Interaction of hTERT with Other Molecules Involved in Epithelial to Mesenchymal Transition

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By

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CERTIFICATE

The research work embodied in this thesis entitled **"Interaction of hTERT with Other Molecules Involved in Epithelial to Mesenchymal Transition"** has been carried out by Mr. **Rishi Kumar Jaiswal** at School of Life Sciences, Jawaharlal Nehru University, New Delhi, India. The work is original and has not been submitted so far, in part or full for any degree or diploma of any other University.

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Rishi Kumar Jaiswal

Dedicated

to

My Parents

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Abbreviations

Commonly used symbols and abbreviations

APS	Ammonium per sulphate
AMP	Ampicillin
Bisacrylamide	N, N-methylene bisacryamide
bp	Base pair
BSA	Bovine serum albumin
BRG1	BRG1, brahma-related gene-1
Conc	Concentration
DEPC	Diethyl pyrocarbonate
DMEM	Dulbeco's modified eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
dsDNA	Double strand DNA
DTT	Dithiothreitol
EMT	Epithelial-mesenchymal transition
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
g	Gram, (also centrifugal force relative to gravity)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GSK3β	Glycogen synthase kinase 3 beta
hrs	Hours
hTERT	Human telomerase reverse transcriptase

hTR/hTERC	Human telomerase RNA
Kb	Kilobase
KCl	Potassium chloride
kDa	Kilo Dalton
LB	Luria broth
M	Molar
MALDI-TOF/MS	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
mg	Milligram
miRNA	MicroRNA
mM	Milimolar
Min	Minute(S)
ml	Milliliter
mRNA	Messenger RNA
MW	Molecular weight
MMP	Matrix metalloproteinases
NCCS	National centre for cell sciences, pune
ng	Nanogram
nM	Nanomolar
nt	Nucleotide
O/N	Overnight
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide

PI3K	Phosphoinositide 3-kinase
PLAU/uPA	Urokinase-type plasminogen activator
PLAUR	Urokinase-type plasminogen activator receptor
pmol	Picomole
RNAi	RNA interference
Rpm	Revolution per minute
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SDS	Sodium dodecyl sulphate
Sec	Second
shRNA	Short hairpin RNA
siRNA	Short interfering RNA
SNAIL	SNAI1, snail homolog 1, a Zn finger protein
TEMED	N,N,N',N'-tetramethyl-ethane-1, 2-diamine
TGF-β	Transforming growth factor-β
Vol	Volume
ZEB1	Zinc Finger E-Box Binding Homeobox 1
μg	Microgram
μl	Microliter
˚C	Degree Celsius
%	Percentage

Introduction

Chapter 1: Introduction

Cancer is a pathological condition where the body's cells begin to grow and reproduce in an uncontrolled way. These cells can invade and destroy healthy tissues and organs. Spread of these cancer cells from one organ to another as a consequence of cancer cell invasion of blood vessels and lymphatics and seeding at loco-regional lymph nodes and distant organs is designated as cancer metastasis. Epithelial to mesenchymal transition is the main cause of metastasis. EMT is a multistage trans-differentiation process in which highly polarized epithelial cells undergo multiple biochemical changes and attain invasive and migratory potential during the pathological and physiological process such as cancer progression, embryonic development and wound healing. One of the properties of cancer cells is unlimited division and this is because of the activation of a specialized reverse transcriptase known as telomerase [1]. Normal cells do not havetelomerase and hence they usually dye after 50 generations. In cancer cells telomerasesolves the end replication problem by adding telomeric repeat at the end of thechromosome [reviewed in 2]. Telomerase is a ribonucleoprotein complex consisting of at least sixsubunits: human telomerase RNA component (hTERC); heat shock protein 90 (hsp90);human telomerase reverse transcriptase (hTERT); telomerase-associated protein 1

(TEP1); p23; and dyskerin [3]. hTERC and hTERT are the core subunits and their in vitro reconstitution leads to telomerase activity. hTERT is a rate-limiting component of telomerase. Involvement of telomerase in cancer progression is not limited to maintaining telomere length only. Many reports came which show that telomerase has many other functions apart from maintaining telomere length. Liu et al reported that hTERT promotes EMT via wnt/β-catenin pathway [4]. Choi et al. reported that TERT expression in mice enhances cancer progression [5]. From the microarray data generated after knocking down hTERT in our laboratory [6], we found genes of plasminogen system such as uPA seemed to have an association with hTERT. The inhibition of hTERT also resulted in regulation of certain other genes such as Phospholipase C, Beta 1 (PLCB1), Fibroblast Growth Factor 2 basic (FGF2), Tissue Factor Pathway Inhibitor 2 (TFPI2), Interferon Regulatory Factor 9 (IRF9), and Kruppel-Like Factor 4 (KLF4). All these findings suggest a key role of telomerase in metastatic cancer progression.

The role of uPA in cancer cell invasion and metastasis is also very well established [7,8]. TGF-β is a well-known inducer of EMT. It is reported that TGF-β induces expression of uPA in cancer cells [9]. Moreover, it is also demonstrated that hTERT is also involved in TGF-β mediated EMT. So, it can be hypothesized that telomerase promotes metastasis by regulating plasminogen activator and it would be interesting to see the regulatory relation between hTERT and plasminogen activator by knocking down hTERT as well as over-expression of hTERT. The molecular mechanism behind hTERT mediated regulation of plasminogen activator will be good to examine.

As we have discussed above that telomerase performs many other functions apart frommaintaining telomere length. Telomerase is also found to be involved in generegulation. It also interacts with many proteins and modulates their functions. So it will
be good to see the molecules regulated by hTERT.

1.1 Aims and objectives

From the above background, we set out the following objectives for the present study.

➢ To validate the relationship between hTERT and plasminogen activator.

➢ Identification of molecules regulated by hTERT.

➢ Phenotypic consequences of knocking down and overexpressing the above molecules.

Review of Literature

Chapter 2: Review of literature

2.1 Telomeres and Telomerase: the guardians of genomic integrity:

Before the discovery of double helical nature of DNA by Watson and Crick in 1953, the presence of special structures at the end of chromosomes was proposed by Hermann Muller and Barbara McClintock, who demonstrated that ends of naturally occurring chromosome are protected while broken chromosomes lead to chromosomal instability [10,11]. Muller called these ends telomeres and suggested that they must be distinguishable from DNA double-strand breaks. However, the exact molecular nature of telomeres remained unknown till Elizabeth H. Blackburn and Jack W. Szostak in 1982 discovered why cellular DNA repair machinery was unable to mistake native chromosome ends as double strand DNA breaks. Blackburn and Szostak found that chromosome ends protected by tandemly repeated sequences rich in guanosine correspond to telomeres and ensure chromosomal stability. After three years, a specialized reverse transcriptase enzyme called telomerase was discovered by Greider and Blackburn which is a unique ribonucleoprotein (RNP) enzyme, it adds telomeric repeats at the extreme ends of the chromosome. The 2009 Nobel Prize in Physiology or Medicine was awarded to Elizabeth H. Blackburn, Carol W. Greider and Jack W. Szostak for their discovery of this unique enzyme.

2.1.1 Replication problem at the end of the chromosome:

In evolutionary terms, the multiple linear chromosomes in eukaryotes are at a great selective advantage, because they permit a high rate of recombination involving the random reciprocal exchange of chromosomal DNA during sexual reproduction. The presence of linear chromosomes, however, has two disadvantages viz., (a) the difficulty with replication of the extreme ends and (b) the need to protect the ends from degradation and fusion with other chromosomes. DNA-dependent DNA polymerase is unable to copy the 3' end of the template because of obligate dependence on primer, which means that the copy strand shortens with each cycle of DNA replication leaving the unreplicated portion of the template strand single stranded. The 3' end of leading strand is continuously synthesized by conventional DNA-dependent DNA polymerase until the end of the template [Fig 1]. This means that the terminal DNA gets shorter through successive cycles of replication, which eventually leads to crisis and loss of

cell viability [Fig 1] [12,13]. The ends of chromosomes or telomeres consist of multiple repeats of a short sequence, which is usually G/T-rich and heterochromatic and thus transcriptionally inactive.
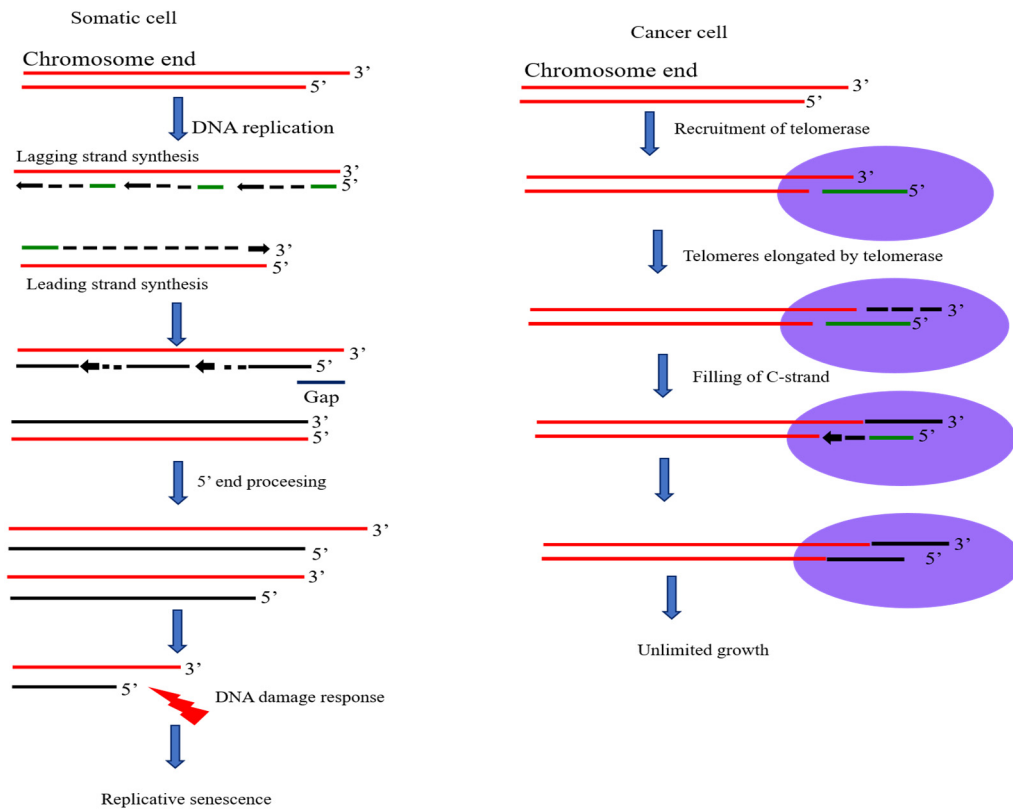
Figure 1: Telomerase expression in cancer cell leads to the immortality of cancer cells. Due to the end-replication problem in normal somatic cells, telomeres shorten with each cycle of DNA replication. In somatic cells during DNA replication DNA-dependent DNA polymerase is unable to copy the extreme 3' end of the template, which means that the copy strand shortens with each cycle of DNA replication, while the 3' end of leading strand is continuously synthesized by conventional DNA-dependent DNA polymerase until the end of the template. Thus the terminal DNA gets shorter through successive cycles of replication, which eventually leads to crisis and loss of cell viability [12,13]. In cancer cells, there is activation of a specialized reverse transcriptase called telomerase which uses its RNA component as a template to elongate telomere [14].

2.1.2 Telomere structures and functions:

Telomeres are DNA-protein complexes with repeats that vary widely between species: ciliate *Oxytricha nova* has 4.5 repeats of G4T4, while *Saccharomyces cerevisiae* has around 500 bp of telomeric repeats [15]. Human telomeres are made up of 5'-

TTAGGG-3' repeat sequences that are bound by a protein complex known as the shelterin complex [16–18]. Telomeric DNA is made up of both double-stranded repeated sequences and single-stranded G-rich overhang which consists of some specialized proteins [19]. Telomeric DNA is supposed to adopt T-loop (telomere loop) structure [Fig 2] where the single-stranded G-rich overhang end loops back on itself and 3′ G-strand overhang invades into the double stranded DNA (the so-called D-loop (displacement loop) [Fig 2] [20]. Telomeric DNA of human are guanine-rich and consists of 5'-TTAGGG-3' repeat sequences which can form a four-strand G-quadruplex structure involving G-tetrads [21]. The function of telomeric G-quadruplex structures inhibit of telomerase-dependent telomere extension, suppress recombination, and protect the telomere [22].

Humans have approximately 10-15 kb of telomeric DNA, while mice have 20-50 kb of telomeric repeats. Most of this telomeric DNA is double stranded but also contains a single-stranded region of several hundred base pairs which is always G-rich [19,23–25]. This single-stranded region basically plays two very important roles: it facilitates extension of the telomere and binds with different components of the shelterin complex to form a cap at the end of the chromosome.

2.1.2.1 Shelterein complex:

Shelterein complex is a protein complex which caps the end of the chromosome by interacting with telomere. This complex consists of six proteins: telomeric repeat-binding binding factor 1 (TRF1; also known as TERF1), TRF2 (also known as TINF2), TRF1-interacting nuclear protein 2 (TIN2; also known as TINF2), TPP1 (also known as ACD), protection of telomerase1 (POT1), and repressor and activator protein1 (RAP1; also known as TERF2IP) [Table 1] [1]. Out of these six proteins, three proteins (TRF1, TRF2 and POT1) interact with telomeric DNA directly, with POT1 binding to the 3' single stranded region of telomere while TRF1 and TRF2 bind with telomeric double-stranded DNA. TRF1 and TRF2 do not interact with each other directly but they interact via TIN2. TRF1 and TRF2 both interact with double stranded region of telomeric DNA specifically with 5'-YTAGGGTTR-3'sequence [1,26–28]. TIN2 acts as a bridge that joins TRF1 and TRF2 and it also connects TRF1 and TRF2 to POT1-TPP1 [Table 2] [17,18,29–31]. TIN2 binds with TRF2 via its N-terminal region and its C- terminal which contains an FxLxP amino acids motifs, facilitates its interaction with

TRF1. N-terminal domain of TIN2 contains a region which interacts with the C- terminal region of TPP1 to recruit it to telomeres [17,18,31,32]. POT1 has high specificity towards the single-stranded region of telomeric DNA sequence 5'TAGGGTTAG-3'and binds to the G-strand 3'overhangs. POT1 interacts with TRF1 via TPP1 and this protein-protein interaction is important for POT1 loading on the G-overhangs of telomeric DNA [Table 1] [15,33–35]. TPP1 interacts with TIN2 through its C-terminal domain while its central domain interacts with POT1 [16,18,32]. Apart from its function to recruit POT1 at single stranded region of telomere, its N-terminal region contains a domain which interacts with telomerase suggesting that TPP1 may be involved in the recruitment of telomerase to telomere [36–38].

RAP1 interacts with TRF2 and this interaction is necessary for RAP1 binding to telomere [39–41]. RAP1 is known to prevent telomere recombination and fragility and unlike TRF1, TRF2, POT1 and TPP1 it is not a telomere protective protein [39,42–45].

Table 1: Human telomere binding proteins:

Single stranded DNA binding protein		
POT1	Similar to cdc13 in yeast; protects the G-strand from degradation by exonuclease1, recruits telomerase to telomere, inhibits telomere elongation of long telomeres	[17,46]
Telomerase TERC TERT	Necessary for telomere elongation, telomere capping function, not necessary for cell for organismal viability for several generations.	[47]
EST1a-c	Three homologs of yeast EST1. EST1a is necessary for unfolding of telomeres and telomere elongation by telomerase	[50]
Double strand telomere binding proteins		
PIP1	Mediates binding of POT1 to the TRF1/TIN2 complex, negative regulator of telomere elongation.	[17]
TRF1	Negative regulator of telomere elongation, telomere independent function necessary for organismal viability	[47,48]
Tankyrase1, Tankyrase2	Poly (ADP-ribose) polymerase that inhibit TRF1 binding to the telomere, positive regulators of telomere lengthening, involved in sister chromatid separation during anaphase	[49]
TIN2	Protects TRF1 from tankyrase action, negative regulator of telomere elongation	[51-54]

PINX2	Negative regulator of telomere elongation	[55]
TRF2	Stabilizes the T-loop, necessary for telomere function, inhibits activation of ATM	[56]
RAP1	Binds to TRF2, negative regulator of telomere elongation	[56]
RIF1	Binds to dysfunctional telomeres and activates ATM and p53bp	[57]

Table 2: Table 2: Non-shelterin proteins at human telomeres [1]**:**

Protein complex	Nontelomeric function	Effects at telomeres	Interactions
PARP-2	BER	not known	TRF2
DNA-PK	NHEJ	deficiency leads to mild fusion phenotype	Associated with shelterin
BLM helicases	branch migration crossover repression	T-loop formation/ resolution?	TRF2
Tankyrases	role in mitosis (tankyrase1)	positive regulator of telomere	TRF1
ERCC1/XPF	NER, crosslink repair 3 flap endonuclease	deficiency leads to formation of TDMs; implicated in overhang processing after TRF2 loss	associated with shelterin
Rad51D	Unknown (HR?)	deficiency leads to mild fusion phenotype	Unknown
WRN helicase	branch migration G4 DNA resolution	deficiency results in chromosome fusions on the lagging strand	TRF2
Mre11/Rad50/Nbs1	recombinational repair DNA damage sensor	T-loop formation/resolution? required for t-loop HR	Associated with shelterin

2.1.3 Telomerase structure and functions:

Cells have evolved a specialized reverse transcriptase called telomerase, which solves the end replication problem by adding telomeric repeats onto the ends of newly

replicated chromosomes. Greider and Blackburn were awarded the Nobel Prize in Physiology and Medicine in 2009 for their discovery of telomerase in *Tetrahymena thermophila* [58]. Almost 90% of cancerous cells have telomerase activity, while most normal differentiated somatic cells do not [59]. Telomerase is a ribonucleoprotein complex consisting of at least six subunits: human telomerase RNA component (hTERC); heat shock protein 90 (hsp90), human telomerase reverse transcriptase (hTERT), telomerase-associated protein 1 (TEP1), p23, and dyskerin [3]. hTERC and hTERT are the core subunits and their in vitro reconstitution is sufficient to restore telomerase activity. hTERT is a rate-limiting component of telomerase. During up- or down-regulation of telomerase, only hTERT expression changes proportionately with the level of telomerase activity, while the levels of the rest of the components remain unchanged and high throughout [3].
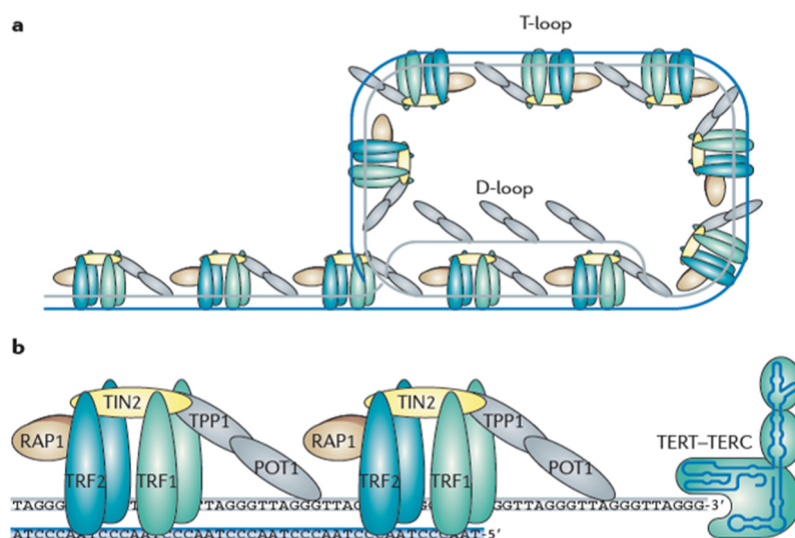
Figure 2: schematic representation of telomere structure and interaction with shelterin complex. a) Telomere bound to shelterin complex in a T-loop arrangement. Telomeres contain both double stranded region of TTAGGG repeats and a single stranded region contains 150-200 nucleotide long G-rich strands. The G-strand overhang (grey strand) invades the doubled-stranded DNA region of the telomere to form a protective telomere T-loop, with a displacement D-loop at the invasion site. b) Telomere bound with shelterin complex and telomerase. Shelterin complex consists of six proteins: TRF1, TRF2, TIN2, TPP1 (also known as ACD), POT1, and RAP1. Out of these six proteins, three proteins (TRF1, TRF2 and POT1) interact telomeric DNA directly, with POT1 binding to the 3' single stranded region of telomere while TRF1 and TRF2 bind with telomeric double-stranded DNA. TIN2 act as a bridge that joins TRF1 and TRF2 and it also connects TRF1 and TRF2 to POT1-TPP1. Telomerase has two core components, the catalytic subunit (TERT) and the RNA template (TERC); it also recognizes the 3'end of the G-rich overhang and elongates the telomere [60]. [Adapted from Nature Reviews Cancer 11, 161-176 (March 2011) doi: 10.1038/nrc3025]

2.1.3.1 Telomerase RNA component (TERC):

TERC acts as a template for telomeric DNA synthesis [61], and its size varies among species. *T. thermophila* has short TERC (159 nucleotides), while mammals have TERCs of intermediate length (murine TERC has 397 nucleotides and human TERC has 451 nucleotides) [62]. Human TERC is encoded by a single copy gene located on the long arm of chromosome 3 (3q26.3) [63]. TERC is one of the factors influencing the processivity of telomerase. It facilitates several rounds of the addition of DNA after only one primer binding step [64,65]. Telomerase RNA shows the divergence of the primary sequence among species, but it also has a remarkably conserved secondary structure in a variety of vertebrate species, which indicates that RNA structure may have an important role in telomerase activity [66]. The 3' end of TERC in vertebrates differs from that in single-cell eukaryotes – certain sequence motifs are absent in the latter [67]. This region of human TERC contains an H/ACA sequence motif that forms a specific class of non-coding RNA [66,68] that facilitates the modification of other cellular RNA. On the basis of localization, H/ACA RNA can be divided into two groups. The H/ACA RNA that accumulates in the nucleolus is known as H/ACA small nucleolar RNA (snoRNA) [69]. It takes part in the modification of ribosomal RNA. The H/ACA RNA that accumulates in the Cajal bodies and takes part in the modification of splicing RNA is known as small Cajal body-specific RNA (scaRNA) [70]. Another sequence motif, known as the Cajal body box or CAB box, is responsible for the difference in cellular localization of H/ACA snoRNA and H/ACA scaRNA. The conserved domain in the hTERC molecule is the binding site for many hTERC-binding proteins that recognize TERC specifically. There are many RNA-binding proteins that interact with hTERC, such as La, hnRNP C1/C1, dyskerin, hStau, L22, hGAR, hNOP10, hTERT and hNHP2 [71,72]. These RNA protein interactions are involved in hTERC accumulation, stability and maturation, and assembly of the telomerase in a functional form. Accumulation of TERC has been seen in human cancer cells, mainly within the Cajal bodies via RNA fluorescence in situ hybridization [73,74]. Of the TERC-binding proteins, dyskerin is an enzyme–a pseudouridine synthetase. It is required for H/ACA RNA stability, which it achieves by interacting with three small associated proteins: GAR1, NHP2 and NOP10. This enzyme complex is directed to its complementary RNA, i.e., ribosomal RNA and splicing RNA, by H/ACA snoRNA and H/ACA scaRNA, respectively. The isomerization of uridine to pseudouridine is mediated by the dyskerin complex. These modifications are required for the proper

function of these target RNAs. The template region is included in the pseudoknot and together makes up the core domain. The core domain and the CR4/CR5 domain independently bind to hTERT. The H/ACA scaRNA domain (including the CR7 domain) binds to the RNP proteins (dyskerin, Gar1, Nop10, and Nhp2) as well as to telomerase Cajal body protein (TCAB1)/WD Repeat domain 79 [75].

