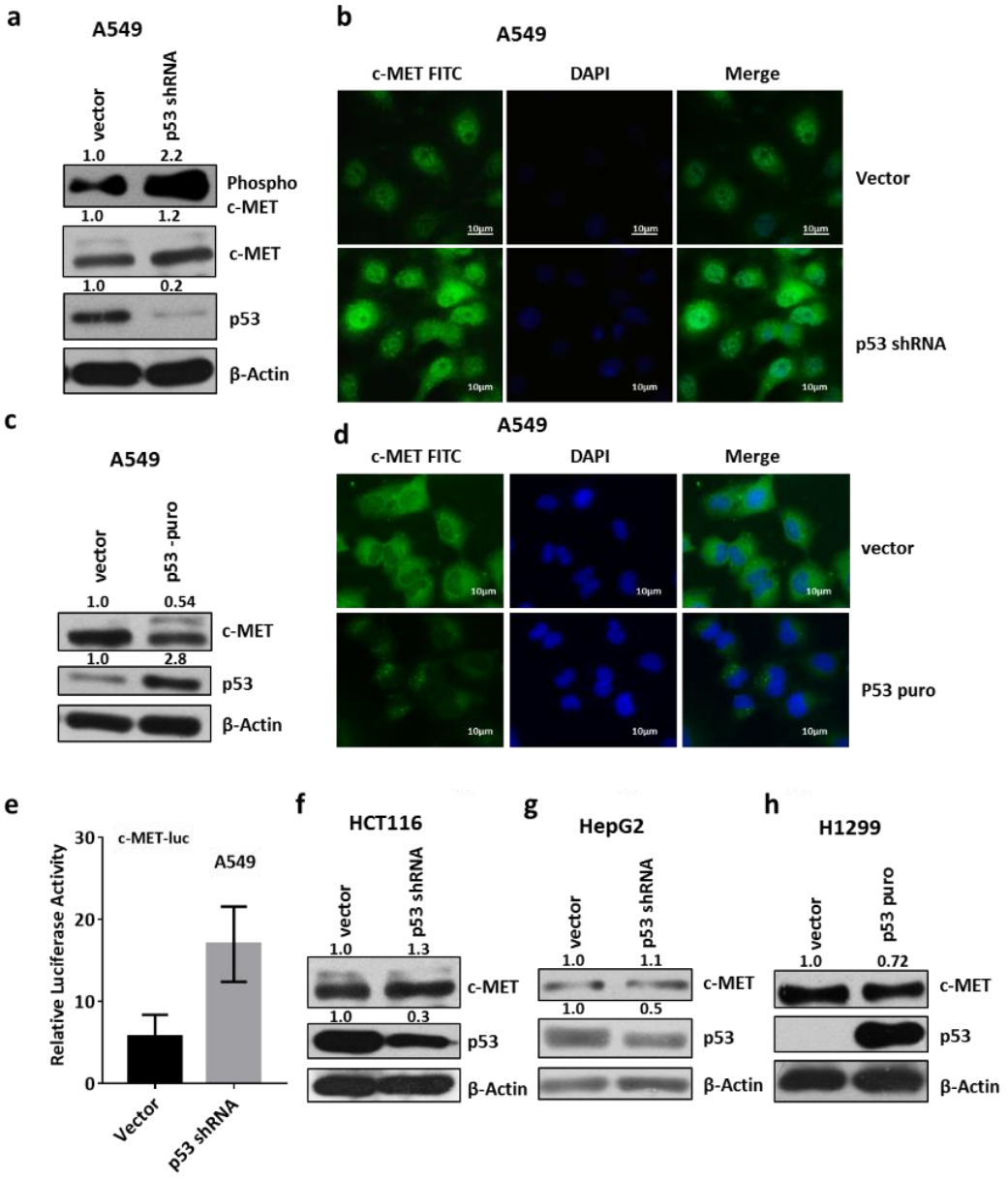

Figure 30: p53 negatively associated with c-MET expression in cancer cells.

a) Immunoblotting of p53, c-MET, and phosho-c-MET in p53 knockdown A549 cell. p53 downregulation promotes the expression and activity of the c-MET protein. b) Immunofluorescence analysis of c-MET protein in p53 knockdown A549 cells confirmed the western result. c) Western blotting and d) immunofluorescence assays for c-MET expression in p53 overexpressing A549 cells. The result shows that p53 has a suppressive effect on c-MET expression. e) Enhanced luciferase reporter expression from c-MET promoter in p53 knockdown A549 cells. f & g) Western blot analysis of p53 and c-MET proteins in HCT116 and HepG2 cells knocked down for p53 expression. h) Immunoblotting of c-MET and p53 protein of H1299 which transfected with the p53 expression vector. p53 overexpression in a null cell for this protein reduces the c-MET expression level.

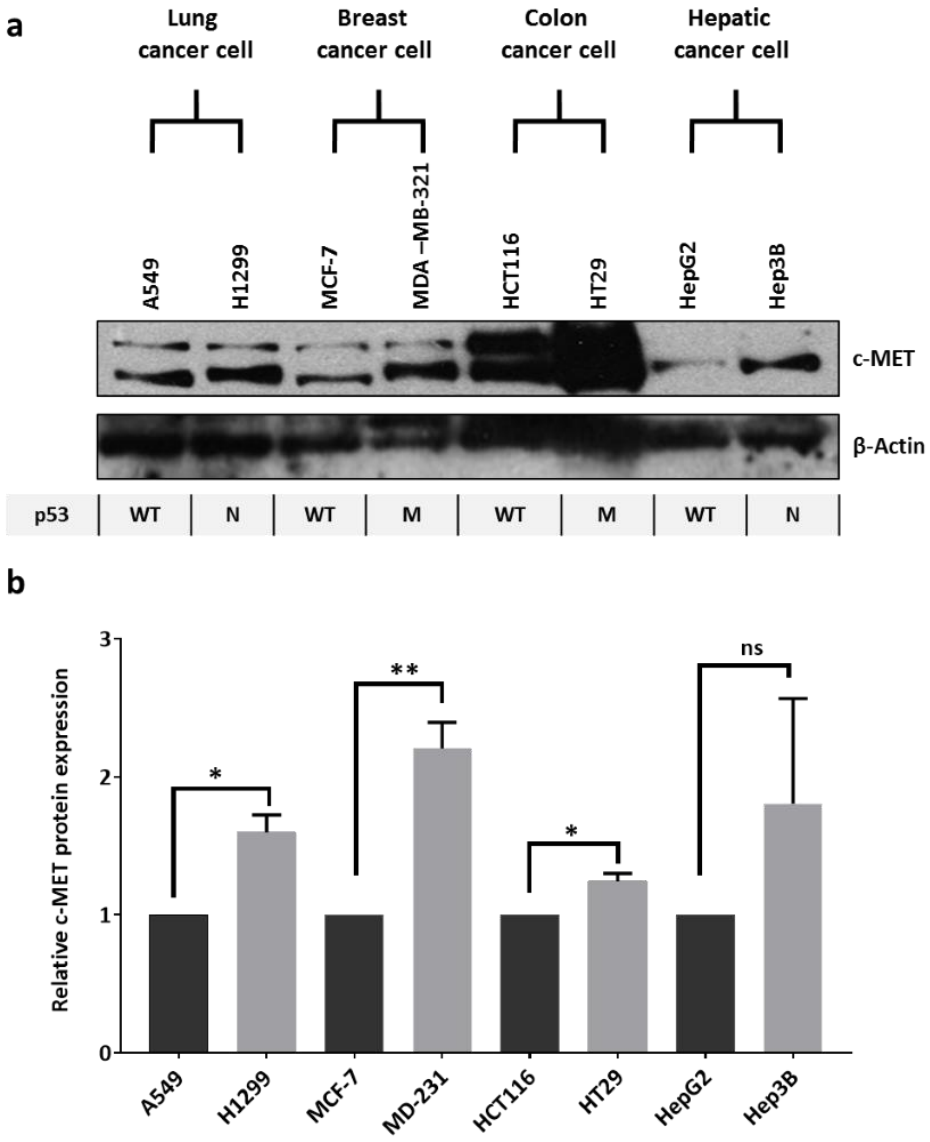

Figure 31: c-MET protein level in different cancer cell lines shows, its p53 dependent expression.