2.1.3.2 Telomerase reverse transcriptase (TERT):

The TERT subunit of telomerase was initially isolated biochemically as p123 from *Euplotes aediculatus* [76]. TERT, an RNA-dependent DNA polymerase, is also a core subunit of telomerase, and it uses its own RNA template for DNA synthesis (Table 3). The gene that codes TERT is located on chromosome 5 (5p15.33). The cDNA and genomic sequence of hTERT (human telomerase reverse transcriptase) revealed that the hTERT gene spans more than 37 kb and contains 15 introns and 16 exons that code for 1132 amino acid residues [77]. The TERT subunit of telomerase is conserved in humans, *Schizosaccharomyces pombe* (Trt1), *Saccharomyces cerevisiae* (Est2) and protozoans [78,79]. The 3D structure of the TERT protein from *Tribolium castaneum* was solved in 2008 by Emmanuel Skordelakes et al. at the Wistar Institute in Philadelphia [80,81]. The protein consists of four conserved domains, the RNA-binding domain (TRBD) and the palm, finger and thumb which organize in a closed-ring tertiary structure with a larger cavity at the center. It is large enough to bind with the primer-template duplex [80]. Structurally and functionally, the TERT protein can be subdivided into three major domains: the reverse transcriptase domain, which contains the finger, palm and thumb subdomains, which may play a role in nucleotide addition and processivity; the telomerase essential N-terminal domain, consisting of 400 amino acid residues; and the TERT RNA-binding domain, which has high affinity for hTERC.

The TERT subunit shows significant conservation with the reverse transcriptase enzyme from retroviruses. Inactivation of this catalytic subunit causes loss of telomerase activity. The expression level of hTERC depends on the telomerase activity in cells and is detectable in all tissues. Cancer cells generally have five times more expression than normal cells, even though only 1 to 5 copies of TERT (mRNA) are found per cancer cell. The level of expression of TERT is low in normal cells and high in immortal cells, showing that TERT rather than TERC characterizes the immortalized cells.

2.1.3.3 Telomere-independent function of telomerase:

Telomerase is a well-known enzyme that maintains the length of the telomere and the physical ends of the eukaryotic chromosome in embryonic stem cells and cancer cells. Telomerase activation extends the lifespan of cells in culture by maintaining the length of the telomere. It has now become clear that the role of telomerase is much more complex than just telomere lengthening. Telomerase influences normal cellular physiology, even in cells that contain long telomeres. Due to its key role in telomere lengthening, alteration in telomerase expression is associated with many degenerative diseases, aging and cancer-related functions. The role of telomerase in cellular immortalization and that of telomere shortening in cellular senescence has been demonstrated by cloning and expression of the TERT gene [82]. Indeed, increased incidence of spontaneous tumors has been found in many independent TERT-transgenic mouse models with constitutive expression of telomerase [83,84]. With oncogenic stress, the proliferative rate of the cells is increased many fold, and the length of the telomere is a factor which limits cell division capacity. The telomere is shorter in cancer cells than it is in normal cells [85]. Replicative senescence (also known as the Hayflick limit or mortality stage I) is the first cellular response to occur at the time of telomere attrition, and its induction needs the proper action of p53 and RB tumor suppressor pathways [86]. Inactivation of these two tumor suppressor pathways extends the replicative potential of the cell, ultimately leading to continuous telomere erosion and loss of telomere capping. Uncapped telomeres are highly recombinogenic, which leads to the formation of dicentric chromosomes and breakage at the time of cell division. They are also prone to a high degree of genomic instability and loss of cell viability during this period of crisis [87]. Of all the cells that undergo crisis, only the 10^{-7} to 10^{-5} fraction emerges from crisis [88], perhaps accompanying enforced expression of hTERT and activation of telomerase, which helps to avoid both senescence and crisis in primary cultured cells. It also causes transformation of primary human cells by its co-expression with SV40 early genes and H-RAS [89-91]. Thus, activating telomerase averts the crisis by capping the telomere and reducing the frequency of dicentric and abnormal chromosomes [92, 94]. Without performing telomere lengthening, stabilization of the telomere can also occur in a TERT-mediated, telomere capping-dependent manner, which increases cellular lifespan [92, 94]. Telomerase can play a role in modulation of chromatin structure and response to DNA

damage [92]. TERT is also known to induce the expression of pro-proliferative genes and inhibit that of anti-proliferative genes. This promotes cell growth and proliferation independent of telomere elongation [93,94].

2.1.3.3.1 Regulation of gene expression by telomerase:

TERT depletion in mouse skin results in a genome-wide transcriptional response in genes involved in signal transduction, epithelial development, the cytoskeleton and adhesion [5,6]. This resembles the transcriptional program regulated by Wnt, a well-known player in stem cell maintenance, cellular transformation and proliferation [95–99]. TERT acts as a transcription factor in β-catenin complexes. It is only directly involved in the modulation of the canonical Wnt pathway; non-canonical Wnt pathways that do not involve the β-catenin complex are not regulated by TERT. Wnt ligands bind to Wnt receptor(s) (the Frizzled family of transmembrane receptors) [99] and the LRP5/6 co-receptor (a low-density lipoprotein receptor-related protein) (Fig. 3) [100]. The co-receptor facilitates the interaction between the Wnt receptor and its ligand. The interaction leads to activation of a cytoplasmic phosphoprotein, Dishevelled (Dvl), which inhibits the activity of glycogen synthase kinase-3β (GSK-3β), which degrades the β-catenin. Wnt signaling allows accumulation of β-catenin, which then translocates into the nucleus and forms a complex with TERT-BRG1 (Fig. 3). The BRG1 is also known as SMARCA4, an SWI/SNFrelated chromatin-remodeling protein that binds to the β-catenin and takes part in Wnt signaling [101]. The β-catenin complex binds at the TCF/LEF site in the promoter/enhancer regions of target genes like Axin2, LEF1, WNT4 and WNT11, and enhances their expression in the canonical pathway (Fig. 3) [102–104]. Furthermore, TERT was found to bind to promoters responsive to Wnt signaling and to promoter elements recognized by BRG1 and β-catenin [98,105]. Studies also indicate a role for TERT in Wnt signaling in collapsing glomerulopathies (characterized by the proliferation of glomerular differentiated epithelial cells, the podocytes) [98,106]. Further analysis has shown that this effect of TERT on kidney cells is independent of its catalytic activity: it is coupled to its Wnt signaling stimulation, with increased expression and nuclear localization of β-catenin. However, increasing evidence implies a bidirectional connection between the Wnt pathway and TERT in both embryonic stem cells and cancer [107,108]. It has been experimentally shown that embryonic stem cells expressing an activated β-catenin show high telomerase activity and have longer telomeres, while in mice lacking β-catenin, the

length of the telomere is short and telomerase activity is also low. Zhang et al. found the same results in human cancer cell lines by inducing or repressing β-catenin expression [108]. In fact, in embryonic stem cells, β-catenin binds with Klf4, a transcription factor expressed by pluripotent cells, and regulates the TERT expression, whereas in human cancer cells TERT appears as a direct target of β-catenin/TCF4-mediated transcription. Therefore, during transformation, the Wnt pathway also participates in some carcinogenic processes via the stabilization of the telomere and stimulation of telomerase activity.

2.1.3.3.2 RNA-dependent RNA polymerase activity of telomerase:

TERT is known for its RNA-dependent DNA polymerase activity in association with TERC. Studies also indicate the role of RNA-dependent RNA polymerase activity of TERT in post-transcriptional gene silencing, which is independent of TERC [Table 3]. TERT is the only RdRP identified in mammals [109]. This function of TERT depends on a mitochondrial non-coding RNA: mitochondrial RNA-processing endoribonuclease (RMRP). Further analysis shows that TERT is associated with two types of RNA in HeLa cells (which overexpress TERT): TERC and RMRP [110]. RMRP is a non-coding RNA, the mutations of which lead to cartilage-hair hypoplasia, an inherited pleiotropic syndrome that is characterized by premature multi-organ failure, mainly in highly proliferative organs, and that involves stem cell dysfunction [111]. The TERT-RMRP complex has RdRP activity, which produces a double-stranded RMRP molecule [98] that is processed into 21 nucleotide siRNA by dicer and RISC (Fig. 3). These siRNA suppress the expression of RMRP. As a result, TERT-RMRP-RDRP regulates the level of RMRP by a negative-feedback control mechanism. The siRNA-mediated suppression pathway demonstrates control of gene expression by TERT. In the same way, the TERT-RMRP complex may amplify other small non-coding RNA and thereby regulate the expression of other genes by producing specific siRNA. It has been experimentally shown that TERT has a role in the control of cellular proliferation. TERT is known to increase cellular proliferation by increasing cell division and decreasing apoptosis in TERT-transductant human mammary epithelial cells (HMECs) [112]. Further analysis shows that the effect of TERT on the proliferation of cells is connected with alterations in cyclin D1, A2, E2F and pRB, which are all cell cycle regulatory proteins, and require the catalytic activity of telomerase rather than activation of Wnt signaling by TERT [112]. Mukherjee et al.

found a reduction in RMRP levels in TERT-transduced HMECs because dicer and RISC process the double-stranded RMRP molecules into 22 nt siRNA that control the level of RMRP. They also showed a connection between the enhancement of cellular proliferation and a decrease in RMRP Brought levels [112]. Knockdown of RMRP using shRNA (short hairpin RNA) results in proliferation of HMECs, which means that both results are comparable in enhancing cellular proliferation and lowering the RMRP levels. Together, these data indicate that TERT has an RNA-dependent RNA polymerase activity that enhances cellular proliferation via small interfering RNA.
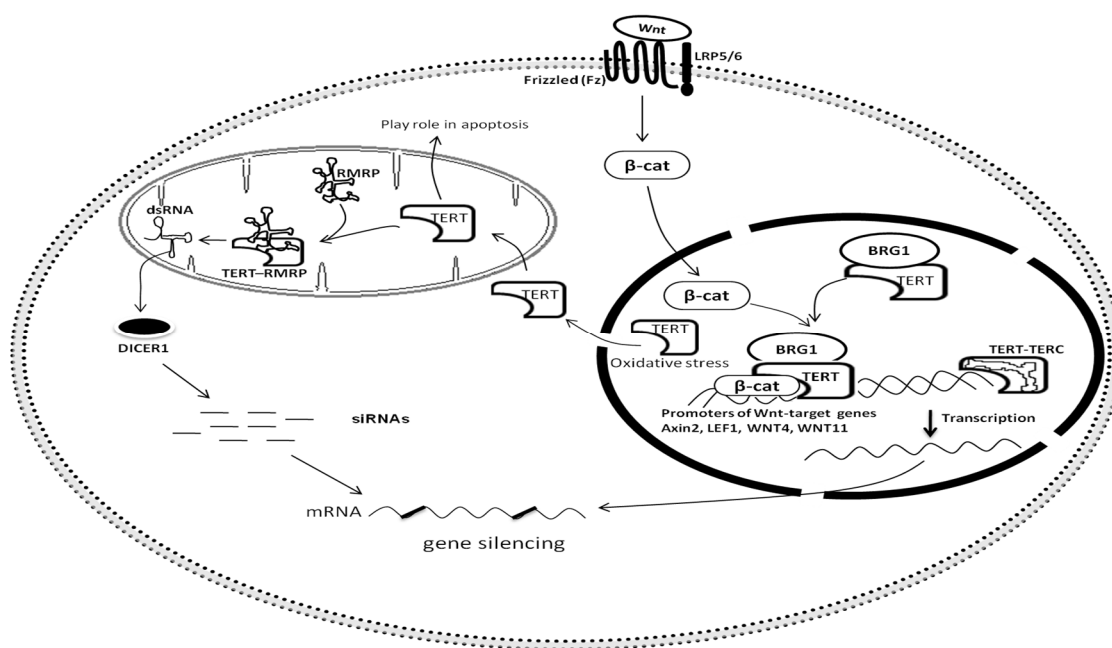
Figure 3: Multiple functions of telomerase. TERT is directly involved in the modulation of the canonical Wnt pathway in which it acts as a transcription factor in β-catenin complexes. Stimulation of Wnt receptor(s) after binding with Wnt on the plasma membrane causes binding of TERT with Wnt transcription factor BRG1 and forms a complex which then binds to the promoters of Wnt-target genes and regulates their expression. TERT also associates with the RNA component of mitochondrial RNA processed into endoribonuclease RMRP and this complex has RdRP activity which produces a double-stranded RMRP molecule further processed in to 22 nucleotide siRNAs by dicer and RISC (RNA-induced silencing complex). These siRNAs suppress the expression of RMRP. As a result, TERT-RMRP-RDRP regulates the level of RMRP by a negative-feedback control mechanism. These siRNAs-mediated suppression pathways mediate control of gene expression by TERT. With oxidative stress, TERT translocates to the mitochondria, from the nucleus. Recent results also show that in the mitochondria TERT also regulates apoptosis.

2.1.3.3.3 RNA-dependent DNA polymerase activity of telomerase:

TERT also has RNA-dependent DNA polymerase activity independent of TERC [Table 3]. TERT is present in the mitochondria and the nucleus (Fig. 4). TERT has an N-terminal mitochondrial targeting signal [113] that helps it to migrate into the mitochondria, probably through the protein complexes known as translocases, which are present on the outer and inner mitochondrial membranes. Translocation of TERT from the nucleus to mitochondria occurs following oxidative stress [98,113–115] and involves the improvement of mitochondrial function and stress resistance, independent of its telomeric function, which finally leads to the survival of tumor cells. It has been experimentally shown that TERC is not present in the mitochondria, which also supports the idea that TERT reverse transcriptase activity is independent of TERC [116]. In the mitochondria, TERT uses tRNA as a template to synthesize cDNA [116]. Human VA13 cells that do not have TERC and use a recombination-based method of telomere lengthening known as alternative lengthening of telomeres (ALT) were transfected with wild-type TERT. This showed that TERT performs its mitochondrial function. Furthermore, in the same cells, a dominant negative form of the enzyme is inactive [116]. RNA-dependent DNA polymerase activity of TERT based on the TRAP assay has also been shown in rabbit reticulocyte lysates (RRLs), in which translation of TERT in the presence of added TERC results in telomeric-DNA synthesis in vitro [116]. The addition of total cellular RNA from TERC-negative VA13 cells or from TERC-positive HeLa cells in the reaction mixture, along with random hexamers to prime the reactions followed by PCR with primers for different mt-tRNA genes demonstrated the synthesis of cDNA in the absence of TERC. It can be concluded that mt-TERT uses tRNA rather than TERC for cDNA synthesis [116]. In the absence of TERT, no products were observed, clearly demonstrating that mt-TERT can act as a reverse transcriptase by using mt-tRNA rather than TERC as a template.

Table 3- Classical and extracurricular activities of telomerase:

Functions	Description	Dependence on catalytic activity of TERT	Reference
Nucleus			
Regulation of gene expression	Acts as a transcriptional modulator	-	[105]
Chromatin organization	Regulates the DNA damage response pathway through its action on chromatin structure	-	[92]
Maintenance of telomere	Acts as a reverse transcriptase by using TERC as a template	+	[117]
Enhancement of cell growth	Affects the expression of growth-promoting genes	-	[118]
DNA repair	Associated with primase, a protein involved in DNA repair; increases the expression level of genes involved in DNA damage response and also physically associated with many DNA repair proteins	+	[119–121]
Mitochondria			
In apoptosis	Sensitizes cells to oxidative stress which can cause apoptotis	-	[113]
RNA-dependent RNA polymerase activity	Gene silencing	+	[109]
RNA-dependent DNA polymerase activity (independent of TERC)	Acts as a reverse transcriptase by using tRNA as a template; plays a role in mtDNA replication and mtDNA repair	+	[116]
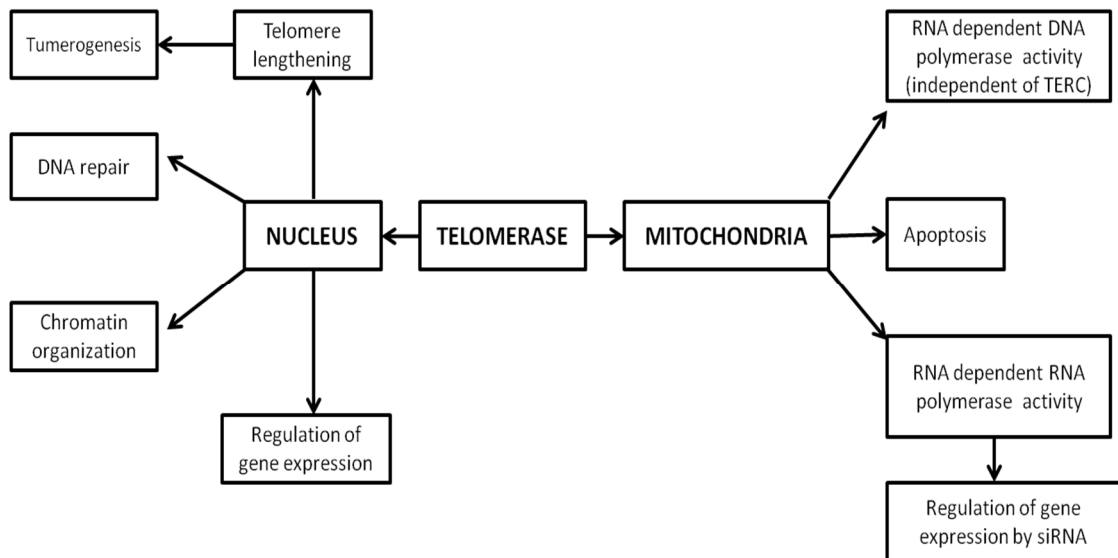
Figure 4: A schematic representation of classical and extracurricular activities of telomerase. Telomerase is found to be active in both mitochondria and nucleus; in mitochondria telomerase shows RNA-dependent DNA polymerase activity which is independent of TERC and uses tRNA as template. It also shows RNA-dependent RNA polymerase activity in mitochondria and it is the only RNA-dependent RNA polymerase known in mammals. It has been experimentally shown that telomerase plays a role in the regulation of apoptosis. Telomerase sensitizes the DNA of mitochondria to H_2O_2 which causes oxidative damage to mt-DNA perhaps through the modulation of metal homeostasis [113]. In the nucleus, telomerase maintains telomere length. Telomerase is found to be directly involved in the modulation of the canonical Wnt pathway in which it acts as a transcription factor in β-catenin complexes. Telomerase is also found to be involved in chromatin re-organization.

2.1.3.3.4 Role of Telomerase in apoptosis:

It has been experimentally shown that telomerase plays a role in the regulation of apoptosis (Fig. 4). This role is independent of its conventional function of telomere lengthening. Further analysis has shown that telomerase sensitizes the DNA of mitochondria to H_2O_2, which causes oxidative damage to mt-DNA, perhaps through the modulation of metal homeostasis [113]. The N-terminal leader sequence of TERT contains a mitochondrial localization signal that targets TERT to the mitochondria. Mutation in this region of TERT causes loss of mitochondrial targeting, and cells with mutated hTERT show decreased levels of mt-DNA damage [115]. These observations suggest the proapoptotic activity of hTERT in the mitochondria and the roles of TERT in the mitochondria are consistent with reports showing that oxidative stress triggers nuclear export of hTERT [122]. By contrast, it has been shown that hTERT overexpression renders cells resistant to apoptosis. This anti-apoptotic effect of TERT

occurs at a pre-mitochondrial step before the release of cytochrome c and apoptosis-inducing factor [123]. The siRNA-mediated down-regulation of hTERT triggers the apoptotic pathway devoid of obvious involvement of telomere erosion, but by post-translational activation of BAX, which induces a CD90-independent mitochondrial pathway of apoptosis [124]. In addition, a recent report indicated that TERT enhances cellular and organism viability independently of its telomerase activity. Cultured cells and a transgenic mouse model expressing wild-type TERT were treated with staurosporin and N-methyl-D-aspartic acid, which both promote apoptosis. Increased resistance against apoptosis was observed in both the cultured cells and the transgenic mice, and this effect of TERT is again independent of telomerase activity [125]. Even though the exact mechanism as to how TERT regulates apoptosis in mitochondria is unknown, TERT may exhibit discrete functions in apoptosis regulation by promoting apoptosis via alteration of the mitochondrial membrane potential or metal homeostasis in mitochondria.

2.1.3.3.5 Role of telomerase in DNA repair:

Telomerase may also play a role in DNA repair independently of its telomere lengthening function (Fig. 4). It has been experimentally shown that hTERT is also associated with primase [119], a well-known protein involved in replication and DNA repair, which indicates a role for telomerase in DNA repair. In addition, studies indicate that ectopic expression of hTERT causes an increase in the expression level of genes involved in the DNA damage response, and this is thought to be associated with a decrease in spontaneous chromosome damage in G1 cells and improvement in the DNA repair kinetics [120]. Moreover, hTERT is also found to be associated physically with many DNA repair proteins and the telomere, thus enhancing the stability of the genome and DNA repair functions [121]. However, increasing evidence is emerging to indicate that the role of telomerase in the DNA damage response is not limited to DNA double-strand break repair but also associated with many other types of DNA repair, including via nucleotide excision [117]. These studies predict a role of telomerase in the DNA damage response independent of its classical activity of telomere length maintenance.

2.1.3.3.6 Telomerase and Cancer:

Expression of telomerase is a hallmark of cancer. Almost the complete spectrum of human cancers was found to be telomerase positive. Generally, the ability of unlimited proliferation and immortality in malignant tumors are characterized by telomerase expression while most benign tumors are telomerase negative which is the cause of its limited proliferative capacity and replicative senescence.

Telomerase expression is necessary to immortalize the cells but it does not by itself induce a transformed phenotype [126,127]. Many factors are required to completely transform telomerase positive fibroblasts, like SV40 large T antigen which blocks p53 and pRb cell cycle check point, SV40 small t antigen to stop phosphatase activity and enhance expression of a mutant version of the H-ras oncogene so that there is constitutive activation of signal transduction pathways [128,129]. hTERT expressing human fibroblast cells show normal characters such as contact inhibition, growth requirements, maintain normal karyotype, adherence and normal cell cycle activities [89]. Entire cancer spectrum required minimum six necessary alterations to be completely transformed and become malignant. They are the resistance to apoptosis, unlimited proliferation, generation of self-stimulatory growth signals, insensitivity to inhibitory growth signals, capacity for angiogenesis, and tissue invasion and metastasis [130]. So to suppress the development of cancer cells, a different cellular mechanism exists and multiplicity of these cellular defense ensure that cancer in human is relatively rare [130].

The expression of telomerase is tightly regulated in normal cells with low expression level and off activity. Moreover, to allow continued proliferation of stem cells and germ cells telomerase is present at low level in these cells and that's the reason researchers have hypothesized the involvement of stem cells in cancer initiation and maintenance [131]. Hence, tight regulation of telomerase expression is a key factor to suppress tumor by stopping uncontrolled growth and division. Telomerase expression is affected by deregulated oncogenes and tumor suppressor genes in a way that cooperatively promotes tumorigenesis. For example, c-Myc is an oncogene and frequently amplified in cancer is also an activator of hTERT [132]. c-Myc is positively regulated by a G2/M cell cycle regulator regularly amplified in epithelial malignancies known as Aurora A kinase [133]. c-Myc activation further activates the telomerase activity [134].

Moreover, a tumor suppressor gene BRCA1 which is lost in ovarian and hereditary breast cancers inhibits hTERT expression by negatively regulating c-Myc [135]. As mentioned in hTERT independent function of telomerase, cell proliferation is promoted by hTERT by modulating expression of genes, either by acting as a transcriptional cofactor or through post-transcriptional mechanism [5,110,118,136,137]. Regarding transcriptional mechanisms, a study in mice suggests that TERT binds with BRG1 (also known as SMARCA4) and acts as a transcriptional cofactor and directly regulate WNT-β-catenin target genes [105,137]. There is also evidence of TERT interactions with p65 subunit of nuclear factor-κB (NF-κB) which suggests the direct role for TERT in NF-κB responsive gene regulation [137]. Furthermore, MYC-dependent transcriptional programs are also modulated by TERT by binding and stabilizing MYC at its target promoter. Cell cycle regulation, cell metabolism, RNA processing and translation are the MYC-driven pathways regulated by TERT expression and all these pathways are involved in tumorigenesis [136].

2.1.3.3.7 Telomerase and epithelial–mesenchymal transition (EMT):

The most common cause of human death by cancer is metastasis, but the principal mechanisms behind this is not very well known. However, emerging evidences suggest the role of epithelial–mesenchymal transition (EMT) in metastasis [138–140]. Epithelial-mesenchymal transition (EMT) is a physiological process which has the key role in embryonic development and characterized by loss of apico-basal polarity, loss of cell-to-cell adhesion, loss of epithelial markers like E-cadherin, the gain of mesenchymal markers like N-cadherin and vimentin, resistance to apoptosis and [136] capacity to invade. There are many developmental transcription factors such as Twist1/2 and Six, Snail ½, TGF-β and Wnt/β-catenin signaling pathways that regulate this EMT program. This transcription factor induces the expression of mesenchymal markers like Vimentin and N-cadherin and represses the epithelial markers like E-cadherin [138,139,141–143]. In 2012 a paper published by Liu et al. in oncogene revealed a potential role of hTERT in EMT [4]. They have shown that hTERT promotes EMT and stem cell like traits in gastric cancer cells when hTERT is ectopically expressed in these cells [4]. TGF-β is a well-known inducer of EMT, but down-regulation of hTERT by siRNA inhibited the TGF-β and β-catenin mediated EMT. This paper demonstrated that hTERT binds with β-catenin in the cytoplasm and once hTERT

interacts with β-catenin it enhances the nuclear translocation and increases its transcriptional activity. Inside the nucleus, this complex interacts with TCF/LEF transcription factor and regulates the expression of many genes such as snail and vimentin [4]. Additionally, expression of different EMT markers and hTERT were positively correlated in gastric cancer samples and cancer cell colonization is stimulated by hTERT in a mouse model [4]. Moreover, the most important thing is that all of these effects of hTERT were independent of its classical activity of telomere lengthening. Furthermore, a Chinese group, demonstrated in 2015, the role of hTERT in EMT using oral squamous cell carcinoma (OSCC) as a model [144]. They found hTERT is overexpressed in oral epithelial dysplasia (OED) and OSCC tissues and correlates with clinical aggressiveness of oral squamous cell carcinoma (OSCC) patients [144]. They have checked hTERT potential to prolong the life span in primary human oral epithelial cells (HOECS). Following overexpression of hTERT in these cells they found hTERT prolongs the life span of this primary cell [144]. They have also demonstrated that overexpression of hTERT is enough to cause EMT by activating Wnt/β-catenin pathway [144]. They have reported that overexpression of hTERT causes cytoplasmic localization of β-catenin, while others suggested nuclear localization of β-catenin when EMT is induced. They have also found the decrease in the phosphorylation of GSK-3β which suggested hTERT upregulation activates the GSK-3β pathway. They demonstrated decreased expression of vimentin, slug and twist while upregulation of E-cadherin when hTERT was knocked down. They also suggested that depletion of endogenous hTERT inhibits the Wnt/β-catenin pathway and hence EMT was also reversed [144]. Matrigel assays confirmed their result because hTERT-overexpressing cells showed increase in invasiveness in comparison to vector transfected cells [144]. Okamoto N et al. reported that hTERT interacts with nucleolar GTP-binding protein nucleostemin/GNL3L and BRG1 and forms a complex to modulate transcriptional programs necessary for the maintenance of tumor-initiating cells and for cancer stem cells [145]. Moreover, the main pathway by which hTERT promotes EMT is wnt/β-catenin pathway and it was unknown if other pathways also existed till a study came in 2015 showing "an hTERT/ZEB1 complex directly regulates E-cadherin to promote epithelial-to-mesenchymal transition (EMT) in colorectal cancer" [146]. This study demonstrated that hTERT interacts with ZEB1 and forms a complex which then suppresses E-cadherin expression by binding to its promoter in colorectal cancer cells

[146]. They found down-regulation of E-cadherin when hTERT was overexpressed in HCT116 and SW480 cells, but E-cadherin expression was back to normal level when ZEB1 expression was impaired even in hTERT overexpressing cells [146]. This suggested that ZEB1 is necessary for hTERT mediated down-regulation of E-cadherin. MJ Choi et al showed that hTERT mediates stress hormone norepinephrine (NE) mediated EMT in ovarian cancer cells [147]. They demonstrated that norepinephrine induces hTERT expression in ovarian cancer, and subsequently aggressiveness of ovarian cancer [147]. Silencing of c-Myc and hypoxia-inducible factor-1α and inhibition of β2-adrenergic receptor 2 and protein kinase A greatly reduced NE-induced hTERT expression [147]. They further demonstrated that ectopic expression of hTERT or norepinephrine stimulation to the cells enhance slug expression and epithelial–mesenchymal transition (EMT) of ovarian cancer [147]. Silencing of either hTERT or slug causes the significant reduction in hTERT and norepinephrine induced EMT and invasion in ovarian cancer [147]. Additionally, they found norepinephrine stimulation activates the src which is a tyrosine protein kinase, activation of src phosphorylates myc and Hif and further activates hTERT and slug [147]. Overall their study indicates that in NE-induced ovarian cancer hTERT links src to slug expression and enhance EMT and invasion [147].

2.1.4 Salient points:

There is a growing mass of evidence for the pleiotropic role of telomerase complex often independently of the classical function of telomere maintenance. All of these telomere-independent roles affect normal cell physiology and promote the proliferation of cancer cells. Further research is needed to clarify the role of TERT as a mitochondrial RNA-dependent DNA polymerase. It is very necessary to develop the tools that would help to study functions of TERT in cellular physiological conditions to provide clear results. There is no direct molecular evidence to explain the role of telomerase in DNA repair, so a testable experimental model is needed to explain the telomere-independent role of telomerase in DNA repair. The present study tries to find associations between hTERT and other players in TGF-β induced epithelial to mesenchymal transition.

2.2 uPA system:

2.2.1 Plasminogen activator system consists of several proteins:

Urokinase-type plasminogen activator (PLAU or uPA), and its receptor receptor (uPAR), the substrate plasminogen (Plg), and the plasminogen activator inhibitor 1 and 2 (PAI1 and PAI2) also known as serpine 1 and serpine 2 together make plasminogen activator system [148]. This system is a key regulator of the tumor microenvironment and is heavily involved in the metastatic process in many cancers. uPA system has wide range of targets along with prominent location in the proteolytic network of tumors and that's why this system attracted attention of many research group [148]. The plasminogen activator system are involved in various physiological process like tissue remodeling [149], but in addition to this, uPA system is involved in pathogenesis of vascular diseases such as atherosclerosis, thromboembolic disorders and stroke [150]. The extensive research on plasminogen system started after urokinase was found in the urine of cancer patients in 1960 [151]. Moreover, another study came in 1988 in breast cancer patients which was first study to reveal uPA as a prognostic marker for survival in cancer [152]. The receptor of uPA i.e uPAR was discovered by Vassalli et al in 1985 and its association with cancer was established in 1991 by Ossowski et al [153,154]. Since then extensive research has been undertaken on its role in cancer invasion and metastasis. Two types of plasminogen activator are found in plasminogen activator system- they are urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). tPA is present in both normal and some malignant tissues and is mainly involved in conversion of plasminogen to plasmin during dissolution of blood clot, while uPA is mainly associated with malignancy of cancer and involved in pericellular proteolysis during cell migration and tissue remodeling [155]. Furthermore, it is well established now that higher expression level of both uPA and uPAR enhance tumor growth and metastasis and correlated with poor prognosis [156,157]. Though both tPA and uPA can activate inactive plasminogen to active plasmin but uPA has been extensively studied in cancer metastasis.

2.2.1.1 Role of stromal cells in plasminogen activation system:

Initially the role of plasminogen activator and other protease systems in cancer were studied in transformed cultured cells or established cell lines [158,159]. Researchers at that time completely believed that cancer cells produced these proteases in tumor

tissues, an observation that was supported by research on transplanted tumors [160,161]. However, these findings were strongly challenged by several studies with human colon cancer. The mRNA and uPA could be shown only in stromal cells in colon adenocarcinomas while uPA expression was not detected in cancer cells [162,163]. This result indicated that, to produce uPA, cancer cells recruit stromal cells in colon cancer. Now, in many types of cancer it is very well established that stromal cells produce component of the uPA system along with other matrix degrading proteases [164–167]. Moreover, each of these molecules are synthesized differently by different types of human cancer, e.g in prostate cancer uPA is mainly synthesized by macrophages [168] while in colon cancer and ductal breast cancer, fibroblasts mainly expressed uPA [169,170]. Moreover, in skin squamous cell carcinomas, uPA expression occurs in the cancer cells [171]. Interestingly, similar to uPA, uPAR expression also differs between different cancers. In ductal breast cancer, uPAR is primarily expressed by macrophages [172] while in colon cancer, both macrophages and cancer cells express uPAR [163,172]. In skin squamous cell carcinomas it is the cancer cells which express uPAR [173] where as in prostate cancer, neutrophils and macrophages both express uPAR [170]. All these studies suggested that uPA and uPAR production by tumor-associated stromal cells enhances invasion and migration of the cancer cells. Studies also indicated that higher uPA and uPAR protein level in blood and tumor tissue are responsible for the poor prognostic in many cancer types [174]. However, this poor prognosis of cancer cells are independent of the type of the cells in which the molecules are produced. For example, in the case of breast cancer uPA and uPAR are separately produced by myofibroblasts and macrophages respectively, but they both strongly associated with poor survival [175,176]. Stromal cells involvement in production of extracellular proteolysis suggested that cancer invasion is not only restricted to cancer cells, but it is process which requires a mixed population of cancer cells and stromal cells [177]. Though, the main initiators of invasion are the cancer cells but the whole process is contributed by each cell type in a distinct way. Additionally, it is also very important to recruit the right combination of stromal cells in the new environment of tumor for successful progression of cancer metastasis [177]. Overall this suggested that stromal cells involvement and recruitment can be the limiting factor in cancer progression and metastasis opening new area of research for prevention of cancer [177].

2.2.2 uPA, uPAR, and Plg:

2.2.2.1 uPA:

uPA is serine protease with molecular weight of approximately 50,000 which is released from the cells as a single chain zymogen pro-uPA, a nonactive 411 amino acid glycoprotein which after cleavage at K158–I159 forms two-chain high molecular weight uPA (HMW uPA) [178]. This conversion is catalyzed by plasmin, blood coagulation factor XIIa, plasma kallikrein, cathepsin B, cathepsin L, T cell–associated serine proteinase, nerve growth factor-γ and prostate specific antigen [161,179–182]. This high molecular weight uPA undergoes additional proteolytic cleavage and is converted into three functionally independent regions: the amino-terminal fragment which contains growth factor domain (GFD) and has high affinity to bind with uPAR, the kringle domain involved in intracellular signaling and cell migration and a carboxyl terminal proteolytically active serine protease domain which retains its plasminogen activator function [183,184]. The serine protease domain of uPA has high affinity for its substrate and cleaved inactive zymogen plasminogen to form activated protease plasmin [Fig 5]. This activated plasmin further degrades various components of ECM showing its pro-invasive and pro metastatic effects [185,186]. The concentration of uPA in blood plasma is around 20 pM and most of it form complex with PAI-1 while rest is present in the pro-uPA form [187]. uPA is very restricted in terms of its substrate specificity with plasminogen as the main substrate [161,188]. However, there are evidences which show that uPA also has other substrates such as hepatocyte growth factor/scatter factor and macrophage stimulating protein (MSP), these substrates have high sequence similarity with plasmin but lack proteolytic activity. They are also secreted as inactive zymogen form and become active after undergoing proteolytic cleavage [189]. Various cytokines and growth factors, including EGF, VEGF and TNF-α can induce release of uPA. However, there is speculation about the enzyme responsible for the activation of pro-uPA to uPA. Many hypothesis were proposed which tell plasmin may be the activator of pro-uPA but then the question arises as to which one of these molecules is activated first uPA or plasmin and how [190]. As mentioned earlier, there are many other activators of pro-uPA such as kallikreins and cathepsins, and they can be also first activator of pro-uPA [191]. Additionally, it is also proved by in vitro experiments that binding of pro-uPA to uPAR facilitates the

activation of plasminogen to plasmin without the activation of pro-uPA. This is not completely surprising because it is already known that uPA plasminogen conversion activity greatly increases by as much as 50 fold after binding with uPAR [192]. This is believed that after binding with pro-uPA, uPAR undergoes some conformational changes that confers protease abilities to the single-chain molecule [193]. It is well established that tumor tissues have elevated expression level of uPA than normal tissues [194–196]. uPA is present at invading front of the cancer cells which facilitate cell invasion and migration in both primary and metastatic tumors [197]. uPA is also found to play role in angiogenesis observed in models of corneal vascularization [198]. It is demonstrated that proteolytic activity of uPA is needed for the migration of endothelial cells which is one of the earliest steps in angiogenesis and also necessary for the earliest stages of tube formation [199,200].

2.2.2.2 Transcriptional regulation of uPA:

Development of cancer and its progression towards the metastatic stage involves the activation and deactivation of many specific genes. Earlier it was believed that cancer is a genetic disease and mutation in the DNA sequence is the sole cause of change in gene expression throughout the cancer progression. However, now it is very well established that epigenetic changes also play a key role in change in gene expression and hence cancer development and progression [201]. Dynamic epigenome is observed by many researchers with some parts of epigenome in a state of flux throughout life while others are inherited or established during embryonic development [202,203]. There are various mechanisms by which epigenetic modifications can be done such as DNA methylation, post-translational modification of histone tails, nucleosome positioning, and non-coding RNA [204]. These epigenetic modifications are brought in by protein machinery which consists histone modifiers, chromatin remodeling complexes, methyl-DNA binding proteins (MBDs), DNA methyltransferases (DNMTs) and proteins which interact with histone modifications [202]. Epigenetic modification of uPA is demonstrated in normal human mammary epithelial cells (HMEC), early stage hormone-responsive breast cancer cell lines (MCF-7 and T-47D), and late stage hormone-insensitive breast cancer cells by exploring the correlation between expression of uPA and hormone (estrogen) sensitivity [205]. The expression of uPA is only found in highly invasive MDA-MB-231 cells [205]. Catherine Leurer et

al demonstrated DNA methylation status of the uPA gene by performing southern blot analysis, they observed that CpG islands of uPA gene of highly invasive breast cancer cell lines are hypomethylated while CpG islands of uPA gene of normal breast cells and early stage breast cancer cells are methylated [205]. They have also performed methylation sensitive PCR to quantify the methylation status of CpG islands in the uPA promoter and found similar results, CpG islands in the uPA promoter of MCF-7 were found around 90% methylated while highly invasive MDA-MB-231 cells had fully demethylated CpGs [205]. To know the reason behind the differences in the methylation status of the uPA promoter between highly invasive MDA-MB-231 and less invasive MCF-7 cells, they checked the expression levels of DNA methylation machinery. They observed that MDA-MB-231 cells have low DNMT1 activity and high DMase activity while MCF-7 cells had reduced DMase activity and high DNMT1 activity. This confirmed their previous data and resulting demethylated uPA promoter in MDA-MB-231 cells while hypomethylated promoter in MCF-7 cells [205]. Additionally, DNA methylation was found as the dominant mechanism for uPA gene silencing because when inhibitor of histone deacetylase i.e Trichostatin A added, it did not induce uPA expression in MCF-7 cells while uPA expression in MDA-MB-231 was enhanced [206]. Most of the methylation mediated repression of uPA gene is studied in breast cancer cells, DNA methylation has also been found to regulate uPA and PAI-1 expression in gastric cancer, meningioma, laryngeal squamous cell carcinoma and prostate cancer where both uPA and PAI-1 were also recognized as epigenetic-based prognostic and therapeutic targets [207,208]. However, to observe the impact of uPA-PAI-1 methylation in cancer, clinical studies still need to be carried out.

2.2.2.3 uPAR:

uPAR was discovered by Stoppelli et al and Vassalli et al in the year in 1985 [153,209]. This is a cell surface receptor which has very high affinity for the A-chain of u-PA. The cDNA of uPAR has been cloned from human and bovine sources [210,211]. uPAR sequence contains three repeats of approx. 90 amino acids, these repeats code for three homologous domains and all are independently folded with respect to each other [212]. A surprising discovery about uPAR is that it does not contain transmembrane domain and all the three domains of uPAR are covalently linked by glycosylphosphatidylinositol (GPI) anchor to the outer layer of the cell membrane

[213]. Human uPAR is heavily glycosylated and its amino acid sequence contains three potential glycosylation sites [210,214]. Researchers have revealed the presence of soluble uPAR variants without any GPI in ascites fluid from ovarian cancer patients [215] and conditioned medium from cell lines [216]. Phospholipase C cleavage or differential splicing of GPI anchor generated these variants [211,217]. The function of these soluble uPAR variants are not very well known. Moreover, in vitro experiments revealed that uPA is also known to cleave uPAR between domains 1 and 2 and truncated variants of uPAR having domains 2 and 3 have been found in extract of murine tumors suggesting that this cleavage can also occur in vivo [218,219]. The binding of uPA to uPAR has Kd value in the range of 0.1 to 0.5 nM but it can vary with the cell type and assay conditions [220]. uPAR can bind with same affinity to both pro-uPA and 2 chain active uPA [221]. uPAR binds with its ligand via domain 1 [212], but other inter-domains also take part and enhance the affinity of uPA and uPAR binding. Isolated N-terminal domain 1 has around 500 fold lower affinity than the intact receptor [222,223]. Domain 1 of uPAR has two sub-regions which is critical for uPA binding [224]. The tyrosine at 57 position in uPAR is involved in the binding [225]. Moreover, apart from localizing uPA to the cell surface for the proteolytic activity, uPAR has many diverse roles in cell phenomena underlying tumor progression and interaction with many key signaling molecules. uPAR interacts with various cell surface proteins such as G-protein coupled receptor (GPCR), VLDLR, receptor tyrosine kinase (including platelet-derived growth factor receptor (PDGFR) and epithelial growth factor receptor (EGFR)) and integrins (including $\alpha v \beta 3$, $\alpha v \beta$, $\alpha 5 \beta 1$ and $\alpha 3 \beta 1$) to alter cell adhesion and cell signaling [Fig1] [226–229]. Additionally, it is reported that uPA-occupied uPAR can leads to the cell binding to vitronectin [Fig 5]. It is also demonstrated by many studies that lateral associations with trans-membrane proteins are formed by GPI-anchor uPAR [230–236]. The latter (uPAR) acts as a nontraditional lateral integrin ligand which is different from the integrin-matrix contact site. Binding of uPAR with integrins may offer a regulatory mechanism to modulate integrin localization and function. Moreover, many hypotheses are include given as a part of a large signaling complex known as signalosome which uses signaling molecules like Src, Akt and FAK (focal adhesion kinase) [237]. It is demonstrated that, binding of uPAR with integrin activates integrin signaling through FAK and/or Src kinases, which further activate MEK/ERK pathways [233,238–240]. uPAR interaction with either $\alpha 5 \beta 1$ or $\alpha 3 \beta 1$

integrin was most extensively studied [227,241]. A point mutation in loop 4 of α3 inetgrin (α3-H245A) impaired its binding with uPAR suggesting uPAR binding site in α3 subunit β-propeller region [242]. Similarly, surface-exposed loop of uPAR (residues 130-142) and a sequence in domain III (residues 130-142) [243] were mapped as integrin-binding sites [244]. Beside α3β1 and α5β1, αMβ2, αvβ5 and αvβ3 are also found to interact with uPAR [244–246]. These data suggested a biochemical mechanism in which uPAR can regulate cytoplasmic signaling pathway through its interaction with transmembrane integrin and hence participate in cell proliferation, regulation of gene expression and migration [241]. Protein-protein interaction between uPAR and transmembrane binding partners are also responsible for regulation of cell proliferation and chemotaxis. For example, it is reported that increased level of uPAR are required for interaction between α5β1 integrin and epidermal growth factor receptor (EGFR), giving a mechanism in which tumor proliferation is controlled by ligand (EGF)-independent growth signaling [247]. EGFR inhibitor AG1478 blocked uPA-dependent activation of ERK [248], and expression of uPAR is essential for a proliferation response to EGF in murine embryonic fibroblasts [249]. Physiologically normal tissue rarely expressed uPAR, though during some pathological process e.g. inflammatory response or wound healing, level of uPAR is upregulated [250,251]. Plasmin plays a key role in fibrin clot lysis and that's why uPAR is also involved in normal hemostasis. Under these conditions, proteolysis of ECM is occurs by plasmin either directly or indirectly through the activation of MMPs [252,253]. Expression level of uPAR is high in cancer cells and it can be expressed by the tumor cells themselves and the cells which are associated with the tumors for example endothelial cells, infiltrating inflammatory cells and stromal cells [190]. There are generally two categories of uPAR-expressing tumors: first one consists of cells in which uPAR is expressed only in tumor-associated cells while in second categories both tumor cells and tumor-associated cells express uPAR [190]. The expression of both uPA and uPAR are not homogenous throughout the tumor, but instead they are generally associated with the interface of tumor tissue-benign tissue or tumor and vascular tissue [254]. To restrict the region of proteolytic activity and to provide directionality, uPAR is mainly expressed on the edge of the migrating and invading cancer cells. This migration of the cancer cells created a path through the ECM, in the direction of cancer cells movement. ECM destruction released chemotactic ECM fragments and latent growth factors which

also create a chemical gradient followed by invading cancer cells [255]. Plasminogen activation system is responsible for the migration of tumor cells, and is also involved in the migration of tumor-associated macrophages. Different effects have been observed of uPA-uPAR binding depending on the state of maturation of the monocytic cells. Binding of uPA to uPAR enhances migration of less mature more monocyte like cells and induce the cells to follow the uPA gradient towards tumor site. After maturation, there are more macrophage-like cells and uPA-uPAR binding enhances adhesion of the cells rather than migration as in the case of less mature monocyte cells [256,257].