a) Western blotting of c-MET protein in different cancer cell lines. b) Relative expression of c-MET protein in different cancer cells. A significant difference in c-MET expression in each pair was calculated by unpaired T-test. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

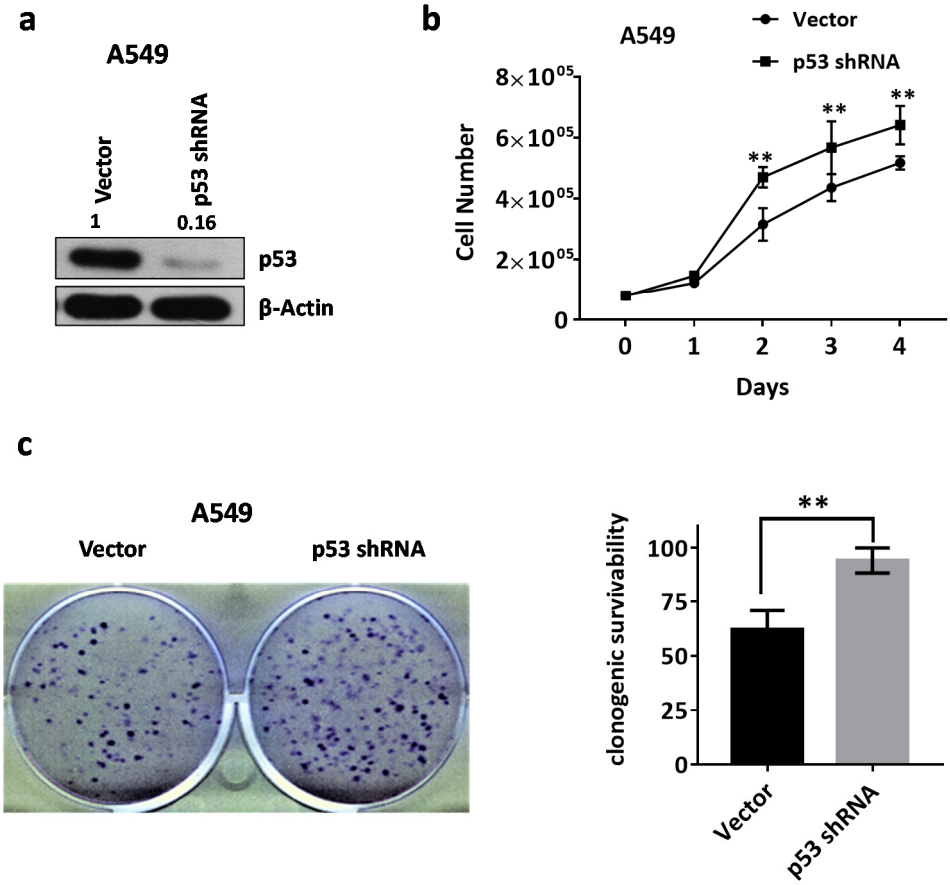

Figure 1: p53 knockdown promotes the growth and survivability of A549 cells.

a) Immunoblotting of p53 protein of A549 cells targeted by p53 shRNA. b) Cell proliferation assay of A549 cells shows increase in cell growth after p53 downregulation. c) Clonogenic survival assay was performed in p53 downregulated A549. Loss of p53 expression promotes the cell survivability. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

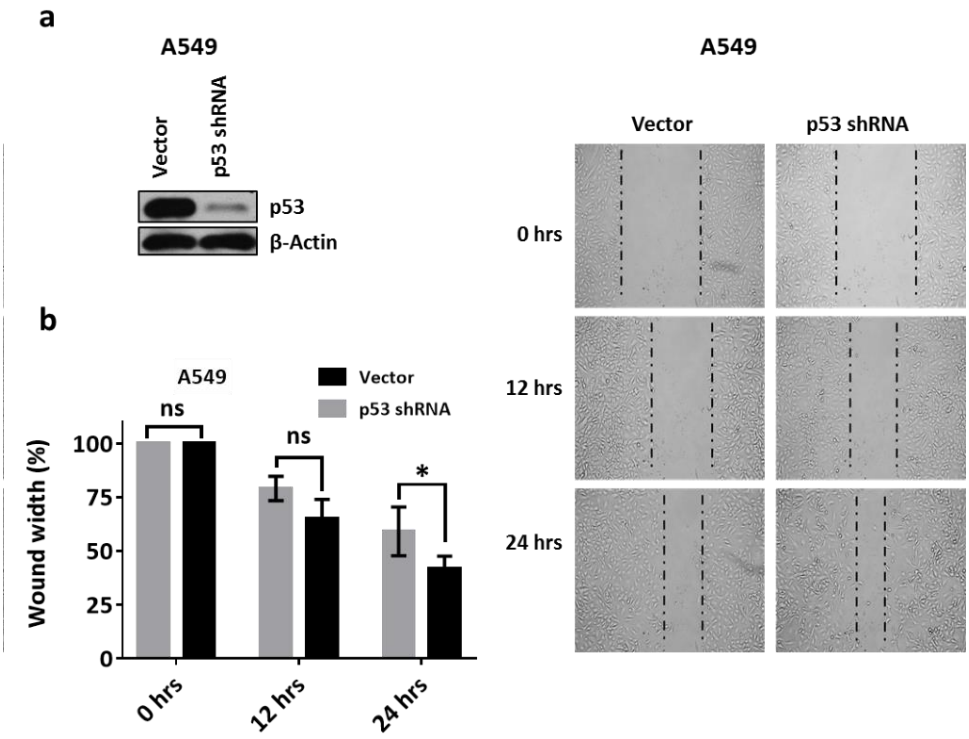

Figure 33: Migratory properties increased in p53 downregulated A549 cells.

a) Western blot of p53 protein of A549 cells transfected with p53 shRNA. b) Wound healing assay used to detect the effect of p53 downregulation on A549 cell migration. p53 knockdown cell shows increased migratory potential compared to control cells. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