2.2.2.4 Plasmin:

Human plasminogen is a single chain glycoprotein of 92 kDa, consisting of 791 amino acids. It has five homologous kringles and 24 disulfide bridges. A single peptide bond between Arg561and Val562 is cleaved by uPA to convert plasminogen to plasmin [258]. Similar to uPA, to form a highly localized point of hydrolysis plasminogen can also bind to a specific cell surface receptor [148]. When sc-uPA binds with uPAR, there is enhanced plasminogen cleavage to produce active plasmin. Moreover, there is a positive feedback mechanism in which activated plasmin further activates latent sc-uPA to an active two-chain uPA (tc-uPA) by cleaving Lya158-Ile159 peptide bond. So, a positive feedback loop is formed by plasmin and uPA which can activate each other. Binding of cell associated plasmin to S100A10 receptor (highly inducible plasminogen receptor) , protected plasmin from rapid inhibition by α2-antiplasmin which further leads to the activation of sc-uPA in to two chain uPA and also helps in the proteolytic activity of focalized plasmin [148,258–260]. Plasmin cleaves a range of ECM components and is necessary for fibrin blot clots degradation and clearance (fibrinolysis) at the time of wound healing. Many matrix metalloproteinases (MMPs), such as MMP2, MMP3, MMP9, MMP12 and MMP13 can be also activated by plasmin [Fig 5] [148,261–263]. uPA-uPAR activated cell associated plasminogen can lead to cell migration through three dimension ECM by accelerating pericellular proteolysis. Presence of uPAR near the front edge of the migrating cells shows control of ECM degradation by guiding uPA and provide a direction of the movement [264]. Additionally, ECM bound growth factors or latent growth factor e.g TGF-β1 can be also released by plasmin and MMPs [265–267]. Migrating cells have coordinated

expression of uPA and uPAR at cell-cell contact site and at cell-substrate [267]. The uPA-uPAR complexes convert plasminogen to plasmin to initiate extracellular matrix degradation and at the same time cell-cell contact is disrupted and increase in cell motility occurs indicating key role of plasmin induced proteolysis in these processes [268,269].

2.2.2.5 Plasminogen activator inhibitors:

Serine proteinase inhibitors (serpins) constitute a superfamily which consist a variety of serine proteinases inhibitors. These inhibitors contain a surface-exposed reactive center peptide loop, having reactive center peptide bond. This peptide bond is actually pseudo-substrate and it helps to trap targeted proteinases in inactive form in a stable complex of 1:1 stoichiometry. Interaction between the large central β-sheet A of the serpin and the reactive center loop results in the formation of this complex [270,271]. Three proteinase inhibitors named PAI-1, PAI-2 and α2AP belong to serpin superfamily. Out of these three α2AP is the main inhibitor of plasmin [272] and PAI-1 and PAI-2 both target uPA as well as tPA proteinases. In vitro, PAI-1 present in inactive, latent conformation is activated by denaturation and folding. Activated form of PAI-1 binds with vitronectin, and this binding of PAI-1 with vitronectin keeps PAI-1 in active conformation [273,274]. PAI-1 and PAI-2 are produced by stromal cells surrounding the tumor cells to neutralize the effect of uPA. PAI-1 and PAI-2 expression leads to internalization of uPA-uPAR complex and is involved in the tight control on proteolysis. Out of these two serine proteinase inhibitors PAI-1 is mainly involve in metastasis while PAI-2 has protective role [275]. Active conformation of the uPA-uPAR-vitronectin complex is maintained by binding of PAI-1 to this complex. PAI-1 binding also interferes with cell matrix interactions and plays a key role as a detachment factor to promote tumor metastasis [276]. Protein C inhibitor (PCI) and proteinase nexin-1 (PN-1) are two other serpins that can bind and inhibit tPA and uPA at physiologically relevant rates. These two proteinase inhibitors are not entirely specific for uPA and tPA and react more slowly than PAI-1 and PAI-2 [277,278].

2.2.2.6 Endocytosis and recycling of uPA and uPAR:

For the effective pericellular proteolysis and cell invasion, an important step is endocytosis and recycling of glycosylphosphatidylinositol (GPI) anchored uPAR [148]. Moreover, it is demonstrated that uPA-uPAR complex alone is quickly endocytosed

and degraded in comparison with uPAR-bound uPA-PAI-1 complex [279]. This was further confirmed by many researchers [264,280,281]. It is also reported that uPAR expressing cells were capable to endocytose the uPA-PAI-1 complex [Fig 5] [282]. A clathrin dependent endocytosis is started, when PAI-1 inhibit uPA bound uPAR, an inactive complex is formed which also involves low density lipoprotein receptor-related protein-1 (LRP1 or a2 macroglobulin receptor) [283,284]. Moreover, it is demonstrated that uPA-uPAR-PAI-1 complex endocytosis occurs by cells expressing α2MR/LRP receptor suggesting an investigation of this endocytosis receptor for their possible role in endocytosis of this complex. This endocytosis results in lysosomal degradation of uPA and PAI-1while uPAR and LPR1 are recycled to the plasma membrane [Fig 5] [284]. The effect of uPA-uPAR on cell migration is controlled by the ability of uPAR to be recycled to the cell membrane. Endocytosis of uPA-uPAR-PAI-1 complex may regulate the focalized pericellular proteolysis to terminate degradation of ECM along with changes in cell adhesion to the ECM, thus facilitating cell migration.

The uPA receptor localizes in adhesion complexes that contain nascent integrin and thereby activates intracellular biochemical cascade by establishing cooperation with integrin as well as other transmembrane partners. Upon being activated by a ligand, uPAR influences cell adhesion that is integrin dependent; it also functions as a non-integrin vitronectin receptor [148,267,285]. Furthermore, interaction of uPAR with recycling collagen receptor of the mannose receptor family that is endocytic receptor 180 (ENDO180) [286] stimulates activation of Cdc42, Rho GTPases and Rac1. Activation of these signaling molecules enhances the reorganization of actin cytoskeleton and give direction to the cell for migration towards the chemotactic gradient of uPA giving a new pericellular proteolysis and new ECM adhesions [284,287]. The Mobility of uPAR in the plasma membrane is high because it is linked to the outer membrane by GPI anchor. Whether a cell is migratory or resting correlates with the location of uPAR in the plasma membrane [174]. Because of endocytosis, the amount of uPAR available at the cell surface for signaling is reduced temporarily and hence uPAR mediated activation of ERK1, ERK2 and Rac1 are inhibited and thus cell migration and chemotaxis are also inhibited [229,288]. This may allow cells to acquire new situation following proteolytic modification of ECM. Therefore, uPA-uPAR regulated pericellular proteolysis, cell adhesion, invasion and migration are complex

and finely tuned mechanisms that make this complex an attractive therapeutic target in metastatic cancer [177].

2.2.3 uPA-uPAR signaling:

Currently, it is very well established that uPA-uPAR complex has many roles apart from the regulation of extracellular proteolysis. When uPA binds to uPAR it activates a cascade of intracellular signaling molecules that promote proliferation, differentiation, adhesion, migration, invasion and cell survival [148,289–291]. Signaling by uPA and uPAR begins when this complex interacts with transmembrane proteins such as integrin family proteins, receptor tyrosine kinases and chemotactic receptors [227,247,292]. The uPAR association with all these receptors are very well documented, but the exact molecular mechanisms underlying these phenomena are not very well understood. Many signaling pathways such as focal adhesion kinase (FAK), p38, Ras-MAPK pathway, Rho family small GTPase Rac1 and Src are activated through uPAR signaling [293–296]. In addition to these pathways, uPA-uPAR signaling can also activate PI3K and JAK1-STAT1 pathways [148,267,297]. Effective activation of intracellular signaling requires binding of uPA to uPAR, but it is not necessary to have proteolytically active uPA. Chemically inactive uPA or nonproteolytic uPA derivatives are able to activate effective intracellular signaling [267,292,298]. Apart from uPA, uPAR has other ligands such as ECM glycoprotein vitronectin. Vitronectin binds to the outer surface of uPAR and its binding site is different than that for uPA, and because of this both ligands can simultaneously bind to uPAR and activate downstream signaling [299]. Moreover, uPA-uPAR can indirectly associate with vitronectin through PAI-1 [92]. Various studies also reported that uPAR is a GPI-linked receptor and it does not have the transmembrane domain to perform its intracellular signaling and hence it is associated with other transmembrane receptors to perform its downstream signaling. The main receptor with which uPAR is associated to perform its intracellular signaling is integrin which is a major family of ECM receptors consisting α3β1, α5β1 and αvβ3 [7,239,300]. Many reports confirm the involvement of uPA-uPAR in intracellular signaling. Rabbani et al reported that, in some cell lines, uPA and its A-chain have the mitogenic effect [301]. Dumler et al reported that, the uPA-uPAR association promotes tyrosine phosphorylation of a protein in U937 cells and also enhances expression of the c-fos gene in OC-7 cell line

by tyrosine phosphorylation mechanism [302,303]. Furthermore, when pro-uPA is given to human epithelial cell line WISH, it enhances serine phosphorylation of cytokeratins 8 and 18 [304]. It is also reported that p56/59 hck tyrosine kinase activity was enhanced by proteolytically inactivated uPA [292]. Involvement of cAMP pathway in uPA signaling is also reported by Li et al [305]. Moreover, proteolytic activation of many growth factors such as transforming growth factor-β, HGF/SF, MSP and basic fibroblast growth factor can also be effected by plasmin and uPA [174]. Interestingly, the effect obtained following exposer of cells to the proteolytic inactive variant of uPA is not necessary because of receptor signaling but it can also be because of receptor masking which results in reduced cell surface uPA activity or enhance soluble uPA activity [174].

2.2.4 Plasminogen activator system and cancer:

Experiments conducted on model systems have revealed that plasminogen activator system plays a key role in cancer progression [306]. Earlier the classical function of uPA was ECM degradation by which it promotes cancer invasion and metastasis. Currently, the function of uPA is not limited to only ECM degradation but there are additional activities indicating its role in the proliferation and spreading of cancer [174,307]. The most important step for invasion and metastasis is to degrade and remodel the ECM many time during cancer progression [307]. The specificity of uPA towards ECM component is restricted to fibronectin only and till date, this is the only substrate known for uPA [307]. Whereas plasmin has the different substrate to digest such as laminin, fibronectin, fibrin and perlecan (a heparan sulfate proteoglycan) [161,174,307,308]. MMPs are other important proteases known to degrade ECM and enhance invasion and metastasis. These MMPs are present in inactivated form as proMMPs and activated by plasmin in MMPs. MMP-13, MMP-12, MMP-9 and MMP 3 are the MMPs known to be activated by plasmin [309]. Additionally, in vitro experiments also revealed that the uPA system has the ability to induce mitogenesis. There are two ways by which uPA system can stimulate metastasis of cancer cells; one is its proteolytic activity in which it degrade ECM and helps in invasion and metastasis, another is nonproteolytic activity in which uPA promotes cell migration by increasing adhesion at the leading edge of the cells, binding of uPAR to vitronectin and binding of uPA with its receptor uPAR result in stimulation of intracellular signaling cascade that

leads to invasion and metastasis. An individual's migrating cells can perform both the mechanisms simultaneously.

Both uPA binding to uPAR and catalytic activity of uPA is required to induce mitogenesis in epidermal tumor lines (CCL.20.2) and melanoma cells [310,311]. While uPA to uPAR binding is only necessary for induction of proliferation in a human ovarian cancer cell line OV-MZ-6 [312]. Moreover, many growth factors such as FGF2, IGF-1, HGF and VEGF activated by plasmin can also stimulate cellular proliferation [313,314]. It is found that growth of epithelial cell is stimulated by HGF and IGF-1 while endothelial cells angiogenesis is stimulated by well-known growth factors such as FGF2 and VEGF [315–317]. Angiogenesis is a very important process and necessary for tumor growth, invasion and metastasis. uPA-uPAR binding plays a crucial role in the multistep process. Activation of pro-angiogenic factors such as VEGF13, FGF2 and TGF-β, ECM remodeling and invasion of the tumor stroma are the main roles of uPA activity through its receptor [318]. PAI-1 plays dual character in cancer, it inhibit the binding of VN-uPAR to VN-integrin that makes it anti-migratory at leading edge. It also inhibits plasmin generation that makes it anti-migratory at ventral surface. But it preferentially protects VN from uPA mediated degradation [319] which would make it pro-migratory at the leading edge. PAI-1 also promotes endocytosis of uPA-uPAR which remove the adhesion signaling maintained by uPA-uPAR association and hence results in detachment at the trailing edge. PAI-1 can control both cell surface expression and internalization of uPA-uPAR which results in inhibition of invasion and metastasis. It is also known to promote tumor growth and dissemination [8]. Invasion and metastasis is stimulated by PAI-1 by modulating cell adhesion, stimulating cell proliferation, playing role in angiogenesis and inhibiting excess degradation of ECM. PAI-1 is inhibitor of uPA and thus it can inhibit the angiogenesis stimulated by uPA. Effects of PAI-1 on angiogenesis is found to depend on its concentration; at micromolar concentration PAI-1 inhibits angiogenesis while nanomolar concentrations were found to be proangiogenic [317]. uPA stimulated cancer cell migration and cell adhesion occur only when uPA is present in excess over PAI-1 [320]. Many evidences concerning their clinical utility in breast cancer confirmed that both PAI-1 and uPA are the first novel tumor biological prognostic factors [321]. Epigenetic modification of uPA and PAI-1 also play very important role in uPA mediated cancer cell invasion and adhesion. Pakneshan P et al. demonstrated that promoter of PAI-1 is hypermethylated

while uPA has hypomethylated promoter indicating reduced expression of PAI-1 as compared to uPA in breast cancer cells [322,323]. It is also demonstrated that uPA can inhibit apoptosis of cancer cells which enhances the survivability of cancer cells at the time of metastasis, and helps in the establishment of secondary lesions. The uPA-uPAR signaling causes elevated basal level of ERK while inhibiting apoptosis, thus indicating an unique mechanism for uPA-uPAR system affecting breast cancer invasion and metastasis [324]. All these functions of uPA system suggest that plasminogen system is an important player of cancer migration, invasion and metastasis.

2.2.4.1 Role of uPA system in cell migration:

It has been extensively studied that expression of plasminogen, uPA, PAI-1 and uPAR was enhanced during migration of various cell types. uPA and uPAR are present at leading edge of the migrating cells [264,325,326]. Inhibition of uPA either with antibodies or with antisense RNA inhibited migration of many cell types [327–329]. Moreover, it is also demonstrated that, uPAR-binding uPA variant which is enzymatically inactive reduced endothelial cell migration [199]. Antibodies which inhibit uPA-uPAR binding can also prevent uPA-induced cell migration [304,330]. Gyetko et al. reported uPA-independent role of uPAR in monocyte chemotaxis in vitro [331]. It is also shown that a mechanical force across the cell membrane to the cytoskeleton is generated by uPAR [332]. Monocyte adhesion to ligands for αMβ2-integrin was inhibited by anti-uPAR antibodies [333]. Transfection of uPAR cDNA in 293 human embryonic kidney cells leads to faster migration [231]. Stefansson and Lawrence et al. found that PAI-1 inhibits cell migration by inhibiting integrin binding to vitronectin without inhibiting the enzymatic activity of plasminogen activators [334]. This effect is counter-acted by uPA because uPA and PAI-1 binding form a complex uPA-PAI-1 and inhibit its binding with vitronectin [334,335]. Petzelbauer et al observed that bovine aortic endothelial cells migration is inhibited by PAI-1 while it promoted bovine aortic smooth muscle cell migration [336].
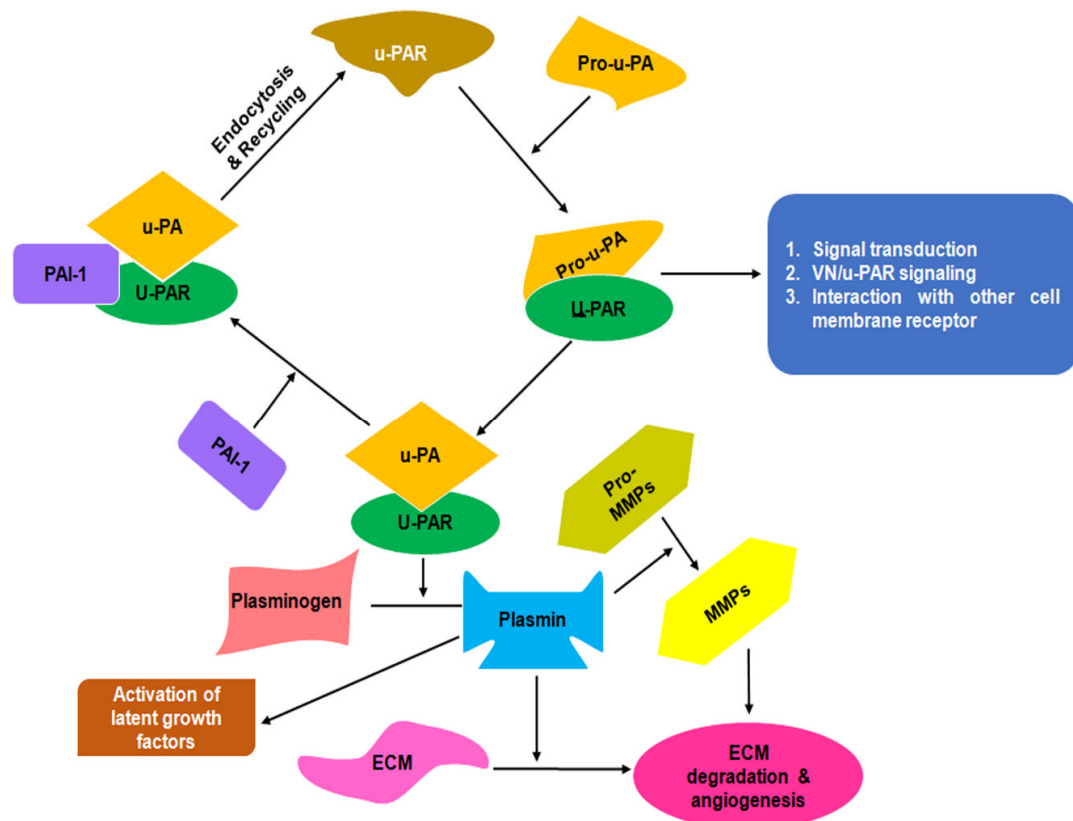
Figure 5: The urokinase-type plasminogen activator (uPA) system in cancer. Inactive urokinsae-type plasminogen activator (uPA) binds with the Urokinsae-type plasminogen activator receptor (uPAR) and cleaves the plasminogen into active plasmin. Activated plasmin then breaks extracellular matrix (ECM) components and helps in cell migration and invasion. Active plasmin can subsequently activate pro-MMPs into MMPs that also degrade ECM. For intracellular signaling uPA-uPAR complex interact with other cell surface receptors such as integrin and EGFR. uPAR also interacts with vitronectin to regulate cell adhesion and cell migration. Plasmin activation by the uPA-uPAR system is inhibited by PAI-1. The binding of PAI-1 forms a trimeric complex PAI-1-uPA-uPAR which is recognized by lipoprotein related protein and internalized for endocytosis.