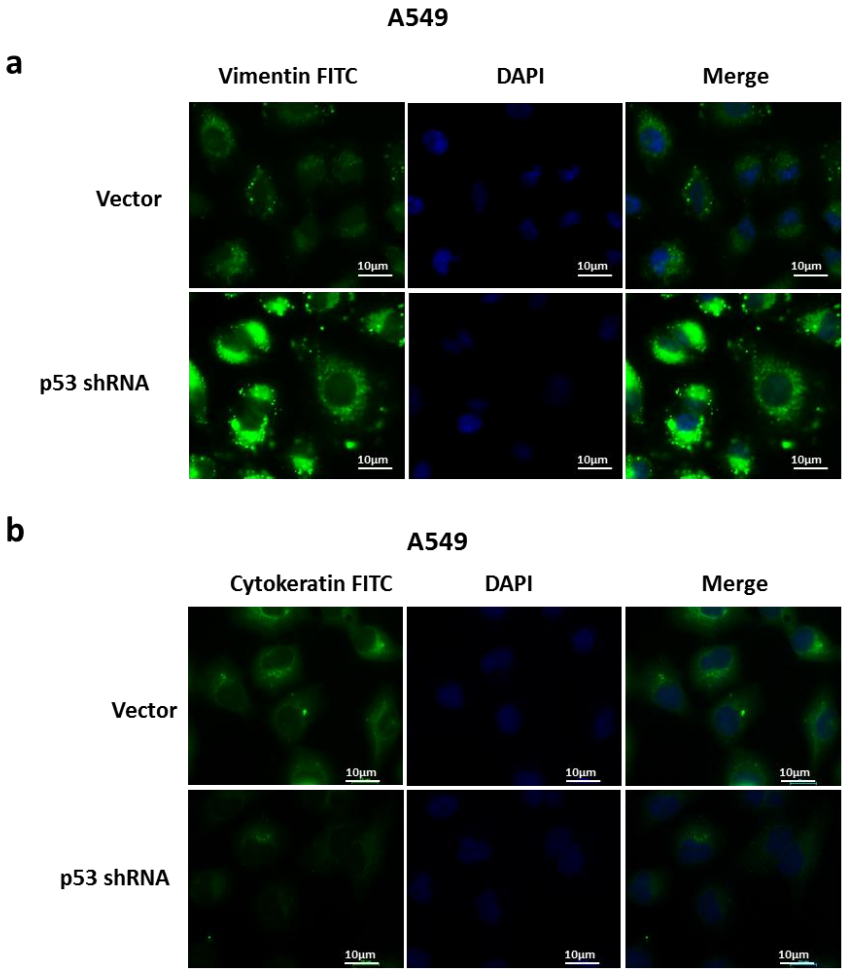

Figure 34: p53 expression is negatively associated with EMT.

a) Immunofluorescence of a mesenchymal marker of vimentin in p53 knockdown A549 cell. p53 downregulation in A549 leads to increased expression of vimentin. b) Cytokeratin is an epithelial and differentiation marker and is decreased in the p53 knock down A549 cells.

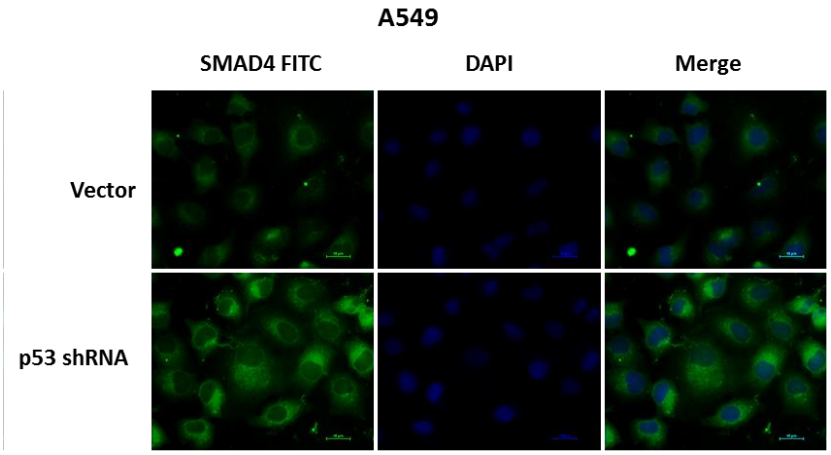

Figure 35: p53 downregulation induces the expression of the SMAD4 protein in A549 cells.

Figure is representing Immunofluorescence of SMAD4 protein in p53 downregulated A549 cells. SMAD4 expression was increased on p53 depletion.

4.2.14 Discussion

hTERT is the protein component of telomerase. It contributes in cancer development by maintenance of telomere length that help cells to overcome from telomere shortening problem. But hTERT inhibition shows very drastic effect on cell survivability. This effect indicated in previous reports and we also get similar kind of effect on hTERT downregulation. hTERT performed telomerase-independent functions but very limited information is known till date. hTERT has functional association with cell growth survivability, migration, and EMT. In the present study, our results strongly suggest the role of hTERT in cancer properties. c-MET is an onocoprotein, its expression and activity closely associated with cancer development. c-MET expression promotes the metastasis and invasion of cancer cells. hTERT and c-MET both proteins are up-regulated in cancer cells and both shows positive influence on cancer development[264,5,]. So, to find out the cross talk between hTERT and c-MET in cancer cells, we overexpressed the hTERT in cancer cells and increased expression of hTERT up-regulates the c-MET level while its down-regulation significantly reduces the expression of c-MET in the cancer cell. Our results show that hTERT and c-MET has a positive association in cancer cells. To understand the mechanism behind the hTERT mediated regulation of c-MET, we searched for putative transcription factor binding sequences in the c-MET promoter, and we got many sites for binding transcription activators like Nf-kB, c-Myc, and SP1 and transcriptional suppressor like p53. Earlier reports suggest the role of p53 in c-MET regulation. So, we checked the effect of hTERT on p53 expression. hTERT down-regulation induces p53 expression in A549. We also investigated the effect of hTERT on Nf-kB (p65) and c-Myc. hTERT knockdown in p53 positive cells induces the expression of Nf-kB (p65) while in p53 null cells it reduces NF-kB expression. We also found that hTERT inhibition reduces the p65 in embryonic kidney cell HEK 293T that is non-cancerous cells. This result shows that hTERT has differential Nf-kB (p65) signaling concerning the p53 expression. We proposed that hTERT knockdown in p53 positive cells induces the expression of p53 protein. p53 inhibits the phosphorylation of IKKγ (NEMO), so Nf-kB (p65) remains sequestered in the cytoplasm that leads to the increased in Nf-kB (p65) protein level. Another possibility is that hTERT mediated increased p53 promotes the apoptosis through Nf-kB (p65), but these two possibilities need further investigation. We also checked the effect of hTERT expression on c-Myc. hTERT

overexpression in H1299 leads to increase the c-Myc protein. For further study, we selected the p53 signaling in hTERT mediated modulation of c-MET expression.

p53 is tumor suppressor protein and loss or mutation in this protein is associated with cancer development in most of the aggressive cancers; the loss or mutation in p53 seems to be a hallmark of cancer[265,266,267]. Wild-type p53 is a transcription suppressor protein, and it targets the genes involved in growth, survivability, angiogenesis, and metastasis[268]. Promoter analysis of c-MET reveals p53 binding site on the c-MET promoter. So, we hypothesized that hTERT acts as a negative regulator for p53 and via p53, it may influence the transcription of c-MET. Moreover, down regulation of p53 in A549, HCT116, and HepG2 cells induces the expression of c-MET, while expression of exogenous p53 in A549 and H1299 cells reduces the c-MET expression. Reporter assays of c-MET promoter confirm the regulation of c-MET expression by p53 at the transcription level. We also checked the expression of c-MET in different cancer cell lines. These cells vary in p53 expression or form. Those cells having mutant form or lack p53 expression, shows higher expression of c-MET.

Further, we checked the effect of p53 on cancer cell growth, survivability, migration, and EMT. P53 down regulation in A549 promotes the cell proliferation and survivability. p53 expression was reduced by shRNA that promotes the wound healing capacity of A549 cells. Expression of this protein also associated with the EMT in cancer cells. p53 down regulation promotes the expression of vimentin and reduces the epithelial protein and marker of differentiation like cytokeratin in A549. Results presented in earlier sections show that hTERT silencing lowers the SMAD4 and induces the p53 expression. Thus, SMAD4 expression was assayed in p53 targeted A549 cells. p53 reduction increased the SMAD4 protein. SMAD4 is a prominent EMT regulator. It is well known to promote the expression of mesenchymal proteins like; E-cadherin and vimentin.