2.2.4.2 Role of uPA system in cell invasion:

The main difference between migration and invasion is that the latter required an additional factor of complexity as along with cell locomotion invasion also requires the cells' penetration in to ECM. Several different in vitro assays have been used to study the role of uPA system in cell invasion e.g., invasion through fibrin gel, matrigel assay and through an isolated human amniotic membrane [337–340]. By using these assays it was found that, bovine pancreas trypsin inhibitor, α2AP and urinary plasmin inhibitor are able to reduce invasion by inhibiting plasmin [337,341–343]. Similar to migration,

uPA inhibition with antibodies also results in reduced invasion of cells [337]. When cDNA of uPA was transfected in a low uPA expressing cells, enhanced invasion was observed [339,344]. Similarly knocking down of uPA with antisense RNA lowered invasion of the cells [345]. Kirchheimer and remold et al. demonstrated variation in invasive activity correlated with variation in uPAR expression in cell lines [338]. uPA treatment of the cells with low uPA expression and high uPAR enhance the invasion of the cells [346]. Schlechte et al found that, invasion of the cells were inhibited by inhibiting uPA-uPAR binding with anti-uPA antibodies, anti-uPAR antibodies or by using uPA-uPAR binding antagonist [345–347]. uPAR cDNA transfection in the cells having low uPAR expression make cells more invasive [157,344]. Furthermore, knocking down of uPAR with siRNA results in reduced invasion of the cells [348].

2.2.4.3 Role of uPA system in cell metastasis:

Anti uPA-antibodies inhibited the metastasis to the lungs of the embryos when human tumor cells were implanted onto the chorioallantoic membrane of chicken embryo [349,350]. By using a series of human melanoma cell line , a direct correlation was found between lung metastasis and uPA expression level with higher uPA expression causing higher metastasis [351]. Growth of human ovarian cancer cells in nude mice was inhibited when antisense RNA against uPA was introduced in mice [352]. Bugge TH et al. correlated the concentration of uPA and the metastatic potential of various cell line and found higher concentration of uPA is positively related with metastatic potential of cancer [353]. It is also observed that inhibiting the uPA expression by siRNA reduced the metastatic potential of cancer cells [353,354]. Mice deficient in uPA or plasminogen show decreased lymph and lung node metastasis of breast cancer [355].

2.2.5 Mechanisms of action of uPA system in cancer invasion and metastasis:

Besides breaking down of ECM uPA system promotes invasion and metastasis employing many other mechanisms [356]. The uPA system can also activate inactive proMMPs into active MMPs which then degrade ECM and decrease the cell to cell and cell to ECM interaction. It was shown in MCF-7 cells and HT1080 fibrosarcoma that cooperation between Ras-Erk and Rho-Rho kinase pathway are necessary for uPA enhanced cell migration [276]. Various signaling molecules such as focal adhesion kinase (FAK), tyrosine kinase Src, extracellular-signal-regulated kinase (ERK)/mitogen activated protein kinase, p130Cas and the serine kinase Raf, are

activated by uPA-uPAR signaling. This protein activation results in enhanced cancer cell adhesion, proliferation and metastasis [149]. uPA and uPAR expression in cancer cells can be enhanced by many factors such as cytokines, the oncogenes v-Src and v-Ras, mitogen, protein kinase C, ligation of integrin with intracellular matrix protein and growth factors [357,358]. Invasion and metastasis by uPA system also depends a lot on signal transduction pathway. uPA-uPAR interaction results in the activation of many signaling pathways including Ras-Raf-MEK-ERK pathway [359]. During signal transduction process, some adaptor proteins and intracellular enzymes play crucial role e.g. the FAK recruits c-Src or other Src family tyrosine kinase to mediate signal transduction events [360]. Tyr-925 of FAK is phosphorylated by Src that facilitates binding of Grb2/Sos to activate Ras. For the recruitment of Grb2, FAK and c-Src may also phosphorylate Shc and act as an adaptor protein. All these three proteins c-Src, FAk and Shc associate uPAR-initiated pathway and integrin-mediated ERK activation [240]. Downstream effectors of uPA-induced Ras-ERK signaling pathway are Raf and MEK [361]. Assimilation of various signaling pathways is required for uPA-uPAR mediated cell migration. Ras-ERK- mediated cell migration progresses in cooperation with Rho-Rho kinase pathway [361]. The p38 MAPK pathway inhibits the MEK/ERK signaling pathway and is also involved in uPA secretion [362].

2.2.6 Plasminogen system and EMT:

Most of the cancer-related deaths are due to metastasis and epithelial-to-mesenchymal transition (EMT) is an integral part of this. EMT is a multistage trans-differentiation process in which highly polarized epithelial cells acquire invasive and migratory potential during pathological and physiological processes accompanying cancer progression, embryonic development and wound healing [138,139]. Many developmental transcription factors such as Twist1/2 and Six, Snail 1/2, TGF-β and Wnt/β-catenin signaling pathways, regulate this EMT program. These transcription factors induce the expression of mesenchymal markers like Vimentin and N-cadherin and repress the epithelial markers like E-cadherin [138,139,141–143]. The role of uPA system in cancer EMT is shown by many researchers. Robin D. Lester et al in 2007 published a paper entitled "uPAR induces epithelial-mesenchymal transition in hypoxic cancer cells", using breast cancer cells cultured in hypoxic conditions (1% O2) undergoing epithelial to mesenchymal transition. Moreover, they found enhanced

expression of uPAR under hypoxia-induced EMT. Hypoxia-induced EMT is stopped by silencing of uPAR gene and mimicked by overexpression of uPAR. Activation of uPAR downstream signaling molecules Akt and Rac1 were also observed [363]. Two different signaling pathways are activated when uPAR binds with two distinct ligands. When uPA binds with uPAR, ERK1/2 and PI3K signaling [364,365] is activated whereas vitronectin binding to uPAR activates Rac1 [296,366]. Minji Jo et al. in 2009 demonstrated whether uPAR knockdown reverse cancer cell EMT. They found reversal of EMT following, by endogenous silencing of uPA (a ligand of uPAR) or by inhibiting uPAR-activated cell signaling molecules such as Src family kinases, PI3k, and ERK in uPAR overexpressing MDA-MB 468 cells [367]. Furthermore, NF-kB is a well-known inducer of EMT [368]. It has also been shown by many researchers that NF-kB enhances the expression of uPA by binding to DNA binding domain of it in many cancer cells [369,370] [Fig 6]. This NF-kB mediated upregulation of uPA suggested existence of another pathway by which EMT is regulated by uPA system. Juan F. Santibanez in 2013 showed that, TGF-β regulates uPA expression in cancer cells [371]. Multiple signaling pathway is activated by TGF-β including SMAD and non-SMAD pathways [372]. Juan F. Santibanez first reported the implication MAPK, Ha-Ras and ERK1 and 2 signaling in TGF-β induced uPA expression in transformed mouse keratinocytes [Fig 6] [371]. Earlier in 2006 Juan F. Santibanez demonstrated that, TGF-β enhanced uPA expression through c-Jun N-terminal kinases (JNK) pathway concomitant with the induction of EMT [373]. Expression of E-cadherin at transcriptional level is repressed by many factors such as Snail, ZEB and Twist families [374]. Among these transcriptional repressor ZEB1 is final downstream effector and in addition to repressing epithelial polarity and adhesion genes, ZEB1 also enhances expression of mesenchymal markers in cancer cells [Fig 6] [374]. Multiple signaling pathways such as Notch signaling pathway, TGF-β signaling pathways and canonical Wnt (β-catenin/TCF4) activate ZEB1 expression [374]. Elke Hiendlmeyer et al reported that uPA is a downstream target of Wnt (β-catenin/TCF4) pathway [9]. Ester Sánchez-Tilló et al. demonstrated that ZEB1 enhances uPA expression by directly binding with its promoter via a mechanism involving the histone acetyltransferase p300 [374]. Overall, this pathway is suggesting that, canonical Wnt (β-catenin/TCF4) pathway activate uPA expression via ZEB1 in concomitant with EMT. Diandra d. randle et al. reported that, snail mediates EMT via the regulation of uPA-uPAR and the MAPK

signaling pathway. They observed that snail overexpression leads to the upregulation of uPA and uPAR [375]. Moreover, knocking down of uPAR in snail overexpressing cells reverse the EMT and invasion in cancer cells [Fig 6] [375].
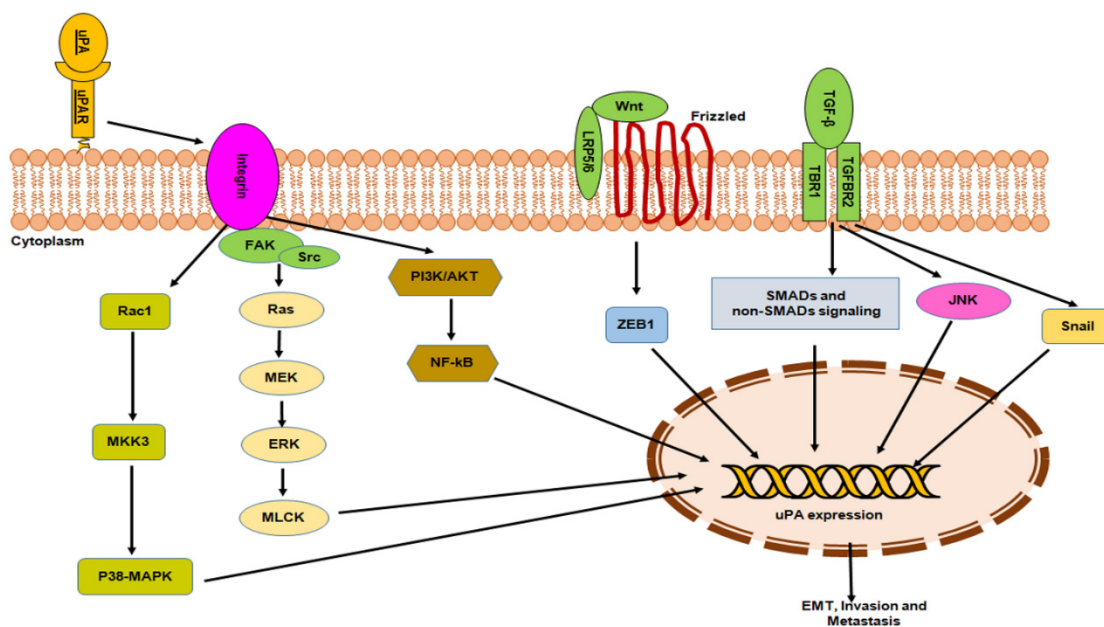
Figure 6: A schematic representation of uPA system in cancer cells EMT, invasion and metastasis. uPA/uPAR complex collaborates with coreceptor integrin to activate cell signaling. These interactions activate the diverse signaling pathways such as RAC1, FAK and PI3K resulting in EMT, invasion and metastasis of cancer cells. The uPA expression is regulated by two different pathways: Wnt/β-catenin pathway upregulates uPA expression via ZEB1 while TGF-β enhances uPA expression through SMADs and non-SAMDs signaling, JNK and snail signaling.

2.2.7 Salient points:

There is substantial evidence in the literature for the important role of uPA system in cancer cell growth, migration, invasion and metastasis. The uPA system is an attractive target for cancer therapeutics because of its crucial role in cancer progression and metastasis. In this review, we have attempted to reveal the role of uPA system in cancer and metastasis. We have also summarized the role of uPA signaling in EMT of cancer cells. We believe that the inhibition of these signaling pathways in cancer cells could stop the EMT, thereby preventing invasion and metastasis.

2.3 Epithelial to mesenchymal transition:

2.3.1 The concept of EMT:

Epithelial to mesenchymal transition is a multistage trans-differentiation process in which highly polarized epithelial cells undergo multiple biochemical changes and attain invasive and migratory potential during the pathological and physiological processes such as cancer progression, embryonic development and wound healing [138]. Epithelial and mesenchymal cells can be differentiated on the basis of their visual appearance. Epithelial cells show adherent properties and form a sheet-like structure by laterally attaching to each other. Epithelial cells are tightly bound and not able to move away from the epithelium because some adherent proteins and cell to cell junctions hold neighboring cells tightly [138]. Epithelial cells contain apico-basal polarity and they are organized in cell layers. In contrast, mesenchymal cells have properties such as spindle shape, a diffuse network, non-polarized and strong migratory potential [138]. Elizabeth Hay (1995) using the chick primitive embryo as a model described "Epithelial-Mesenchymal Transformation" for the first time [376]. Hay demonstrated that during embryonic development, epithelial cells undergo dramatic changes and transform into mesenchymal cells. Transformation of epithelial cells to mesenchymal needs deep changes in epithelial cell organization such as loss of E-cadherin which mediate cell to cell connection and gain of N-cadherin which provide weak adhesive force to the cells resulting in adaptation of mesenchymal phenotype. Completion of EMT is marked by underlying basement membrane degradation and migration of mesenchymal cells away from the epithelial layer where it is originated [138]. EMT initiation and completion is controlled by many factors such as expression of specific cell surface protein, activation of some transcription factors, reorganization and expression of cytoskeletal proteins, changes in the expression of specific microRNAs and production of ECM-degrading enzymes. During EMT progression expression of epithelial markers such as E-cadherin, laminin, occludin and claudin go down whereas expression of mesenchymal markers such as N-cadherin, fibronectin and vimentin goes up [377]. EMT is a reversible process and mesenchymal cells can revert back to epithelial cells, a process known as Mesenchymal-Epithelial Transition (MET), hence the term transformation has been changed to transition and instead of Epithelial-Mesenchymal Transformation it is known as Epithelial-Mesenchymal Transition. EMT can be categorized into three different types [141]. EMT during embryo formation,

implantation, and organ development is called as type 1 EMT. Type 2 EMT occurs during inflammation processes and plays a key role in wound healing and tissue regeneration and type 3 EMT involve in cancer progression [138].

2.3.1.1 EMT during embryonic development:

Type 1 EMT occur during embryonic implantation, gastrulation, and organ development [378]. During the implantation of the embryo, the trophectoderm cell goes EMT to invade the endometrium and anchor in the placenta [379]. During gastrulation, formation of three germ layers viz., the ectoderm, the endoderm, and mesoderm take place from the initial epithelial layer-epiblast. The first EMT during gastrulation is the degradation of basement membrane underlying the epiblast. The occurrence of EMT in the cells present in the primitive streak leads to ingression of these cells within the primitive streak. After ingression, the ingressing cells either remain mesenchymal or form mesoderm or it undergoes MET to form endoderm. Another very good example of type 1 EMT underlies neural crest formation. A group of migratory neural crest cells generated when neuroectoderm (epithelial cells) undergoes EMT [380]. Different types of cells such as pigment cells, cells of the adrenal medulla, and the neuron of the peripheral nervous system are formed when migratory neural crest cells disperse throughout the embryo.

2.3.1.2 EMT during tissue regeneration and organ fibrosis:

Inflammatory cells and fibroblasts mediate the release of a variety of inflammatory signals as well as components of the extracellular matrix such as elastin, fibronectins, collagen and tenacins. It is demonstrated that these cells tend to release inflammatory signals which lead EMT to occur in normal epithelial cells under pathological conditions. Importantly, such EMT is known to be associated with fibrotic disease occurring in liver, kidney, intestine and lung [381–384]. Moreover it is also found that α-SMA, fibroblast-specific protein 1 (FSP1) and collagen 1 are the mesenchymal markers for the EMT that happens during fibrosis [385,386]. FSP1 mesenchymal marker and α-SMA expressing cells still display epithelial markers such as E-cadherin and show epithelial morphology. This kind of behavior of epithelial cells suggests that epithelial cells display the different degree of EMT known as "partial EMT" [387].

2.3.1.3 EMT during cancer progression:

Metastasis is a process in which cancer cell spread and leave the primary site and disseminate to the distant site. The Death of cancer patients occurs mainly because of metastatic tumor instead of the primary tumor. Metastasis is a multistep process which involves loss of fixed intercellular contact, acquisition of motility and invasion of the basement membrane and surrounding tissues, intravasation of blood vessels, circulation through the blood stream, extravasation from the blood vessels and colonization of the secondary site [Fig 7] [388,389]. Many studies demonstrated that activation EMT program is the key mechanism for metastasis [390]. EMT is characterized by loss of apico-basal polarity, rearrangement of cytoskeleton, gain in higher migratory potential and invasiveness and expression of mesenchymal markers [Fig 7]. EMT of cancer progression is not very much different from EMT of embryonic development and wound healing. Similar pathways regulate EMT of all types. The major difference is during development the steps of EMT are well defined and mainly associated with cell fate while EMT of cancer and fibrosis is not clearly defined and specific to each pathology. Developmental EMT typically proceeds stepwise and is controlled by many morphogenetic signals whereas oncogenic EMT occurs with unpredictable genetic changes present in cancer cells and local tumor microenvironment.
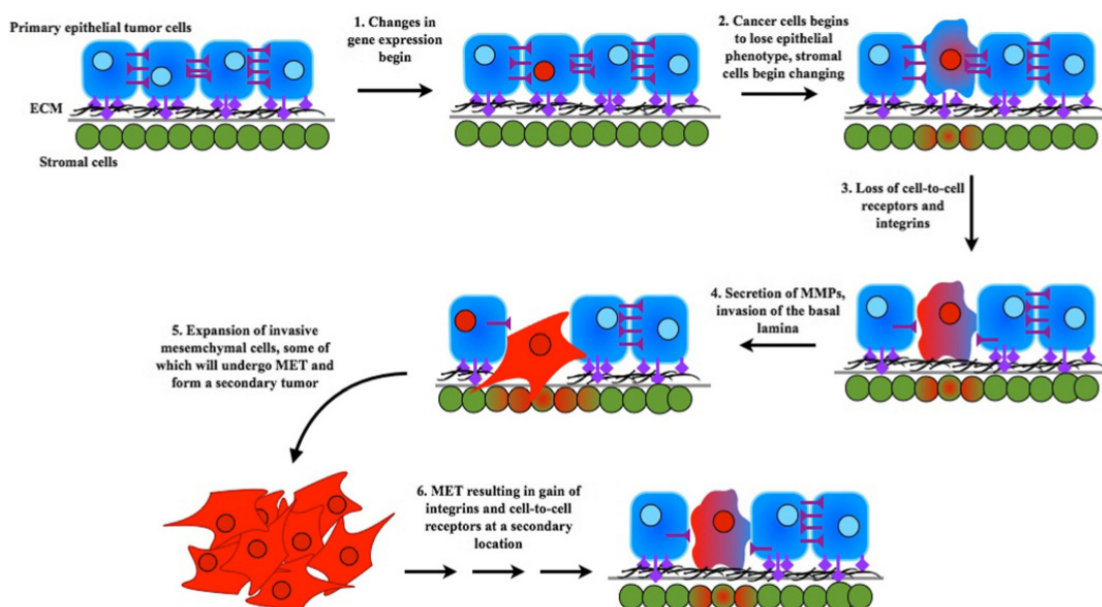
Figure 7: Changes that occur as tumor cells undergo EMT and then metastasize at secondary locations (Adapted from Clinical and Translational Medicine review [391])

EMT is induced by many factors such as (TGF-β), Wnt, Twist, Snail/Slug and Six1. Studies indicate that these factors induce EMT and enhance migratory and invasive properties in various cell lines and mouse model. Hence, EMT is considered as a very critical mechanism for cancer progression and metastasis. However, it is very difficult to demonstrate EMT in vivo because not all tumor cells but only a subset of tumor cells undergo EMT at any one time.

2.3.2 Molecular mechanism of EMT:

Regulation of E-cadherin- The most critical step for initiation of EMT is transcriptional repression of E-cadherin. E-cadherin basically acts as tumor suppressor gene and plays the diverse roles in regulating cell polarity, migration, differentiation and stem cell-like properties. Loss of cell-cell adhesion and decreased expression of E-cadherin is very important for EMT. Chan et al. (2003) reported that loss of E-cadherin leads to cancer progression, metastasis and poor prognosis in many cancers [392]. Studies indicated that loss of E-cadherin expression is subject to controls at genetic, epigenetic and transcriptional level [393]. Among all three reason, transcriptional repression is very well studied. E-box response element present in the promoter region of E-cadherin is determined as the binding site for various repressors [393,394]. The main transcription factors which directly bind to E-cadherin promoter and decrease its expression are snail family of zinc finger domain (snail, slug), ZEB1, ZEB2 and Twist [395,396]. Among these transcription factors, snail is the key repressor of E-cadherin. Studies indicated that snail overexpression in the cells does not reduce only E-cadherin expression but it also upregulates many mesenchymal markers such as N-cadherin and vimentin [397]. It is also reported that snail overexpression results in higher migration and invasive potential of cancer cells [397]. Additionally, many signaling pathways are demonstrated which upregulate snail expression e.g. epidermal growth factor receptor (EGF) pathway, transforming growth-β (TGF-β) factor receptor pathway and fibroblast growth factor (FGF) pathway. Becker et al. (1994) in gastric cancer and sarrio et al. in lobular breast cancer (2003) reported mutations in E-cadherin gene resulting in decreased expression of E-cadherin [398,399]. Lin et al (2006) reported polymorphism of E-cadherin promoter as an important marker for the risk of bladder cancer recurrence [400]. Graff et al (1995) found hypermethylation in the promoter region of E-cadherin in many human cancers leading to loss of E-cadherin expression [401]. He further

observed that degree of methylation in the promoter region of E-cadherin is unstable and heterogeneous during cancer progression which indicates methylation may induce EMT to enhance cancer progression and metastasis [401]. The microRNAs are also known to regulate E-cadherin expression and EMT. Gregory et al found that miR-141, miR-200b and miR-205 families regulate EMT by controlling the expression of ZEB1 and ZEB2 [402]. Knocking down of these miRNAs results in reduced expression of E-cadherin while overexpression of these miRNAs results re-expression of E-cadherin and MET in mesenchymal cells. The exact mechanism by which microRNAs regulate EMT is not very well studied and further study is required to elucidate their role in EMT.

2.3.3 Signaling networks of EMT:

EMT can be induced by cytokines, growth factors or ECM proteins secreted by the tumor microenvironment. Tumor microenvironment interaction with cancer cells affects the behavior of cancer cells. Tumor microenvironment consists of cancer associated fibroblast, ECM, immune cells and myofibroblast. Lewis et al. (2004) reported TGF-β signaling by stromal myofibroblasts can enhance hepatocyte growth factor secretion which stimulates cancer cell progression and metastasis [403]. As discussed above, multiple signaling pathways such as epidermal growth factor receptor (EGF) pathway, transforming growth-β (TGF-β) factor receptor pathway and fibroblast growth factor (FGF) pathway are the inducers of EMT. Out of these pathways, the most potent and well-study pathway is transforming growth-β (TGF-β) factor receptor pathway and TGF-β is the best studied inducer.

In our studied, we were mainly focused on TGF-β mediated induction of EMT. TGF-β superfamily contains four different isoforms viz., TGF-β 1, TGF-β 2, TGF-β 3 and TGF-β 4. Multifunctional TGF-β family of cytokines bind with TGF-β receptor and activate cytoplasmic smads. Once activated, smads move into the nucleus and activate snail which is an E-cadherin repressor. Studies indicated that TGF-β promotes EMT in many types of cancer cells with a gain of mesenchymal characteristics [404,405]. The wnt/β-catenin pathway is another very well study pathway implicated in EMT of cancer and development [96,406]. It is demonstrated that Wnt/β-catenin pathway activation stimulates snail expression concomitant with EMT in various cell lines [407]. Lieu et al. (2012) reported the potential role of hTERT in EMT [4]. They have shown that

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hTERT promotes EMT and stem cell like traits in gastric cancer cells when hTERT is ectopically expressed in these cells [4]. TGF-β is a well-known inducer of EMT, but downregulation of hTERT by siRNA inhibited the TGF-β and β-actenin mediated EMT. This paper demonstrated that hTERT binds with β-catenin in the cytoplasm and once hTERT interacts with β-catenin it enhances the nuclear translocation and increases its transcriptional activity. Inside the nucleus this complex interacts with TCF/LEF transcription factor and regulates the expression of many genes such as snail and vimentin [4]. Additionally, expression of different EMT markers and hTERT were positively correlated in gastric cancer samples and cancer cell colonization is stimulated by hTERT in a mouse model [4].

Materials and Methods

Chapter 3: Materials and methods

3.1 Materials:

All biochemicals were procured from Sigma-Aldrich, St Louis, USA, Promega, NEB, Genei, Merck GR, Qualigen-AR, Millipore GS and Qualigen-AR unless mentioned otherwise. Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum and Penicillin Streptomycin solution were purchased from Sigma Aldrich, USA. Trypsin-EDTA solution was purchased from Invitrogen Life Technologies, USA. Tissue culture plastic ware was from Corning International, USA and Greiner, Germany. All other plastic wares were from Tarsons, Germany. All restriction enzymes, DNA modifying enzymes and DNA ladder (100 bp and 1 kb) were procured from Thermo Fisher Scientific USA or New England Biolabs, USA. Bacterial growth media (LB and Agar) was purchased from HiMedia. Plasmid Isolation kit and gel extraction kit were from Qiagen, Germany. Pre-stained protein molecular weight marker was obtained from Biorad, India. RNA Isolation reagent was TRI reagent from Sigma, USA. Reverse Transcription kit and Power SYBR® Green Master Mix was procured from Applied Biosystems, Inc., USA. Lipofectamine 300 Transfection Reagent was purchased from Invitrogen, USA. PVDF membranes were purchased from Amersham Biosciences, USA. X-Ray Films were purchased from Kodak. Filter Papers were purchased from Whatman Ltd. (Madistone, England). Enhanced Chemiluminescence (ECL) detection reagent was purchased from Millipore, USA.

Established protocols were used to prepare standard buffer and stock solutions by using autoclaved Milli-Q water. All buffer and solutions used for RNA work were prepared in diethylpyrocarbonate (DEPC)-treated water (1.5ml/L)[408].

3.1.1 Chemicals, stock solutions and reagents: Please see Table 4.

Table 4: List of chemicals used:

Chemicals	Company name
Acetic acid glacial	Merck-GR
Acetone	Qualigen
Acetonitrile	Applied Biosystems
Beta-Mercaptoethanol	Sigma
Bis-acrylamide	Sigma
Bromophenol blue	Plus One

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cDNA Synthesis Kit	Thermo Fisher Scientific
Chloroform	Merck
Diethylpyrocarobonate	Sigma
Dithiothreitol (DTT)	Fluka Analytical
DMEM: Dulbecco's Modified Eagle's Medium	Hyclone™
Dimethylsulfoxide (DMSO)	Sigma
Enhanced chemiluminescence kit (ECL)	Millipore
Ethanol	Merck
Glycerol	Qualigens
Iodoacetamide	Sigma
IPG Buffer	GE healthcare
Lipofectamine 3000	Invitrogen
Methanol	Qualigen
Mineral oil	plus one
Opti-MEM	Invitrogen
Penicillin/Streptomycin antibiotic	Himedia
Phenylmethanesulphonyl fluoride (PMSF)	Sigma
Propidium Iodide	Sigma
Puromycin	Sigma
PVDF Membrane	Millipore
Protease inhibitor cocktail	Sigma
Restriction enzymes	New England Fermentas, USA
RIPA Buffer	Sigma
Skimmed Milk	TM Media
SYBR™ Green Master Mix	Thermo Scientific
Transforming growth factor beta 1 (TGF-β1)	Peprotech
N, N, N', N', Tetra methyl ethylene diamine (TEMED)	Sigma
Thiourea	GE healthcare
Triton X-100	Sigma
Trizol	Sigma
Trypsin	Sigma
Urea	Sigma

Acrylamide 30%: 30% acrylamide was prepared by dissolving 29 g Acrylamide (Sigma, A-9000) and 1 g N, N-methylene-bis-acrylamide (Sigma, M-7256) in 60 ml of MilliQ water. The solution was kept on the magnetic stirrer to dissolve. Final volumewas made up to 100 ml and the solution was filtered through Whatman 1MM paper toremove any particulate matter in the solution. It was stored at 4°C in dark brown bottle.

Agar plate: Agar plate was prepared by dissolving 1.5 g of agar powder and 2.5 g of Luria broth in 100 ml of water. The medium was autoclaved and cooled for at least 15 minutes at room temperature, and ampicillin (100 μg/ml) was added before pouring into the plate.

Agarose gel: Agarose gel was used to visualize DNA or RNA. Type V high melt agrose (Sigma A-3768) was prepared by boiling or micro-waving appropriate amount of agarose in 1XTAE or 0.5X TBE buffer containing 0.5 μg/ml final concentration of Ethidium bromide. The gel-casting tray was held at room temperature/refrigerator for complete setting of the gel.

Ammonium Bicarbonate (Sigma, S5761): 79 mg of ammonium bicarbonate was dissolved in 10 ml of MiliQ water to prepare a 100 mM stock solution which is further diluted to 25 mM.

APS 10%: 1 g of ammonium persulphate (Sigma, A-9164) was dissolved in 10 ml of MilliQ water and the solution was stored at -20°C.

Ampicillin stocks: In 1 ml of MiliQ water 50 mg of ampicillin was dissolved and kept it in -20°C for future use.

Antibodies: List of antibodies used (Please see Table 5).

Table 5: List of antibodies used:

S.No.	Antibodies used	Company provided	Catalog number
1	Urokinase type plasminogen activator (uPA)	Santa Cruz, USA	(H-140): sc-14019
2	Urokinase type plasminogen activator receptor (uPAR)	Santa Cruz, USA	(FL-290): sc-10815
3	Human telomerase reverse transcriptase (hTERT)	Santa Cruz, USA	Sc-393013
4	β-Actin	Santa Cruz, USA	Sc-47778
5	Heat shock protein 90 (Hsp90)	Enzo-Life sciences	ADI-SPA-844-F
6	Heat shock protein 70 (Hsp70)	Enzo-Life sciences	ADI-SPA-757-F
7	Heat shock protein 60 (Hsp60)	Enzo-Life sciences	ADI-SPA-806-F
8	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Cell signaling technology	#2118
9	E-cadherin	Cell signaling technology	#3195
10	Vimentin	Cell signaling technology	#5741
11	β-catenin	Cell signaling technology	#8480

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Bacterial strains:

Escherichia coli **DH5α and/or DH10β [mcrAΔ (mrr-hsdRMS-mcrBC)]:** Two strain DH5α and DH10β were used for plasmid transformation.

Bradford's reagent: Bradford reagent was prepared by mixing 100 mg of Coomassie brilliant blue G-250 (S.D. Fine Chem Limited, B.No. F04Y/0603/1101/64) in 50 ml of 95% ethanol. Following this 100 ml of 85% phosphoric acid was added and final volume made up to 1 liter with MiliQ water.

BSA 10mg/ml: 100 mg BSA (Fraction V, Sigma, A-9647) was dissolved in 10 ml of MiliQ water and stored at -20°C for future use.

Calcium chloride (0.2 M and 0.1 M): In 70 ml of MiliQ water 4.376 g of $CaCl_2.6H_2O$ was dissolved, and the final volume made up to 100 ml. It was stored at 4°C after filter-sterilization.

CBB-R Stain: 0.25 g of CBBR-250 (Sigma, B-0149) was dissolved in 50 ml methanol, 10 ml glacial acetic acid and 40 ml of MiliQ water.

Cell Culture Plates were obtained from Genetix.

Cell lines: A549 (lung carcinoma cell) HeLa cell line (Cervical cancer cells) and U2OS (osteosarcoma cells) were purchased from NCCS Pune.

Colloidal staining solution: 0.025% of CBBG-250 was dissolved in 10 % acetic acid.

Destaining solution: Destaining solution was prepared by mixing 120 ml of water, 60 ml of methanol and 20 ml of glacial acetic acid.

DNA loading dye (6X): In 7 ml of MiliQ water: 25mg (0.25% w/v) bromophenol blue, 25 mg (0.25% w/v) Xylene cyanol and 3 ml (30%) sterile glycerol were dissolved in water and the volume made up to 10 ml.

Ethidium Bromide (10 mg/ml): 100 mg of ethidium bromide (Sigma, E-8751) was dissolved in 10 ml of MiliQwater. The working concentration of it is 0.5 μg/ml

Equilibration buffer for 2D- gel electrophoresis: For 20 ml

Equilibration buffers composition for 2D- gel electrophoresis (Please see Table 6).

Equilibrium buffer 1: Dissolved 10 mg DTT in 1 ml of equilibrium buffer.

Equilibrium buffer 2: Dissolved 25 iodoacetamide in 1 ml of equilibrium buffer.

Table 6: Composition of equilibration buffer for 2D- gel electrophoresis:

S.No.	Chemical name	Amount required
1	Urea	7.2 gm
2	10% SDS	4 ml
3	100% glycerol	6 ml
4	1.5 M Tris-Cl (PH 8.8)	5 mg
5	Bromophenol blue	Traces
7	MilIQ water	Maintained to 20 ml

FBS: 10 % heat-inactivated fetal bovine serum (FBS from GIBCO) was used to prepare cell culture medium.

Luria Broth: 2 g of Luria Bertani medium (Himedia) was dissolved in In 100 ml of MiliQ water and sterilized by autoclaving.

Magnesium chloride 10 mM: 30.5 mg of MgCl2 (Qualigen AR) was dissolved in a mixture of the 15ml solution of Tris-Cl pH 7.5, 1mM DTT and water.

Membrane Filter Paper: Millipore, Durapore, 0.22μm, 0.47μm, CAT NO: GVWP04700.

PBS (10X): 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g KH2PO4 were dissolved in 70 ml of MiliQ water and maintained the final volume of 100 ml after checking the PH of 7.4 It was stored at room temperature after filter sterilization.

PBST: PBST was prepared by adding Tween-20 in PBS.

Plasmid Constructs: List of plasmids used (Please see Table 7).

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Table 7: List of plasmids used:

S.No.	Plasmids Name	Functions	Marker
1	pBABE-puro- vector	Mammalian expression vector	Puromycin
2	pBABE-puro-hTERT	Mammalian expression vector	Puromycin
3	pMKO.1-puro vector	shRNA transfer vector	Puromycin
4	pMKO.1-shRNA-hTERT	shRNA transfer vector	Puromycin
5	pLKO.1-puro vector	shRNA transfer vector	Puromycin
6	pLKO.1 shRNA-PLAU	shRNA transfer vector	Puromycin
7	psPAX2	Lentivirus packaging	Ampicillin
8	pMD2.G	Lentivirus packaging	Ampicillin

Primers used for PCR-based assays (Please see Table 8).

Table 8: Primers used for PCR-based assays.

Gene name	Real-time primers sequences (5'-3')
HSP60	Forward primer- TGCCAATGCTCACCGTAAG
	Reverse primer- ACTGCCACAACCTGAAGAC
HSP70	Forward primer- ACCAAGCAGACGCAGATCTTC
	Reverse primer- CGCCCTCGTACACCTGGAT
HSP90	Forward primer- ACTACACATCTGCCTCTGGTGATGA
	Reverse primers- TGTTTCCGAAGACGTTCCACAA
hTERT	Forward primer- CGGCGACATGGAGAACAAG
	Reverse primers- CCAACAAGAAATCATCCACCAAA
GAPDH	Forward primer- GTCTTCACCACCATGGAGAAGGCT
	Reverse primers- CATGCCAGTGAGCTTCCCGTTCA
ACTIN	Forward primer- GGCACCCAGCACAATGAAG
	Reverse primers- GCCGATCCACACGGAGTACT

Protein lysis solution for 2D-gel electrophoresis (Please see Table 9).

Table 9: Protein lysis solution composition for 2D-gel electrophoresis.

S.No.	Chemical name	Amount required
1	7M urea	210 mg
2	2m thiourea	77 mg
3	4% CHAPS	20 mg
4	1% DTT	5 mg
5	1mM PMSF	5μl
6	Protease inhibitor cocktail	5μl
7	MilIQ water	Maintained to 500μl

Rehydration Buffer for 2D-gel electrophoresis

Rehydration Buffer composition for 2D-gel electrophoresis: For 500 μl **(Please see Table 10).**

Table 10: Rehydration Buffer composition for 2D-gel electrophoresis: For 500 μl.

S.No.	Chemical name	Amount required
1	7M urea	210 mg
2	2m thiourea	77 mg
3	4% CHAPS	20 mg
4	1% DTT	5 mg
5	IPG buffer	1μl
6	Bromophenol blue	Traces
7	MilIQ water	Maintained to 500μl

SDS 10 %: In 100 ml water, 10 g Lauryl sulfate-sodium salt (SDS, L-4390) was dissolved. It was stored at room temperature.

Sodium Acetate 3 M: 40.8 g $CH_3COONa.3H_2O$ (Qualigen-Excel AR) was mixed in 80 ml of water and PH was adjusted to 8 before making the final volume of 100 ml.

Syringe-driven Filter Unit: Millipore, MillexR – GV, PVDF, 0.22 μm.

TAE Buffer (50X): 24.2 g Tris base was dissolved in 5.7 ml of glacial acetic acid and 10 ml of 0.5 M EDTA and the final volume made up to100 ml by MiliQ water.

TBE Buffer (5X): 27.5 g boric acid, 20 ml of 0.5 M EDTA and 54 g Tris base were dissolved in 1 Liter of MiliQ water to make the 5X buffer.

Tris-HCl 1 M: 121.1 g tris was dissolved in 800 ml of MiliQ water and pH was maintained by using concentrated HCl. Once pH was adjusted to 6.8 the final volume was made up to 1L.

Tris-HCl 1.5 M: In the same way 181.65 g Tris was dissolved in 800 ml of MiliQ water and PH was maintained by using concentrated HCl. Once pH was adjusted to 8.8, the final volume was made up to 1L.

Transfer Buffer: Transfer buffer was prepared by mixing 20 ml of methanol, 20 ml of 5X SDS running buffer and 70 ml of MiliQ water for semi dry transfer in western blotting. For wet transfer, the buffer consisted of 192 mM glycine, 25mM tris-HCl and 20% (v/v) methanol.

Water: MilliQ water (Millipore deionizer) or double distilled water or diethyl pyrocarbonate (DEPC, Sigma-Aldrich) treated water, autoclaved.

Whatman Paper: Whatman 3 MM paper (3030917) was stored at the clean dry place.

3.2 Methods:

3.2.1 Cell culture:

Cell lines viz., 1) U2OS (an hTERT negative human osteosarcoma cell line), 2) HeLa cells and 3) A549 cells were obtained from National Centre for Cell Science, Pune and grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, South Logan Utah,) with 10% fetal bovine serum (FBS) (Himedia). Cells were maintained at 37˚C and 5% CO2 in a humidified CO2 incubator.

3.2.2 Transfection of Cells and establishment of stable cell lines overexpressing hTERT:

One day before transfection HeLa and U2OS cells were seeded in 6 well plates and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and

antibiotics (penicillin/ streptomycin). Two micrograms each of pBABE-puro empty vector and pBABE-puro hTERT obtained from Addgene were transfected in to cells by using lipofectamine 3000 (Invitrogen). After 48hrs, transfected cells were selected by using 2μg/ml of puromycin and maintained in 1μg/ml of puromycin. Total protein was extracted at passage number 5 for two-dimensional gel electrophoresis.

3.2.3 Transfection of Cells and establishment of stable cell line expressing shRNA targeting hTERT:

One day before transfection A549 cells were seeded in 6 well plates and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics (penicillin/ streptomycin). Two micrograms each of pMKO.1-puro empty vector and pMKO.1-shRNA- hTERT were transfected in to A549 cells by using lipofectamine 3000 (Invitrogen). After 48hrs, transfected cells were selected by using 2μg/ml of puromycin and finally maintained in 1μg/ml of puromycin. Total protein was extracted at passage number 5 for two-dimensional gel electrophoresis.

3.2.4 Lentiviral transfection of cloned shRNA-PLAU into HeLa cells and establishing stable cell line of shRNA-uPA:

2 μg of each of pLKO.1-puro empty vector and pLKO.1-puro PLAU shRNA and 1 μg each of pSPAX and pMD2G were transfected in to 293T cells with the help of lipofectamine 3000 (Invitrogen). After 48hrs, cells were selected for resistance to 2 μg/ml of puromycin and after 7 days virus particles were collected. 600 μl of this virus preparation were again transfected in HeLa cells and after 48hrs, cells were selected for resistance to 2 μg/ml of puromycin and finally maintained in 1μg/ml of puromycin. Real-time PCR (Fig 3) and western blots (Fig 4) confirmed upto 70% knocking down of PLAU.

3.2.5 Wound healing assay:

Cell migration required for healing artificially created wound was assayed at 0, 12, 24, 36 and 48 hrs for hTERT overexpressing HeLa and U2OS cells, hTERT knockdown HeLa and A549 cells and uPA knockdown HeLa cells. Briefly cells were separately seeded in 2 wells of a 6 well plate and cultured until confluency. Then by using a pipette tip we made a straight scratch, simulating a wound. The plates were washed gently and

fresh DMEM replaced with supplements (serum, antibiotics). The cells were observed by phase contrast microscopy.

3.2.6 Colony formation assay:

Cells were trypsinized, counted, and seeded for colony formation assay in 6-well plates by seeding 500 cells/well. During colony formation, the culture medium was replaced every 3 days. On the 10th day after seeding, the media of the plates were removed and the cells were washed with 1xPBS. After washing cells were fixed by adding chilled methanol into the plates and an incubation of 30 minute at 4^0C. Following that fixation solution was discarded and cells were again washed with 1xPBS. Staining of colonies were done by adding 0.5% of crystal violet for 5 min at room temperature. Cells were washed with MiliQ water and colonies were counted by using an inverted microscope (NIKON SMZ 1500).

3.2.7 Two-dimensional gel electrophoresis:

We profiled cellular proteins after the overexpression of hTERT in HeLa and U2OS cells. 2D-Gel electrophoresis was performed as reported by Diao S et al [409]. Briefly, isoelectric focusing (IEF) was performed using an Ettan IPGphore 3 apparatus (GE healthcare) and using the nonlinear IPG strips of 13 cm in the pH range of 3.0-10.0. A total of 250 µg protein was diluted to 250 µl in a rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 1% DTT, 0.5% IPG buffer and some traces of bromophenol blue) and the rehydration step was continued for 16 h at room temperature. IEF was performed following a step-wise voltage increase procedure in the following order, 500 V for 5 hrs, 1000 V for 1 h and 8000 V for 3.5 h. After IEF, the IPG gel strips were placed in an equilibration buffers (5M urea, 10% SDS, 10 % glycerol, 1.5 M Tris-HCL, pH 8.8 and some traces of bromophenol blue) 1 and 2 for 15 min each and then kept in SDS-page running buffer for 5 min. Equilibration buffer 1 contained 1% DTT while equilibration buffer 2 contained 2.5 % iodoacetamide. Separation in the second dimension was performed by SDS-polyacrylamide (12 %) gel elctrophoresis at the constant voltage of 120 volts till the bromophenol blue dye front reached the lower end of the gels. Gels were fixed for 1 h in fixing solution (50% methanol and 10% acetic acid) and stained with colloidal Coomassie G-250 stain (that is compatible with downstream MS analysis, as previously described [410] for 3 h, and then destained with 10 % acetic acid. The images were scanned with a scanner (Ettan

IPGphor3). Images were analyzed by using ImageMaster 2D Platinum v7.0 gel analysis software-(GE Healthcare Life Sciences). After analysis of spots and normalizing for the background, we excised 28 spots of our interest from the U2OS and 23 from HeLa and submitted them for mass analysis.

3.2.8 In-gel protein digestion and MALDI-TOF-TOF/MS analysis:

Trypsin digestion of the excised bands was done according to Shevchenko et al. [411]. Briefly, after destaining, the gel was washed twice with mili-Q water and the spots of interest excised from the gel and cut into 1mm cubes. These small 1mm cubes of gels were transferred to a 1.5 ml microcentrifuge tube pre-rinsed with 100% acetonitrile. Gel particles were further washed with a solution of 100 mM ammonium bicarbonate in 100% acetonitrile and water. After 15 minutes of incubation on a rotory shaker, supernatant was discarded and this step was repeated till completion of destaining. After destaining, all the remaining liquids were removed and enough acetonitrile was added to cover the gel particles which let the particles to shrink together. Acetonitrile was removed completely and gel particles were dried down in a vacuum centrifuge at room temperature. Further gel particles were swelled in a solution of 50 µl each of 10 mM DTT and 100 mM ammonium bicarbonate and incubated for 45 minutes at 56 °C. After this incubation, tubes were cooled to room temperature and excess liquid removed and replaced quickly by the same volume as above of freshly prepared solution of 55 mM iodoacetamide in 100 mM ammonium bicarbonate and further incubated for 30 min at room temperature. The gel was washed again with a solution of acetonitrile and ammonium bicarbonate and dried down in a vacuum centrifuge. Enough sequencing grade modified trypsin (20g/ml) was added to the tube and incubated at 37°C for 30 minutes. 5µl of 25 mM ammonium bicarbonate was added to keep the gel moistened. Trypsin added tubes were further incubated at 37 °C overnight. Next day supernatants were collected in new microfuge tubes and 10 µl of 1% TFA and 10 µl of 100% acetonitrile were added to the gel and the mix sonicated for 20 min at room temperature. Supernatants were taken and pooled and further dried in the Speed vac and submitted for mass analysis.