Here based on the results, we were concluded that hTERT controls cancer growth, survivability, motility and EMT by modulating the expression of c-MET. c-MET has the binding sites for tumor suppressor p53 on its promoter. hTERT is regulating the expression of c-MET by modulating the expression of p53.

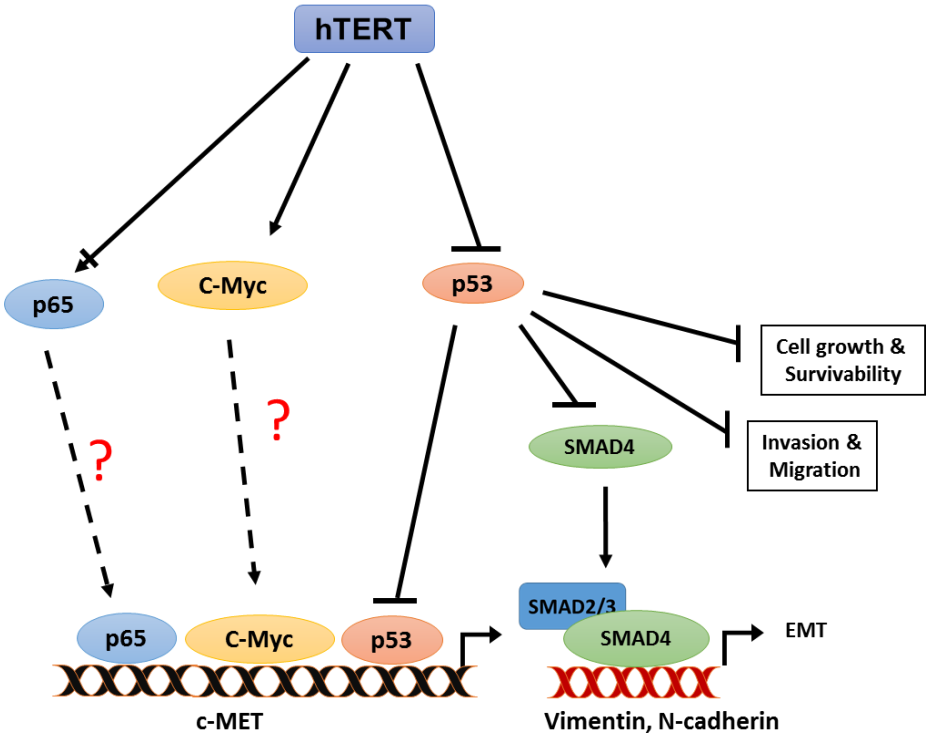

Figure 36: A schematic representation of possible hTERT mediated regulation of c-MET and hallmarks of cancer cell via p53.

Objective 3

hTERT modulated HGFR/c-MET signalling in cancer cell invasion and migration

4.3 Objective 3: hTERT-modulated HGFR/c-MET signaling in cancer cell invasion and migration

4.3.1 c-MET is associated with regulation of growth and survivability of cancer cells

To find out the role of c-MET in cancer cell proliferation, c-MET expression was knocked down by shRNA, and cell proliferation assayed (Fig. 37a & b). As expected, cell proliferation was impeded following c-MET down regulation in A549 cells (Fig. 37c). Further, the role of c-MET in cell survivability was evaluated by colony forming assay. The results show that c-MET down regulation significantly reduces the colony formation by A549 cell (Fig. 37d).

4.3.2 Down regulation of c-MET reduces the migration potential of A549

Furthermore, we tested the impact of c-MET knockdown on cancer cell migration. Cells were transduced with c-MET and shRNA virus, and positive transductant cells were selected for puromycin (2µg/ml) resistance and used for wound healing assay. To minimize the effect of cell growth on migration, vector control, and c-Met shRNA transfected A549 cells were treated with mitomycin c (5 µg/ml) for 2 hours. Then cells were left to migrate for 24 hours and cell migration efficiency was estimated regarding percentage wound healing by the cells. We found that c-MET inhibition in A549 reduces cell migration (Fig. 38 a & b).

4.3.3 c-MET and EMT have positive correlation in cancer cells

EMT is a crucial step in the process of cancer development. EMT helps cancer cells to gain metastatic potentials and resistance against chemotherapy. To find out the role of c-MET in EMT, shRNA was used to target the c-MET transcript. EMT involved change in expression of proteins that provide the mesenchymal features to cells. Diminishing c-MET expression reduces the mesenchymal marker, vimentin and increases the epithelial character by increasing the expression of E-cadherin in A549 cell (Fig. 39 a-c).

4.3.4 Inhibition of c-MET in cancer cells promotes the expression of differentiation marker

c-MET is a mitotic factor that promotes the mesenchymal feature in cancer cells. c-MET restricts the cell differentiation in cancer cells that help the cells to divide uninterruptedly. To know the role of c-MET in differentiation process, cytokeratin level was monitored in A549 cells knocked down for c-Met. Cytokeratin is an epithelial marker which is increased on c-MET knockdown (Fig. 40 a & b).