3.2.9 Isolation of RNA:

Following the chosen time periods of experimental treatment, the culture media was removed from 6 well plates. 600 µl of Trizol reagent was added into the each well and

the cells were lysed directly in the culture dish by pipetting. After that, the culture plates were kept at room temperature for 5 min. The lysed cells were now transferred into in a clean RNase free tube. 125 µl of chloroform was added in to the tubes and mixed properly by pipetting and incubated for 10 min at room temperature. Following that, tubes were centrifuged at 12000 rpm for 15 min at 4°C. After centrifugation an aqueous phase is formed; 150 µl of this was transferred into a new RNase free tube. After that, 250 µl of isopropanol was added and mixed by pipetting. The tubes were now incubated for 10 min at room temperature and centrifuged for 10 min at 12000 rpm. The Supernatant was discarded and the pellet was washed twice with 70% ethanol and precipitate retained by centrifugation at 7500 rpm at 4°C. The pellet was dried at 37°C till complete removal of ethanol. Pellets were further resuspended in 30 µl of DEPC water at 50°C for 10 min in water bath.

3.2.10 Spectrophotometric estimation of nucleic acids:

The quantification of isolated RNA and plasmid were done by measuring absorbance at 260 and 280 nm. The ssRNA concentration was calculated by taking the Abs260 = 1 = 40 µg/mL. The dsDNA concentration was determined by taking the Abs260 = 1 = 50 µg/mL.

3.2.11 RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from cells using TRIzol reagent (Sigma). cDNA was synthesized by using reverse transcription kit (Thermofisher) according to the manufacturer's protocol. 1 µg cDNA was used as template for PCR reaction using gene specific primers. The real-time primers sequences are given in Table 1. Real Time PCR conditions were: 15 sec at 95°C for denaturation, 1 min at 60°C for both annealing and elongation over 40 cycles in Applied Biosystems 7500. Data were normalized with reference to actin used as endogenous control. Primers used for PCR-based assays are presented in Table 9.

3.2.12 Protein Isolation:

Following experimental treatments the media was removed from the dish and cells were further washed once with PBS. Cells were now trypsinized and pelleted at 2000 rpm for 2 minutes. Pellet was dissolved in RIPA buffer (Sigma-Aldrich) with

phenylmethylsulphonyl fluoride (PMSF) and protease inhibitor cocktail and 30 minutes incubation on ice was given to completely lyse the cells. After that tube was centrifuge at 4°C and 13000 rpm for 30 minutes. The supernatant was collected in new tube and quantified by Bradford method. The protein was stored at -80^0C for future use.

3.2.13 Western blotting:

Western blotting was performed as previously described [412]. Stable cells were lysed in RIPA buffer (GCC biotech). Cell lysates were quantified by Bradford assay and 40 μg of total protein was separated by SDS polyacrylamide gel electrophoresis. Proteins resolved on SDS-PAGE gels were further transferred by making sandwich of (-ve pole) transfer pads-two Whatman filter paper-gel-Polyvinylidene difluoride (PVDF) membrane (Millipore)-two Whatman filter paper-transfer pads (+ve pole) clamped between transfer sheets. The whole assembly was fitted in the transfer apparatus in a way that the membrane should be on the positive pole while the gel should be on negative pole which facilitates the migration of negatively charged proteins towards positive pole. Whole assembly is run at 80 volts of constant voltage at 4 degree for 2 hrs. The blots were kept in blocking buffer (5% skimmed milk in 1xPBST) for two hours on a reciprocating shaker. After blocking, blots were incubated with primary antibody (usully rabbit IgG) against hTERT, β-actin, HSP90, HSp70, GAPDH, uPA, uPAR and HSP 60, followed by incubation with secondary antibodies i.e., Goat anti‑rabbit immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked antibody and Goat anti‑mouse immunoglobulin G (IgG), horseradish peroxidase (HRP) -linked antibody. Luminata^{TM} Forte western HRP substrate was used for band visualization according to the manufacturer's protocol. β-actin was used as an internal control for protein expression. Quantification of protein expression was done by ImageJ software.

3.2.14 Immunofluorescence assay:

Experimental cells were grown on sterilized cover slips. After proper incubation cells were washed twice with 1XPBS and cells were fixed by a solution of (1%) chilled acetone and methanol at 4°C for 20 minutes. Cells were again washed twice with 1XPBS after removing fixative. Cells were now permeabilized with a solution of 0.2% Triton-X 100 in 1XPBS at room temperature for 5 minutes. Permeabilization solution

was removed and the cells washed twice with 1XPBS. Cells were blocked by using 5% skimmed milk for 2 hours. After blocking cells were incubated with primary antibody (1:1000 dilution) for 1hr at room temperature. After primary antibody incubation cells were washed 3 times, 10 minutes each. From here rest of the steps were done in dark. Cells were now incubated with secondary antibody with same dilution of 1:1000 for 45 minutes at room temperature. Again cells were washed 3 times with 1XPBS for 10 minutes each. At this stage, nuclear staining was done by incubating cells with DAPI (1mg/ml) for 5 minutes at room temperature. Cells were again washed twice with 1XPBS for five minutes at room temperature. Clean slides were taken and 20 µl of mounting solution (50% glycerol) was added to each slide. The coverslip were picked with forceps and kept on mounting solution by taking care the cell must be downward. Slides were further viewed with the help of a confocal microscope (NIKON ECLIPSE TIS).

3.2.15 Polymerase Chain Reaction:

Polymerase chain reaction was performed in 20 µL reaction volume with 1X buffer, 2 ng/µL primers, 5 ng of template, 0.2 mM dNTPs, and 1U of Taq Polymerase (NEB).

Cycling condition was as follows:

1. 95°C for 5 min   Initial Denaturation

2. 95°C for 1 min   Denaturation

3. 55°C -60°C for 30 sec   Annealing

4. 68°C for 1-6 min   Extension

5. Steps 2-4 were repeated over   30-35 cycles

6. 68°C for 5 min   Final Extension

3.2.16 Restriction digestion:

Restriction digestion was performed using standard protocol [413].

3.2.17 Ligation reaction:

Both vector and insert were digested with the same set of enzymes and purified from agaroge gel. After their quantification by spectrophotometer they were ligated

according to the following approximation: [Amount of insert = (Size of insert/Size of vector) X 100 ng of the vector] in 20 μL volume in 1X Ligase buffer and 5U of T4 DNA ligase (NEB, USA) at 16°C overnight.

3.2.18 Competent Cell Preparation:

DH5α or DH10β cells were incubated in 5 ml of LB media at 37°C for overnight in the shaker incubator. The next day secondary culture was prepared by inoculating 250 μl of primary culture in 200 ml of autoclaved LB media for 3 hours at 37°C to an OD at 600 nm of 0.4 to 0.5. Cells were chilled on ice for 1 hour and all the tubes glycerol, tips and $CaCl_2$ kept in -20°C. Cells were now pelleted down for 10 minutes at 4500 rpm in a clean 50 ml Falcon tubes. The pellets were resuspend in 25 ml of 0.1M filtered $CaCl_2$ and kept on ice for 30 minutes. Cells were again pelleted down at 4500 rpm for 10 minutes at 4°C. Cells were now resuspended in 20% glycerol and 2 ml of 0.2 M $CaCl_2$. 200μl aliquots were prepared and kept at -80°C for future use.

3.2.19 Bacterial Cell Transformation:

10 to 50 ng of plasmid DNA or equivalent amount of ligation mixture was added to a vial containing 100 μl of competent cells. Tubes were gently mixed by tapping and kept on ice for 30 minutes followed by heat shock treatment at 42°C for 90 sec and chilling on ice for 5 minutes. 800 μl of LB medium was added and the cell suspension shaken at 37°C for 1 hour to revive. Cells were now centrifuged at 4500 rpm at room temperature and 800 μl of media was removed from the tube. Cells were resuspended in remaining media and plated on LB agar plate containing appropriate antibiotic. The plates were kept for 12-14 hour at 37°C.

3.2.20 Plasmid Isolation:

Plasmid DNA was isolated by using PureYield™ Plasmid Miniprep System (Promega) according to the manufacturer's protocol.

3.2.21 TGF-β1 treatment:

For TGF-βtreatment, cells were seeded in 6 well plates and incubated overnight at 37°C and 5% CO_2 overnight. Cells were starved for 14-16 hours in serum-free DMEM media. After starvation 5ng/ml TGF-β was given added and PBS treated cells were used

as a control. Cells were harvested and used for different assays based on real-time PCR, immunofluorescence and western blotting.

3.3 Microarray after hTERT overexpression in U2OS cells:

3.3.1 RNA quality assessment using the agilent 2100 bioanalyzer:

Total RNA was extracted using Trizol reagent (Sigma, USA) according to the manufacturers' protocol. RNA purity and integrity were evaluated by ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA), and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA) to ensure that the sample have a quality that will give usable reproducible endpoint data. Both RNA sequencing and microarray are heavily dependent on high quality intact RNA to produce reliable data. The 2100 bioanalyzer uses microfluidic channels on a lab-on-chip platform to evaluate the quality and quantity of RNA. These chip based assays with specifically designed software produce a standard of RNA quality assessment. At least 200pg/μl is needed for analysis on this instrument. Degradation of RNA can be easily detected using the agilent 2100 bioanalyzer.

3.3.2 RNA Integrity Number (RIN): Standardization of RNA Quality Control:

To estimate the integrity of total RNA samples RNA integrity number (RIN) software was used. This software automatically assigns an integrity number of total eukaryote RNA samples. Sample integrity using this tool is determined by the electrophoretic traces of RNA samples and no longer determined by the ratio of the ribosomal bands. The provided RIN is independent of instrument, analyst and sample concentration hence becoming a genuine standard for RNA integrity. The RIN number ranges between 1 and 10, with 10 being the best. RIN number greater than 6 is considered to be good for real-time PCR for downstream analysis. Fleige S et al. (2006) reported that RIN numbers greater than 5 have no effect on downstream analysis as shown by real-time PCR [414]. However, for microarray RINs greater than 7 is acceptable. In our case, we sent four sample to Macrogen for microarray, two of which represent pBABE vector transfected and remaining two represent hTERT overexpressing cells. The quality control result of RNA is produced by Macrogen which is given below in [Table 11] [Fig 8].
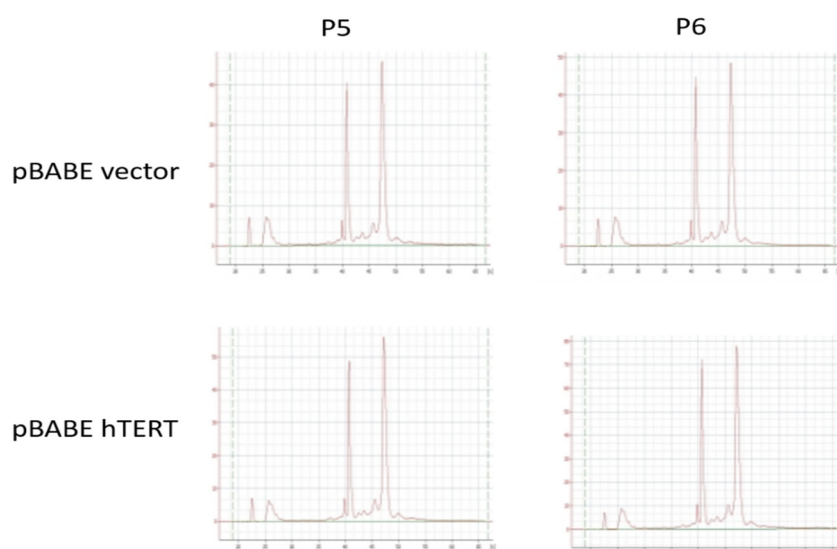
Figure 8: Electropherogram output we obtained from the Agilent 2100 Bioanalyzer.

Table 11: Quality control result of RNA.

Sample name	Conc. (ng/µl)	Purity (A260/ A280)	Purity (A260/ A230)	Volume	Total amount (µg)	rRNA ratio	RIN	Result
Vector P5	2161.46	2.1	2.29	30	64.84	1.8	9.3	Pass
Vector P6	2119.29	2.07	2.26	30	63.58	1.8	9.3	Pass
hTERT P5	1729.01	2.09	2.3	30	51.87	1.9	9.5	Pass
hTERT P6	1949.24	2.11	2.13	30	58.48	1.8	9.3	Pass

3.3.3 Labeling and purification:

Total RNA was amplified and purified using Target Amp-Nano Labeling Kit for Illumina Expression BeadChip (EPICENTRE, Madison, USA) to yield biotinylated cRNA according to the manufacturer's instructions. Briefly, 500 ng of total RNA was reverse-transcribed to cDNA using a T7 oligo (dT) primer. Second-strand cDNA was synthesized, *in vitro* transcribed, and labeled with biotin-NTP. After purification, the cRNA was quantified using the ND-1000 Spectrophotometer (Nano Drop, Wilmington, USA).

3.3.4 Hybridization and data export:

750 ng of labeled cRNA samples were hybridized to each Human HT-12 v4.0 Expression Beadchip for 17h at 58°C, according to the manufacturer's instructions (Illumina, Inc., San Diego, USA). Detection of array signal was carried out using Amersham fluorolink streptavidin-Cy3 (GE Healthcare Bio-Sciences, Little Chalfont, UK) following the bead array manual. Arrays were scanned with an Illumina bead array Reader confocal scanner according to the manufacturer's instructions.

3.3.5 Raw data preparation and Statistic analysis:

The quality of hybridization and overall chip performance were monitored by visual inspection of both internal quality control checks and the raw scanned data. Raw data were extracted using the software provided by the manufacturer (Illumina GenomeStudio v2011.1 (Gene Expression Module v1.9.0)). Array probes were transformed by logarithm and normalized by quantile method. Statistical significance of the expression data was determined using LPE test and fold change in which the null hypothesis was that no difference exists among groups. False discovery rate (FDR) was controlled by adjusting p value using Benjamini-Hochberg algorithm. For a DEG set, Hierarchical cluster analysis was performed using complete linkage and Euclidean distance as a measure of similarity. Gene-enrichment and Functional Annotation analysis for significant probe list were performed using gene ontology (www.geneontology.org/) and KEGG (http://kegg.jp). All data analysis and visualization of differentially expressed genes was conducted using R 3.1.2 (www.r-project.org).

3.3.6 Differential gene expression analysis:

An important feature of gene array data analysis is to produce lists of differentially expressed genes in the test sample with respect to the reference sample and identifying patterns in gene expression that can be correlated with the phenotype. Identification of differentially expressed genes from transcriptomics data is firstly based on the using cut-off value for the change of a gene in the test sample as compared to reference one [415]. Normalized data were subjected to pair-wise overall comparison between hTERT overexpressing to vector transfected samples. Gene lists generated after each pair-wise comparison is the only genes that showed a fold change of 1.5 or higher.

We have used LPE (local-pooled-error) test to visualize significant differentially expressed genes. This is important when the number of samples is small. The basis of LPE estimation is pooling errors within genes and between replicate arrays for those genes consisting similar expression values. This is motivated by the fact that many gene expression research is applied with a small number of replicate arrays and by the observations that errors between duplicates vary as a function of the average gene expression intensity [416].

Results and Discussion

Results and discussion

Objective 1: To validate the relationship between hTERT and plasminogen activator.

4.1 Background:

Cancer is a large group of diseases involving unregulated cell growth. Cancer cells divide and grow uncontrollably and in many cases form malignant tumors, and invade nearby and distant parts of the body. Cancer cells have unlimited proliferation potential. One of the ways of acquiring this involves reactivation of a specialized reverse transcriptase called telomerase which solves the end replication problem by adding telomeric repeats at the ends of newly replicated chromosomes. Almost 90% of cancerous cells have telomerase activity while most of the normal differentiated somatic cells do not. The classical activity of telomerase is to maintain telomere length, permitting chromosome stability and cellular proliferation. However, there has been increasing evidence to implicate telomerase in many functions in addition to synthesizing telomeric repeats [2]. These functions are independent of TERC being used as a template. Telomerase has an RNA-dependent RNA polymerase (RdRP) activity in mitochondria [109]. It also plays a role in the Wnt signaling pathway by acting as a transcriptional modulator [105]. Telomerase acts as a reverse transcriptase independent of its core subunit TERC [116]. In addition, studies indicate that telomerase also plays a role in apoptosis and DNA repair [113,119]. In general, telomerase activity remains high in metastatic cells [417].

The most common cause of human death by cancer is metastasis, but the principal mechanisms behind this are not very well known. However, emerging evidence suggest the role of epithelial–mesenchymal transition (EMT) in metastasis [138–140]. Epithelial-mesenchymal transition (EMT) is a physiological process which has a key role in embryonic development and is characterized by loss of apico-basal polarity, loss of cell-to-cell adhesion, loss of epithelial markers like E-cadherin, the gain of mesenchymal markers like N-cadherin and vimentin, resistance to apoptosis and capacity to invade [141]. There are many developmental transcription factors such as Twist1/2, Snail ½, TGF-β and Wnt/b-catenin signaling pathways that regulate this EMT program. This transcription factor induces the expression of mesenchymal markers like Vimentin and N-cadherin and represses the epithelial markers like E-cadherin [138,139,141–143]. In 2012 a paper published by Liu et al. in Oncogene revealed a

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potential role of hTERT in EMT [4]. They have shown that hTERT promotes EMT and stem cell like traits in gastric cancer cells when ectopically expressed in these cells [4]. TGF-β is a well-known inducer of EMT, but down-regulation of hTERT by siRNA inhibited the TGF-β and β-actenin mediated EMT. This paper demonstrated that hTERT binds with β-catenin in the cytoplasm and once hTERT interacts with β-catenin it enhances the nuclear translocation and increases its transcriptional activity. Inside the nucleus, this complex interacts with T-cell factor and elymphoid enhancer factor (TCF/LEF) and regulates the expression of many genes such as snail and vimentin [4]. Additionally, expression of different EMT markers and hTERT were positively correlated in gastric cancer samples and cancer cell colonization is stimulated by hTERT in a mouse model [4]. Significantly, all of these effects of hTERT were independent of its classical activity of telomere lengthening. Furthermore, a Chinese group demonstrated in 2015, the role of hTERT in EMT using oral squamous cell carcinoma (OSCC) as a model [144]. They found hTERT is overexpressed in oral epithelial dysplasia (OED) and oral squamous cell carcinoma (OSCC) tissues and correlates with clinical aggressiveness of OSCC patients [144]. They have checked the potential of hTERT to prolong the life span in primary human oral epithelial cells (HOECS). Following overexpression of hTERT in these cells they found hTERT prolongs the life span of these cells [144]. They also demonstrated that overexpression of hTERT is enough to cause EMT by activating Wnt/β-catenin pathway [144]. They have reported that overexpression of hTERT causes cytoplasmic localization of β-catenin, while others suggested nuclear localization of β-catenin when EMT is induced [418]. They also found decrease in the phosphorylation of GSK-3β which suggested activation of the GSK-3β pathway by hTERT upregulation. Knocking down hTERT expression led to decreased expression of vimentin, slug and twist end upregulation of E-cadherin. They also suggested that depletion of endogenous hTERT inhibits the Wnt/β-catenin pathway and hence EMT was also reversed [144]. Matrigel assays confirmed their result because hTERT-overexpressing cells showed increase in invasiveness in comparison to vector transfected cells [144]. Okamoto et al. reported that hTERT interacts with nucleolar GTP-binding protein nucleostemin/GNL3L and BRG1 and forms a complex to modulate transcriptional programs necessary for maintenance of tumor-initiating cells and for cancer stem cells [145]. Moreover, the main pathway by which hTERT promotes EMT is wnt/β-catenin pathway and it was

unknown if other pathways also existed till a study came in 2015 showing that "an hTERT/ZEB1 complex directly regulates E-cadherin to promote epithelial-to-mesenchymal transition (EMT) in colorectal cancer" [146]. This study demonstrated that hTERT interacts with ZEB1 and forms a complex which then suppresses E-cadherin expression by binding to its promoter in colorectal cancer cells [146]. They found down-regulation of E-cadherin when hTERT was overexpressed in HCT116 and SW480 cells, but E-cadherin expression was back to normal level when ZEB1 expression was impaired even in hTERT overexpressing cells [146]. This suggested that ZEB1 is necessary for hTERT mediated down-regulation of E-cadherin. There are many other genes known to be involved in metastasis such as matrix metalloproteinase9 (MMP9), ras homolog gene family member c (Rhoc) [419], Tissue plasminogen activator (PLAT), Urokinase-type plasminogen activator (PLAU) and Urokinase-type plasminogen activator receptor (PLAUR) etc. [420,421]. The plasminogen activator/ plasmin system is an enzymatic cascade involved in the control of fibrin degradation, matrix turnover, EMT, cell invasion and metastasis.

The role of uPA system in cancer EMT is shown by many researchers. Robin D. Lester et al (2007) published a paper entitled "uPAR induces epithelial-mesenchymal transition in hypoxic cancer cells", showing that, breast cancer cells cultured in hypoxic conditions (1% O2) undergo epithelial to mesenchymal transition. Moreover, they found enhanced expression of uPAR under hypoxia-induced EMT. Hypoxia-induced EMT is stopped by silencing of uPAR gene and mimicked by overexpression of uPAR. Activation of uPAR downstream signaling molecules Akt and Rac1 were also observed [363]. Two different signaling pathways are activated when uPAR binds with two distinct ligands. When uPA binds with uPAR, ERK1/2 and PI3K signaling [364,365] is activated whereas vitronectin binding to uPAR activates Rac1 [296,366]. Minji Jo et al. (2009) demonstrated that in uPAR overexpressing MDA-MB 468 cells, endogenous silencing of uPA (a ligand of uPAR) or inhibiting uPAR-activated cell signaling molecules such as Src family kinases, PI3k, and ERK, resulted in reversal of EMT [367]. Furthermore, NF-kB is a well-known inducer of EMT [368]. It has been also shown by many researchers that NF-kB enhances the expression of uPA by binding to DNA binding domain of it in many cancer cells [369,370]. This NF-kB mediated upregulation of uPA suggested existence of another pathway by which EMT is regulated by uPA system. TGF-β regulates uPA expression in cancer cells [371].