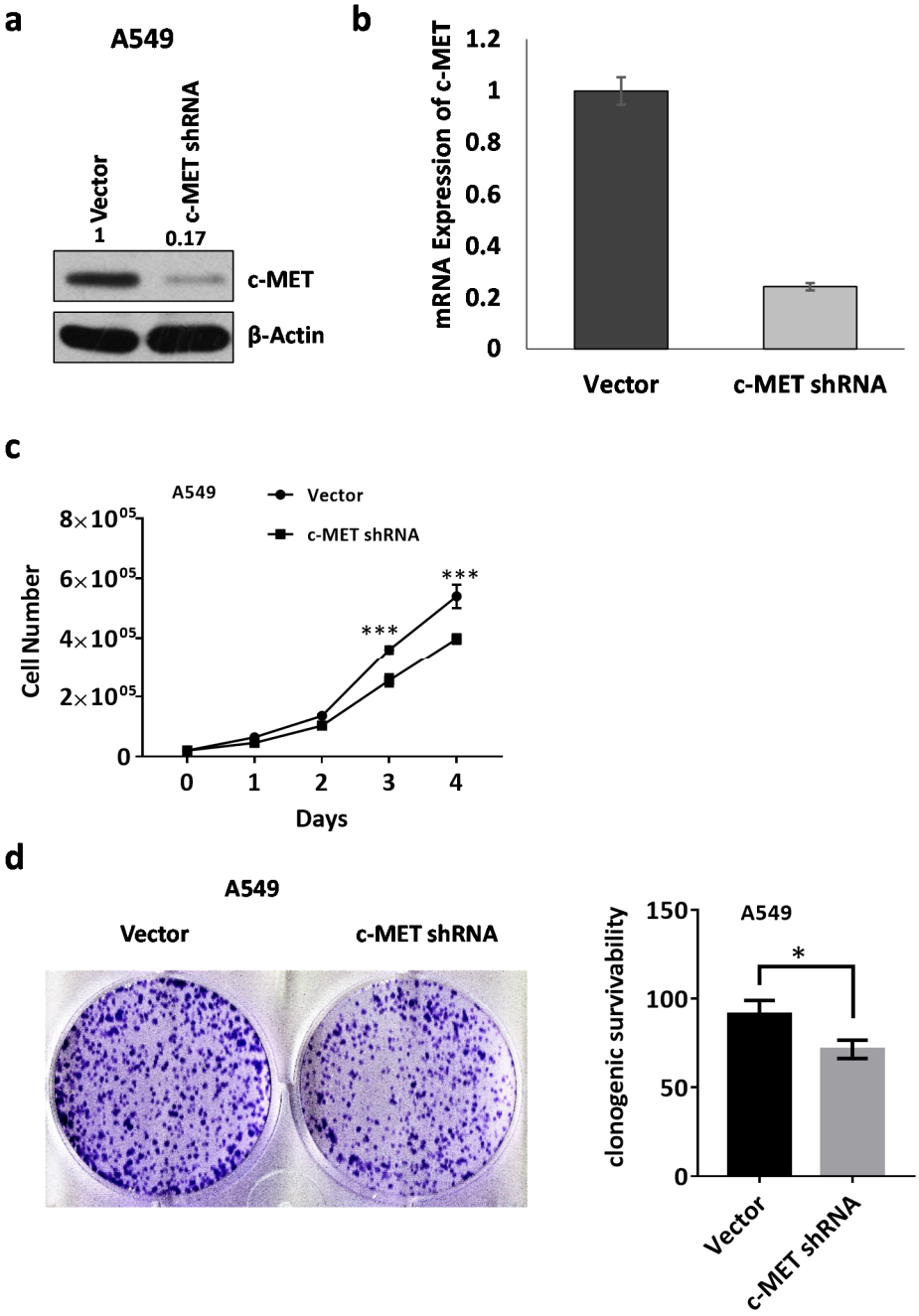

Figure 1: c-MET is associated with regulation of growth and survivability of cancer cells.

a & b) The targeted shRNA reduces the expression of c-Met at both protein and transcript level in A549. c) Growth kinetics of c-MET downregulated A549 cells showing reduction in cell proliferation following c-Met knockdown d) c-MET knockdown also reduces the A549 cell survivability assayed by clonogenic assay. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

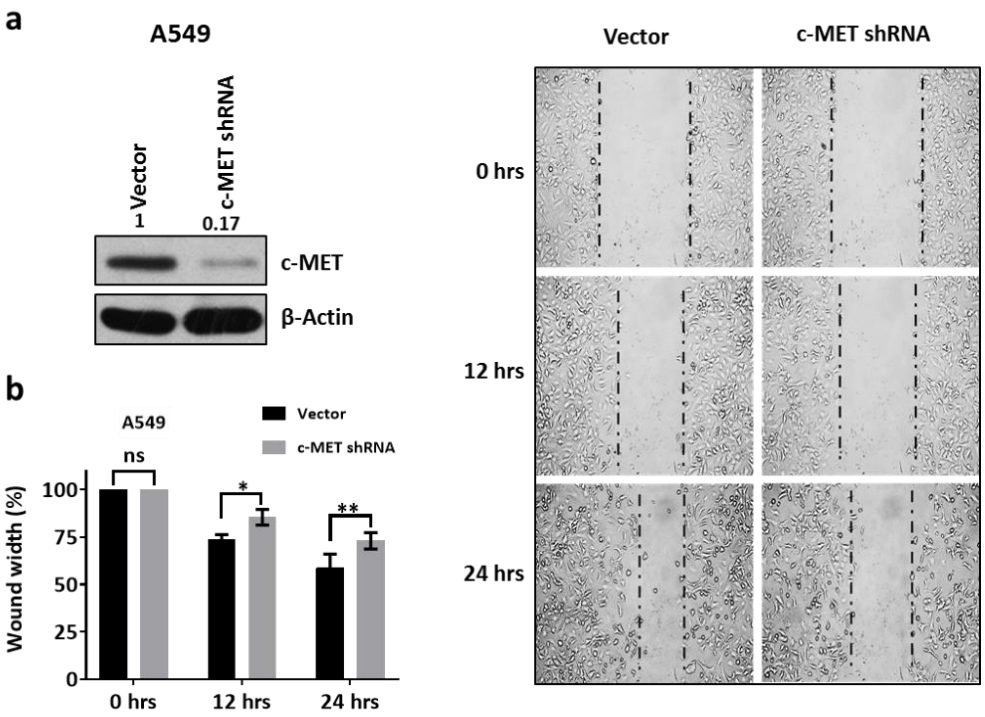

Figure 38: Downregulation of c-MET reduces the migration potential of A549.

a & b) wound healing assay used to observe the effect of c-MET knockdown on A549 cell migration. c-MET downregulation reduces the migratory potential of A549 cells. Represent p-value <0.05, *p > .01, **p= 0. 0.0029, ****p>0.0001, NS= not significant

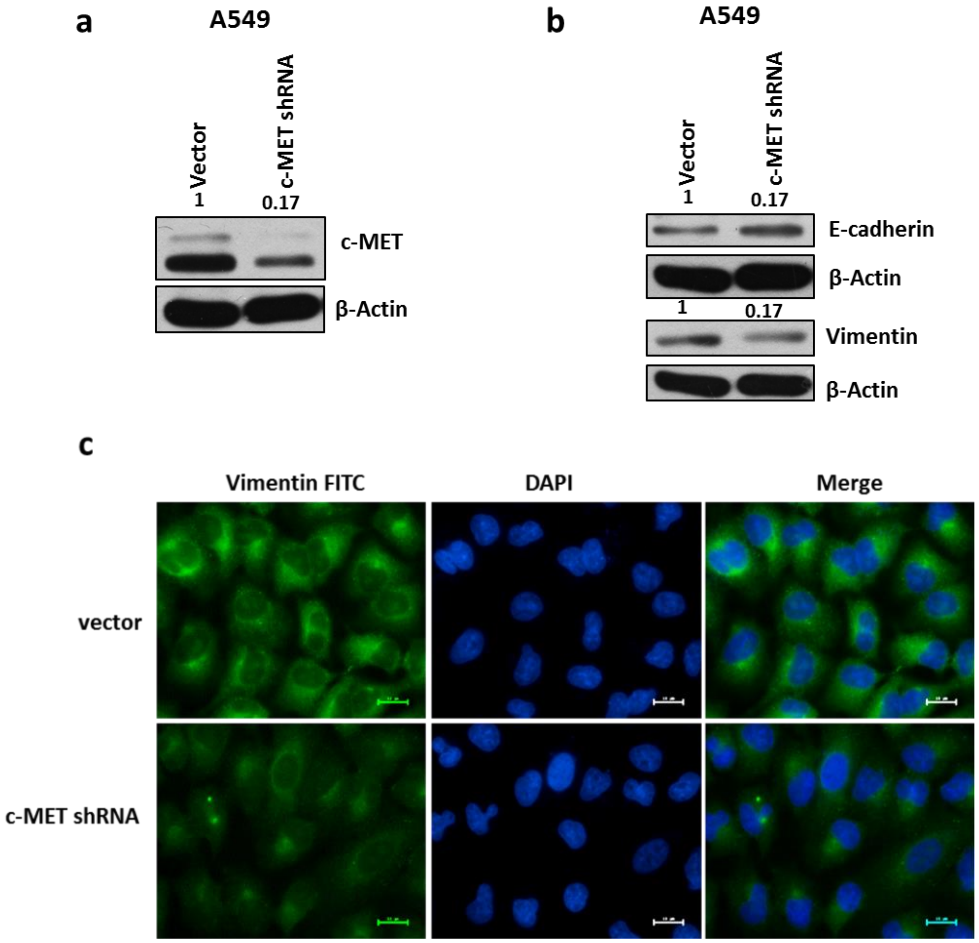

Figure 39: Expression of c-MET and markers of EMT have a positive correlation in cancer cells.

a & b) western blot analysis of vimentin and E-cadherin following c-MET knockdown in A549 cells. c-MET downregulation induces the expression of E-cadherin and reduces the vimentin expression. c) Immunocytochemistry of vimentin shows the reduce expression in c-MET knockdown A549 cells.

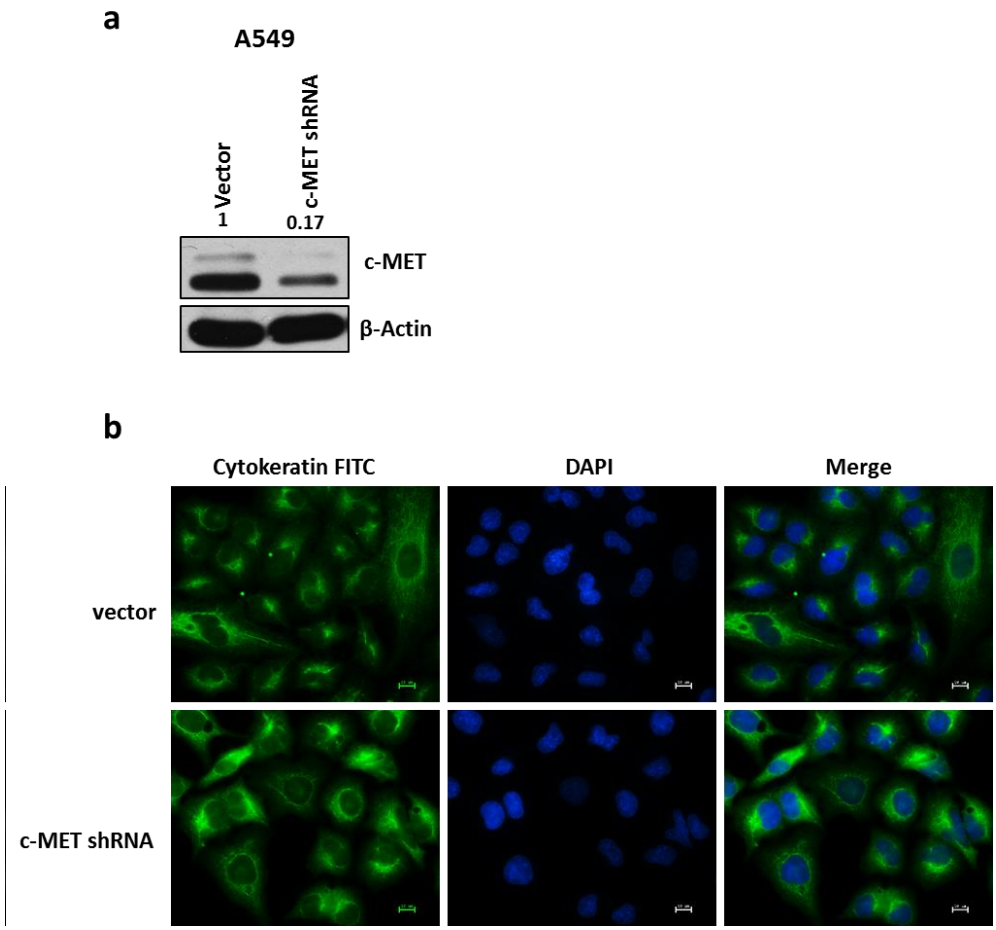

Figure 40: Inhibition of c-MET in cancer cells promotes the expression of differentiation marker.

a & b) immunocytochemistry of cytokeratin c-MET downregulated A549 cells. c-MET transcription inhibition induces the expression level of epithelial differentiation marker cytokeratin in the cell.

4.3.5 Discussion

HGFR/c-MET is an oncoprotein, containing 1390 aa long disulfide linked heterodimer which consists of extracellular α and β domains, transmembrane helix, and a cytoplasmic domain. c-MET is a receptor tyrosine kinase plays a critical role in cell migration during embryonic development and organ regeneration[269]. c-MET signaling also associated with cancer development. Activation of c-MET signalling is which started by binding of HGF ligand in an autocrine or paracrine manner that induces the MAPK, PI3K and STAT3 pathways. These pathways are well recognized in the process of cytoskeleton reorganization, survivability, growth, and migration process. In the present investigation, we validated the role of c-MET in the process of cell proliferation, survivability, migration, and EMT. c-MET down-regulation by RNA interference in A549 cells lowers cell proliferation. Cells survivability in an isolated environment is also an important feature of cancer cells. c-MET/HGF signaling induces the Ras/Raf pathways in cancer cells which reduce apoptosis. This signaling also promotes the expression of cytoprotective haem oxygenase (HO-1) and anti-apoptotic protein Bcl-2/Bcl-xl[270] to promote cell survivability. Our result is also suggestive of a role of c-MET in survivability. c-MET inhibition in A549 reduces the survivability as assayed by the colony forming assay. c-MET expression in the cancer cell is associated with increased invasion and migration[271]. We also observed the reduction in the A549 cell migration on c-MET down regulation. Further, we investigated the role of c-MET in EMT process. Vimentin is a mesenchymal marker protein and was reduced in c-MET knockdown cells while E-cadherin, an epithelial marker, was found up regulated. Cytokeratin, a marker of differentiation, was up regulated in the c-MET knockdown A549 cells thus showing a negative association of c-MET with the differentiation process. *In-vitro* studies demonstrate the role of c-MET in the regulation of different steps of hallmarks of cancer.

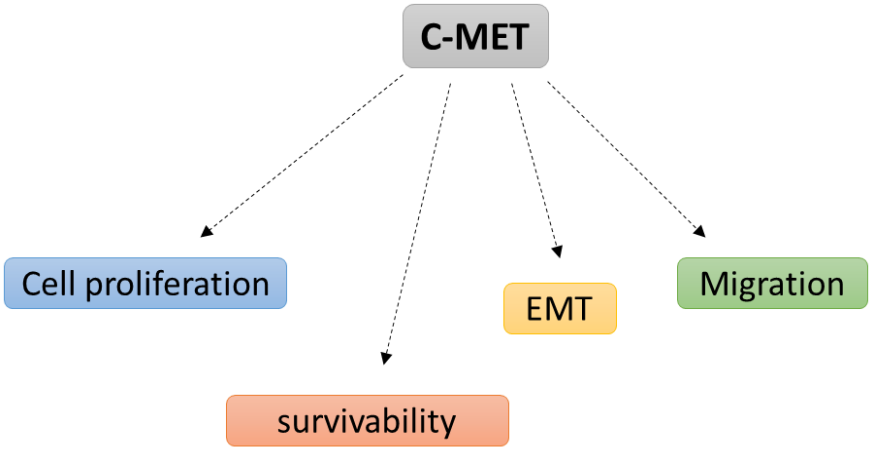

Figure 41: A schematic view of c-MET mediated regulation of different steps of hallmarks of cancer.

SUMMARY

5 SUMMARY

hTERT expression is associated with cellular immortalization, cell growth, and cancer malignancy[254]. However, very limited evidences are available about mechanisms of extracurricular activities of hTERT and its association with cancer cell malignancy[255]. In the present study, our results strongly suggest for the role of hTERT in cancer growth survivability, migration, and EMT. c-MET is an oncoprotein, highly expressed in most of the malignant cancer cells. Its activity is associated with increased metastasis and invasion of cancer cells[233]. TERT and c-MET, both proteins are up-regulated in cancer cells [264,5]. Hence to find out the cross talk between hTERT and c-MET, we modulated the expression of hTERT and found that the increase in hTERT expression up-regulates the c-MET level while its down-regulation markedly reduces the expression of c-MET in the cancer cell. Our result suggests that hTERT was actively associated with transcriptional regulation of c-MET in cancer cells.

hTERT is the protein component of core telomerase enzyme. It interacts with RNA component of telomerase and is involved in the telomere length maintenance[272]. hTERT is not a common transcription factor that translocates to nucleus and regulates the gene expression, but some recent reports suggest that hTERT is acting as a transcription factor or transcription modulator to regulate the expression of other genes[273]. hTERT had shown interaction with BRG1 that is chromatin remodelling protein. BRG1 is important regulator of the Wnt/beta-catenin target genes[274]. hTERT also interacts with beta-catenin and perhaps facilitates its nuclear localization[9]. hTERT shows the interaction with Nf-kB p65 subunit to facilitates the nuclear localization and binding with target gene promoters such as cytokines, TNF-alpha and IL-6[143]. These genes are essential for inflammation and cancer progression[275]. hTERT-p65 interaction regulates the expression of MMP-2 and MMP-9 in cancer cells independent of telomerase activity[147]. hTERT promotes heparanase expression via increased binding efficiency of c-Myc protein on heparanase promoter. It enhances invasion and metastasis in gastric cells by interacting with c-Myc and binding to the heparanase promoter to enhance the heparanase expression[276]. These reports suggest the role of hTERT beyond telomerase activity and its involvement in cancer invasion and metastasis. We transfected the hTERT expression vector in H1299 cells and observed the effect on c-MET expression, and found that hTERT expression up-regulates the expression of c-MET in cells while down-regulation of hTERT in A549 cells reduces the c-MET expression. To understand

the mechanism behind the hTERT mediated regulation of c-MET, we searched for transcription factor binding sequences in the c-MET promoter, and got binding sites for may transcription activators like Nf-kB, c-Myc, and SP1 and some transcriptional suppressor like p53 have the binding site on the c-MET promoter. Previous reports suggest a role of p53 in c-MET regulation. So, we checked the effect of hTERT on p53 expression. hTERT downregulation induces the expression of p53.

p53 is a tumor suppressor protein and loss or mutation in this protein promotes cancer development[265,266]. Variation in expression and form of p53 is observed in most of the aggressive cancers and it seems to be a hallmark of cancer[267]. Wild-type p53 is transcription suppressor protein, and it targets the growth and survival associated genes[268]. Promoter analysis of c-MET reveals p53 binding site on c-MET promoter. So, we hypothesized that hTERT acts as a negative regulator for p53 and via p53, it regulates the transcription of c-MET. Moreover, down-regulation of p53 in A549, HCT116 and HepG2 induces the expression of c-MET, while exogenous expression of p53 in A549 and H1299 reduces the c-MET expression. Reporter assay with c-MET promoter following p53 knockdown in cells confirms the involvement of p53 in transcription regulation of c-MET.

c-MET is a receptor tyrosine kinase crucially involved in the growth and cell migration during embryonic development and organ regeneration[269]. c-MET signaling also plays a significant role in cancer development. Activation of c-MET signaling is started by binding of HGF ligand and induced MAPK, PI3K and STAT3 pathways and leads to activation of cytoskeleton remodeling, survival, growth, and migration associated protein[277,278]. Cytoskeleton reorganizing protein molecules modified the pattern of intracellular filaments to promote the cell invasion and migration. Our results also support the previously reported function of c-MET. We found that down-regulation of c-MET in A549 lung cancer cells reduces the cell proliferation and survivability. Further, we checked the role of c-MET in migration and EMT process. C-MET knockdown decreases the A549 cell migration and it shows its effect on EMT markers; mesenchymal marker vimentin was reduced, and the epithelial marker (E-cadherin) increased. C-MET inhibition also leads to increase of differentiation marker cytokeratin.

hTERT promotes the expression and activity of ITGB1, VEGF, growth response (EGR)-1, MMPs that are associated with cancer progression[279,280]. This may not be the only pathway by which hTERT executed its role in cancer development. Here we propose another new significant mechanism in which hTERT is regulating expression of c-MET via modulating the expression of p53 in cancer cells. hTERT mediated regulation of c-MET promotes the aggressiveness of cancer cells. In view of the aforesaid, it seems likely that targeting of hTERT mediated c-MET pathway may offer a new strategy for treatment of cancer.

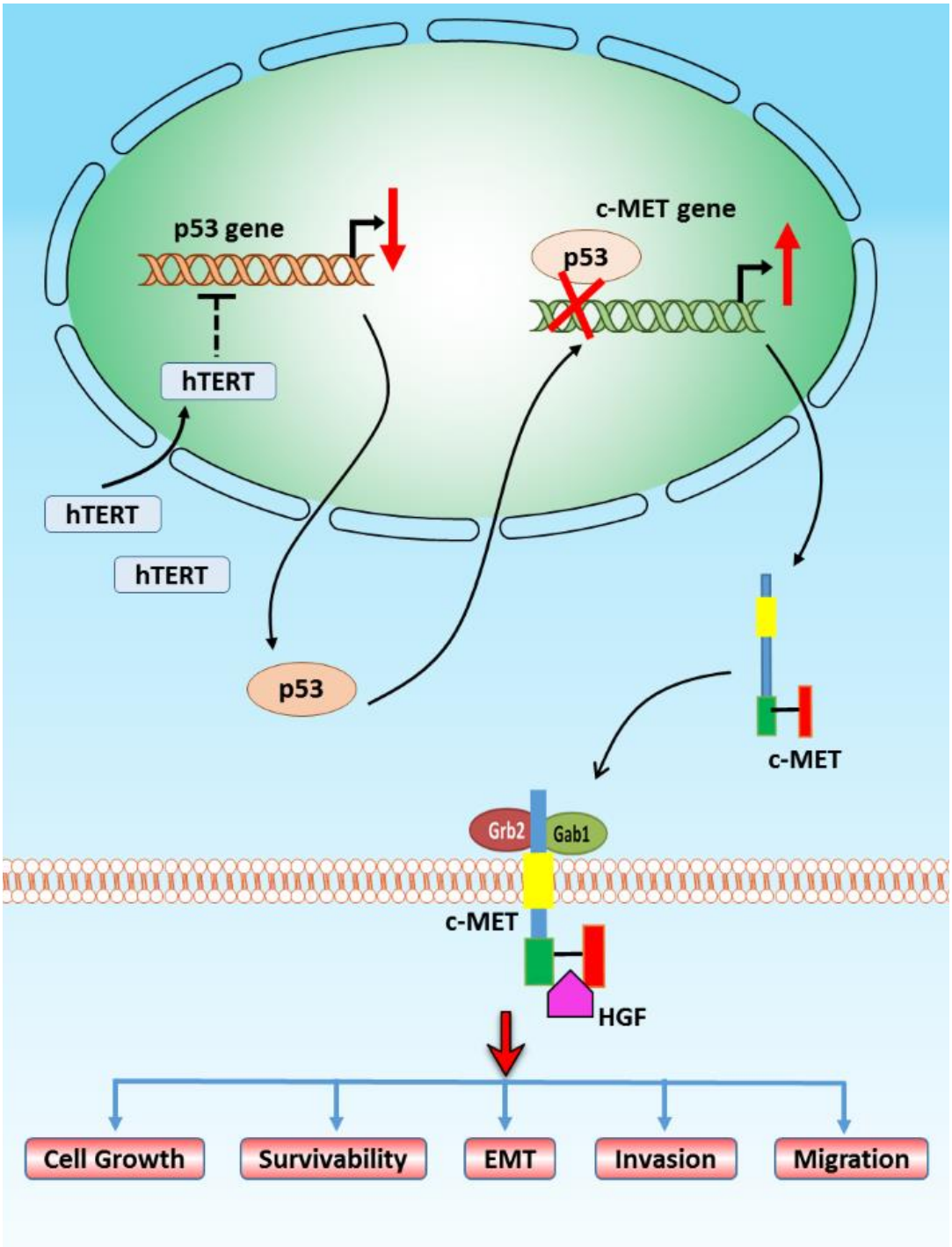

Figure 42: A schematic representation of hTERT mediated regulation of c-MET via p53.

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APPENDIX

7 APPENDIX

Table A1: Phosphate-Buffer Saline (PBS)

Components	Concentration	Amount
NaCl	137mM	8 g
KCl	2.7mM	0.2 g
Na_2HPO_4	10mM	1.44 g
KH_2PO_4	2mM	0.24 g

All the components were dissolved in 800ml and pH was adjusted to 7.4 before making final concentration 1 litter.

Table A2: Tris Acetic Acid Electrophoresis (TAE) buffer

Components	Amount for 50X stock (1 litter)
Tris-base	242g
Glacial acetic acid	57.1 ml of glacial acetic acid
EDTA	100 ml of 0.5 M EDTA (pH 8.0)

Table A3: Ethidium Bromide stock (10 mg/ml)

0.01g of ethidium bromide was dissolved in 1ml double distilled or MQ water.

Table A4: 30% Acrylamide solution:

Components	Amount
Acrylamide	29.22g
N, N'-methylenebisacrylamide	0.78g
Water	to 100 ml

Solution was dissolved in dark and filtered Whatman filter paper and stored in at 4℃in a dark bottle.

Table A5: 10% (w/v) Ammomium Persulphate (APS)

1g of APS was dissolved in 10 ml of MQ water and stored at -20℃.

Table A6: 1X SDS Running Buffer

Components	Amount
Tris-base	3.02g
Glycine	14.26g
SDS	1g
Water	Up to 1000 ml

Table A7: 1X Transfer Buffer

Components	**Amount**
Tris-base	3.02g
Glycine	14.26g
Water	800ml

Tris and glycine were dissolved in of water then 20% methanol was added.

Table A8: 20X Tris Buffer Saline

Components	**Amount**
NaCl	160g
KCl	4g
Tris-base	60g
Water	1000ml

Table A9: LB Medium (Luria-Bertani medium) preparation

Components	**Amount**
LB broth powder	2.5 g
Deionized water	100 ml

The dissolved LB media was autoclaved and stored at 4℃.

Table A10: Antibiotics stocks and working dilutions

Antibiotic	**Stock solution**	**Working**	**Storage**
Ampicillin	100 mg/ml	100 µg/ml	-20°C
Spectinomycin	100 mg/ml	50 µg/ml	-20°C
Penicillin/Streptomycin mix	100X	1X	-20°C

Table A11: C**omplete Dulbecco Modified Eagle's Medium (DMEM)**

Components	**Amount (1 Litter)**
DMEM powder high glucose (4.5 g/l)	13.48 g
Sodium bicarbonate	3.7 g
Penicillin Streptomycin mix (100X)	1X
Fetal Bovine Serum (FBS)	10 % of volume

Table A12: Roswell Park Memorial Institute medium (RPMI) preparation

Components	**Amount (1 Litter)**
RPMI powder	13.48 g
Sodium bicarbonate	2.8 g
Penicillin Streptomycin mix (100X)	1X
Fetal Bovine Serum (FBS)	10 % of volume

Table A13: Preparation of 2.5% Trypsin-EDTA solution

Components	**Amount (100 ml)**
Trypsin	250 mg
EDTA	20 mg
Glucose	100 mg

Table A14: List of real time primers

Gene		5’ to 3’ Sequence
hTERT	F	CGGCGACATGGAGAACAAG
	R	CCAACAAGAAATCATCCACCAAA
Actin	F	ATGTGGCCGAGGACTTTGATT
	R	AGTGGGGTGGCTTTTAGGATG
c-MET	F	GAGAAGACTCCTACAACCCGAATACT
	R	CGGGACACCAGTTCAGAAAAG
SP1	F	AGCCAACCCCTGTGAATGC
	R	ATGGATTCCCCCCCCTTT
NF-kB	F	ACCGGATTGAGGAGAAACGTAA
	R	GGGAAGGCACAGCAATGC
c-Myc	F	AATGAAAAGGCCCCCAAGGTAGTTATCC
	R	GTCGTTTCCGCAACAAGTCCTCTTC
E-cadherin	F	CGGGAATGCAGTTGAGGATC
	R	AGGATGGTGTAAGCGATGGC
Vimentin	F	AGGAAATGGCTCGTCACCTTCGTGAATA
	R	GGAGTGTCGGTTGTTAAGAACTAGAGCT

Table A15: List of real time primers

Cloning primers

pGL3-HGFR	F	ATCTCGAGTGTCCATAAATAGGCCCAGTGC
	R	ATAAGCTTGCAATTTTACCTTTCGGTGCCC
pLVX –hTERT-HA	F	CGGTGGGAGGTCTATATAAGCA
	R	TTTCTAGATCAAGCGTAATCTGGAACATCGT ATGGGTAGTCCAGGATGGTCTTGAAGTC

8 LIST OF PUBLICATION

1. Interaction of Piriformospora indica with Azotobacter chroococcum. Soubhagya Kumar Bhuyan, Prasun Bandyopadhyay, Pramod Kumar, Deepak Kumar Mishra, **Ramraj Prasad**, Abha Kumari, Kailash Chandra Upadhyaya, Ajit Varma & Pramod Kumar Yadava, *Nature Scientific Reports*, 09 September 2015.

2. TCTP is required for TGF-β1 induced epithelial to mesenchymal transition and influences cytoskeletal reorganization. Deepak K Mishra, Pratibha Srivastava, Amod Sharma, **Ramraj Prasad**, Soubhagya K Bhuyan, Rahul Malage, Pramod Kumar, Pramod Kumar Yadava. (Manuscript under revision)

3. Human telomerase reverse transcriptase regulates c-MET expression via p53. **Ramraj Prasad,** Deepak K Mishra, Manoj Kumar, Amod Sharma, Pramod Kumar Yadava. (Manuscript under submission)

Poster presentation

- **R. Prasad**, D.K. Mishra, M. Kumar, A. Sharma, P.K. Yadava. hTERT mediated c-MET expression is p53 dependent in cancer cells. Submitted in Asia ESMO 2017 congress, Singapore.

- Mishra D.K., Rahul D., Sharma A., **Prasad R**, Yadava P.K. Modulation of TGF-β induced cancer cell reorganization by TCTP. (2016) in EMBO-EMBL symposium: Tumor microenvironment and signalling, Heidelberger Germany.

Conferences attended

- 5th international conference on stem cells and cancer. Jawaharlal Nehru University, India, November 2014.

- International symposium on current advances in radiobiology, stem cells, and cancer research. Jawaharlal Nehru University, India, February 2015.

- International conference on mitochondrial in health and disease. Jawaharlal Nehru University, India, February 2017.