Results and discussion

Multiple signaling pathways are activated by TGF-β including SMAD and non-SMAD pathways [372]. Juan F. Santibanez first reported the roles of MAPK, Ha-Ras and ERK1 and 2 signaling in TGF-β induced uPA expression in transformed mouse keratinocytes [371]. TGF-β was shown to enhance uPA expression through c-Jun N terminal kinase (JNK) pathway concomitant with the induction of EMT [373]. Inhibition of E-cadherin is a very critical process for the EMT to begin. Expression of E-cadherin at transcriptional level is repressed by many factors such as Snail, ZEB and Twist families [374]. Among these transcriptional repressor ZEB1 is the final downstream effector and in addition to repressing epithelial polarity and adhesion genes, ZEB1 also enhances expression of mesenchymal markers in cancer cells [374]. Multiple signaling pathways such as Notch signaling pathway, TGF-β signaling pathway and canonical Wnt (β-catenin/TCF4) activate ZEB1 expression [374]. uPA is a downstream target of Wnt (β-catenin/TCF4) pathway [9]. ZEB1 enhance uPA expression by directly binding with its promoter via a mechanism involving the histone acetyltransferase p300 [374]. Overall, this suggests that canonical Wnt (β-catenin/TCF4) pathway activates uPA expression via ZEB1 concomitant with EMT.

From the microarray data generated after knocking down hTERT in our laboratory [6], we found an association of genes of plasminogen system such as PLAU with hTERT. The inhibition of hTERT also resulted in regulation of certain other genes such as Phospholipase C, Beta 1 (PLCB1), Fibroblast Growth Factor 2 basic (FGF2), Tissue Factor Pathway Inhibitor 2 (TFPI2), Interferon Regulatory Factor 9 (IRF9), and Kruppel-Like Factor 4 (KLF4). All these findings suggest a key role of telomerase in metastatic cancer progression.

So, it can be hypothesized that telomerase promotes metastasis by regulating plasminogen activator and it would be interesting to see the regulatory relation between hTERT and plasminogen activator by knocking down hTERT and/or over-expressing hTERT. The molecular mechanism behind hTERT mediated regulation of plasminogen activator is worth examining.

Results:

4.1.1 Overexpression of hTERT in U2OS and HeLa cell line*:*

Overexpression of hTERT in HeLa and U2OS cells was confirmed by qRT-PCR and western blotting. Remarkably, hTERT mRNA expression was upregulated to approximately 180 fold in U2OS cells transfected with pBABE-puro-hTERT in comparison to vector transfected U2OS cells (Fig 9A) while in HeLa it is upregulated to 36 fold [Fig9D]. Overexpression of hTERT in U2OS and HeLa cell lines was further confirmed by western blotting [Fig9B-C, 9E-F].
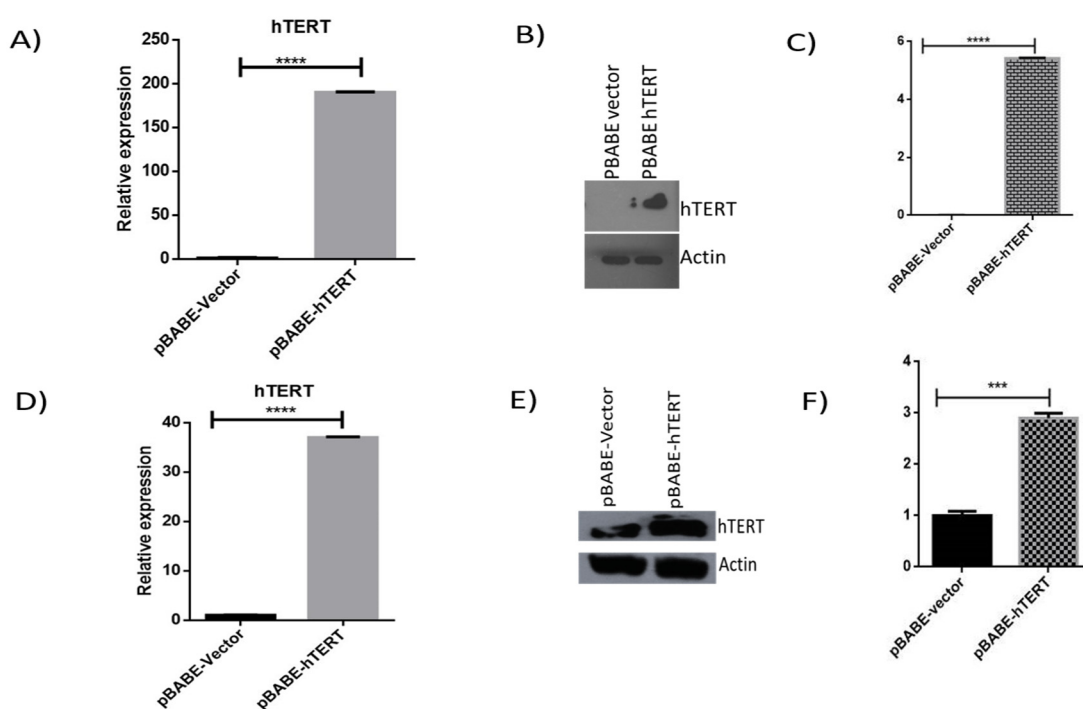
Figure 9: hTERT overexpression in U2OS and HeLa cell lines. hTERT is overexpressed in U2OS and HeLa cell lines. A & D show mRNA level of hTERT in U2OS & HeLa cell lines as determined by quantitative real-time PCR. B & E are Western blots confirming overexpression of hTERT in U2OS and HeLa cell line. C & F are the histograms presenting densitometric quantification of the hTERT overexpression of three corresponding independent Western blot experiments in U2OS and HeLa cell lines.

4.1.1.1 hTERT overexpression leads to the upregulation of urokinase-type plasminogen activator (uPA) in HeLa cell line:

To know the effect of hTERT overexpression on uPA in cancer cells we demonstrated the expression level of uPA in HeLa cells. We found hTERT overexpression enhances the uPA expression at protein level in HeLa cell line [Fig 10A-C]. Anomalously, at mRNA level we observed reduced expression (~60%) of uPA in HeLa cells overexpressing hTERT as compared with control [Fig. 10D].
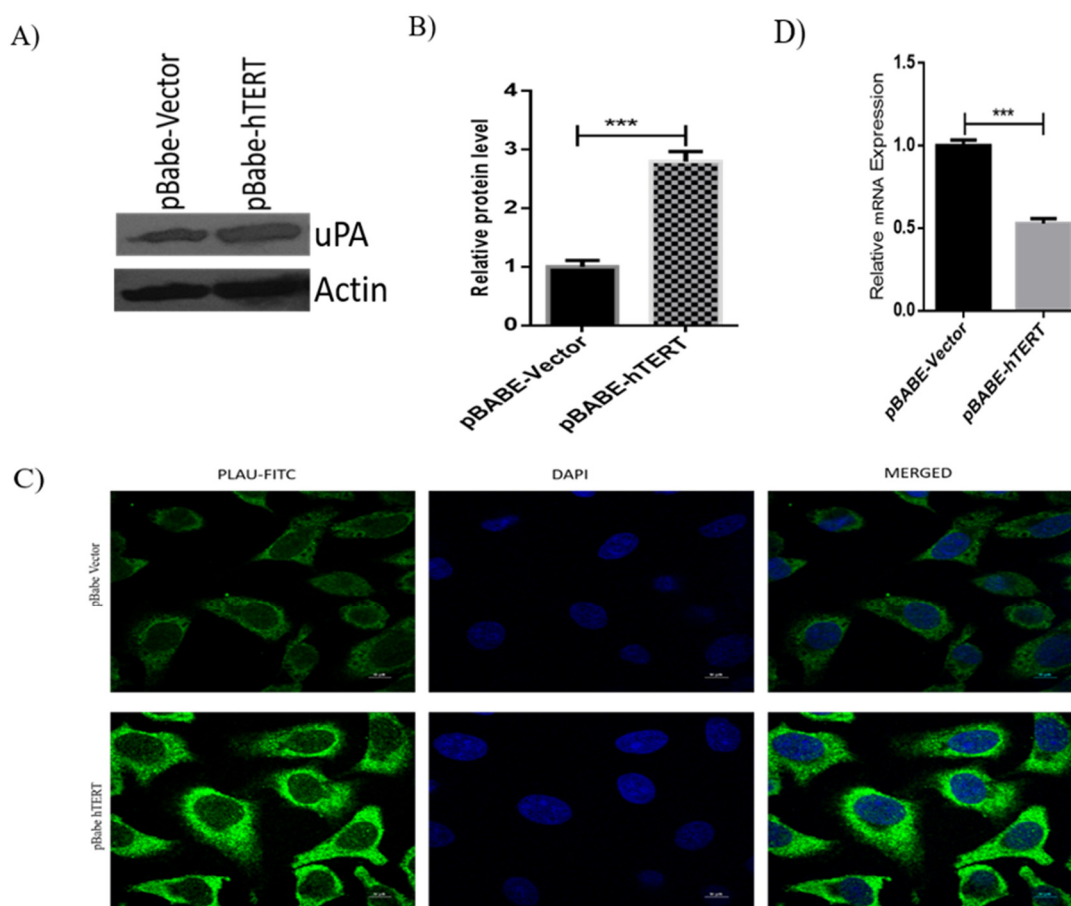
Figure 10: hTERT overexpression enhances the uPA expression in HeLa cells. (A) The hTERT induced upregulation of uPA at the protein level in HeLa cells was confirmed by western blotting. (B) Histogram shows the result by applying ImageJ software. (C) Immunofluorescence also confirmed the induced expression of uPA in HeLa cells. (D) Reduced expression of uPA was observed at mRNA level by qRT-PCR.

4.1.1.2 hTERT overexpression leads to the upregulation of urokinase-type plasminogen activator (uPA) in U2OS cell line:

HeLa is telomerase positive cell line. We have also demonstrated uPA expression in a telomerase negative cell line U2OS after overexpressing hTERT in it. The U2OS cells are telomerase negative osteosarcoma cells. Overexpression of hTERT caused increased expression of uPA at both protein and mRNA level which is confirmed by western blotting, immunofluorescence and qRT-PCR [Fig 11A-D].
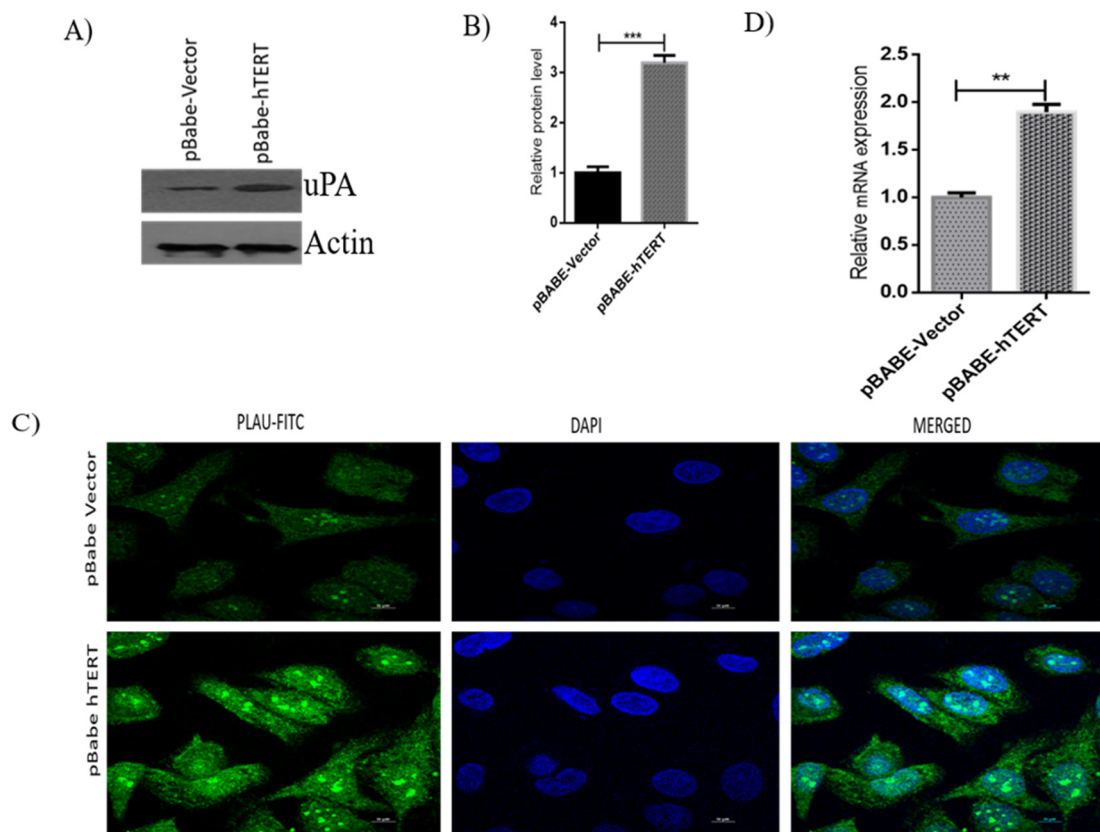
Figure 11: hTERT overexpression elevated the uPA expression in U2OS cells. (A) The hTERT induced upregulation of uPA protein in U2OS cells as confirmed by western blotting (B) Histogram shows the result of western blotting by applying ImageJ software. (C) Immunofluorescence also confirmed the induced expression of uPA in U2OS cells. (D) qRT-PCR also confirmed enhanced expression of uPA at mRNA level.

4.1.1.3 hTERT overexpression enhances the urokinase plasminogen activator receptor (uPAR) expression in HeLa cell line:

We have also checked uPAR expression in hTERT overexpressing HeLa cells. We found significant overexpression of uPAR following ectopic expression of hTERT in HeLa cells. Western blotting, immunofluorescence and qRT-PCR were confirmed upregulation of uPAR in hTERT overexpressing HeLa cells [Fig12A-D].
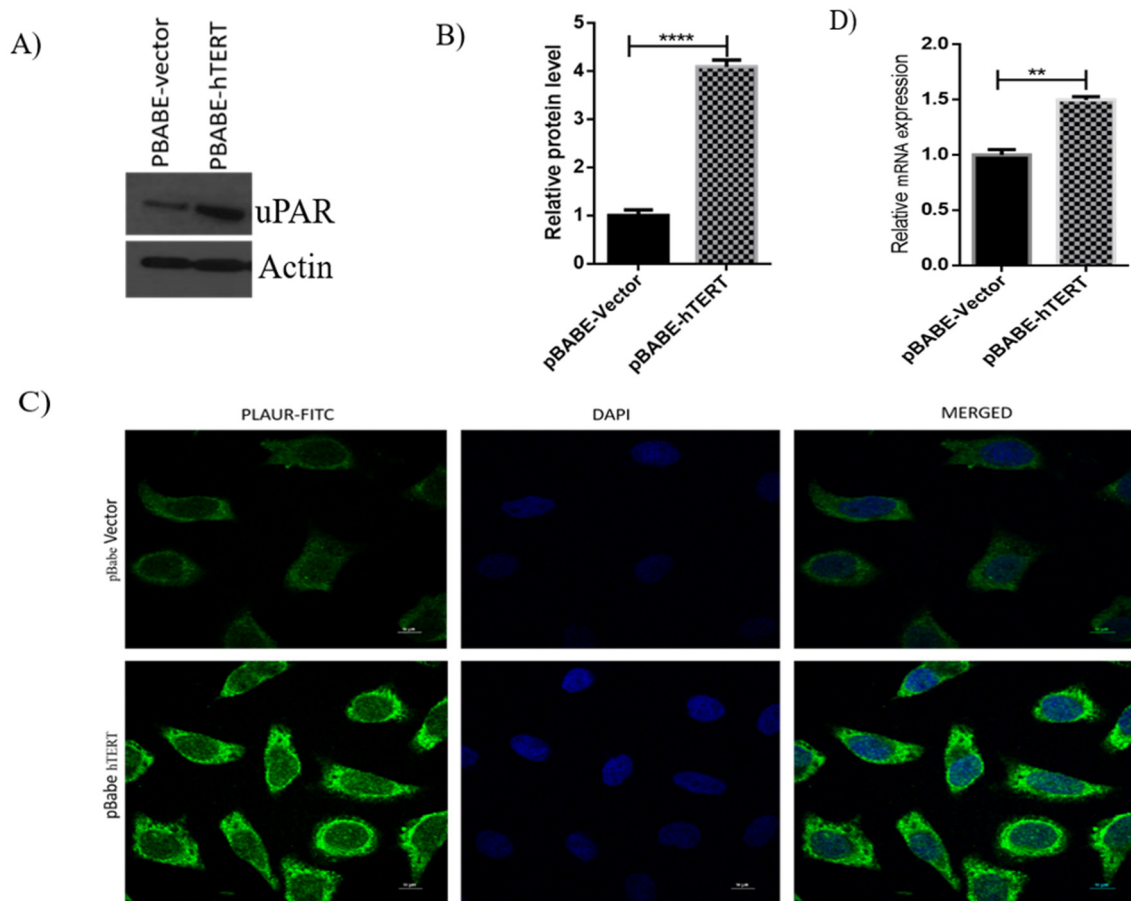
Figure 12: hTERT overexpression enhances the uPAR expression in HeLa cells. The uPAR expression was found upregulated in hTERT overexpressing HeLa cells. uPAR expression at protein and mRNA level in hTERT overexpressing HeLa cells was confirmed by western blotting (A-B), immunofluorescence (C) and qRT-PCR (D).

4.1.1.4 hTERT overexpression leads to the upregulaton of urokinase type plasminogen activator receptor (uPAR) in U2OS cell line:

The U2OS cells are telomerase negative osteosarcoma cells. uPAR expression was also checked in this telomerase negative cell line. We observed that hTERT overexpression led to elevated expression of uPAR in U2OS cells. Upregulation of uPAR in U2OS cells was confirmed by western blotting, immunofluorescence and qRT-PCR [Fig13A-D]. The immunofluorescence images show enhanced expression of uPAR in cytosolic as well as nuclear compartments. The hTERT overexpressing U2OS cells showed 3-fold increase in expression of uPAR both in terms of mRNA as well as protein.
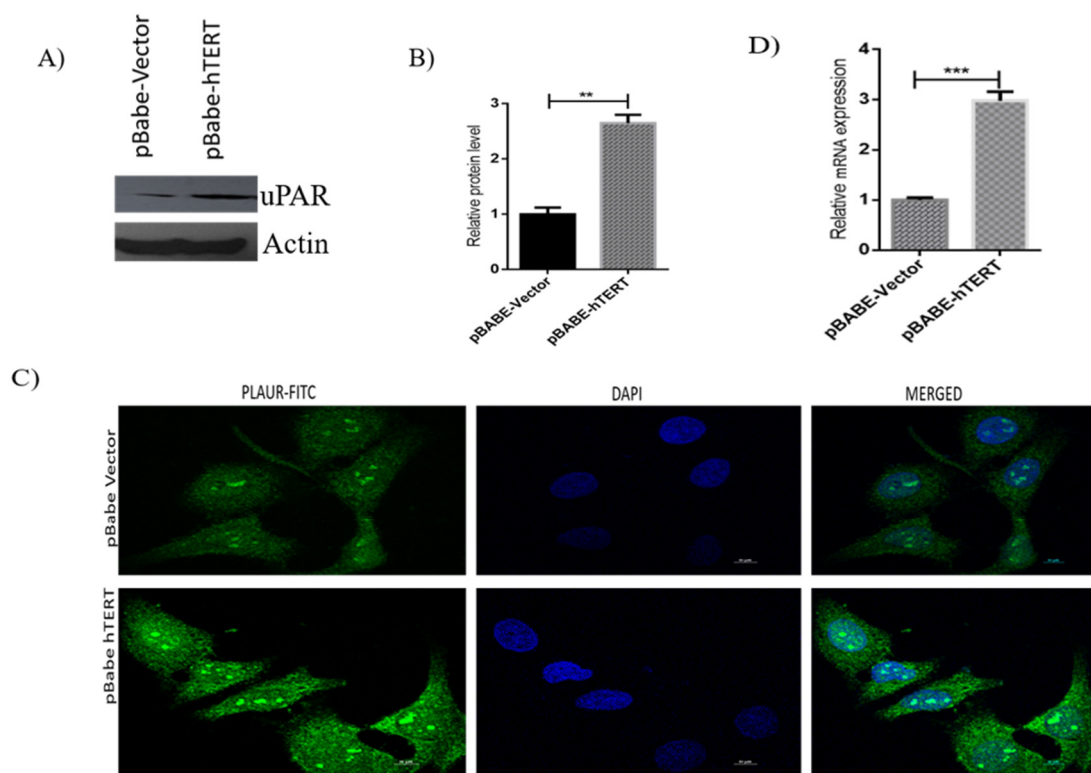
Figure 13: hTERT overexpression elevated the uPAR expression in U2OS cells. To know the effect of hTERT on uPAR in hTERT expressing telomerase negative cells, we have probed for expression of uPAR in U2OS cells ectopically expressing hTERT. (A) Western blotting confirmed that hTERT enhanced uPAR expression at protein level in U2OS cells (B) Histograms depict densitometric quantification of uPAR (C) Immunofluorescence showed the induced expression of uPAR in U2OS cells. (D) qRT-PCR also confirmed enhanced expression of uPAR at mRNA level.

4.1.1.5 hTERT overexpression enhances the mesenchymal character in cancer cells:

It is reported by many researchers that hTERT stimulates expression of mesenchymal property in cancer cells. To confirm this we have checked the expression level of some epithelial and mesenchymal markers in both hTERT expressing HeLa and U2OS cells.

4.1.1.5.1 hTERT overexpression led to increased expression of mesenchymal markers in HeLa cells:

Expression of mesenchymal markers was checked in hTERT expressing HeLa cells. It is found that hTERT overexpression in HeLa cells increases the expression of mesenchymal markers vimentin [Fig14A-D] and β-catenin [Fig15A-B].
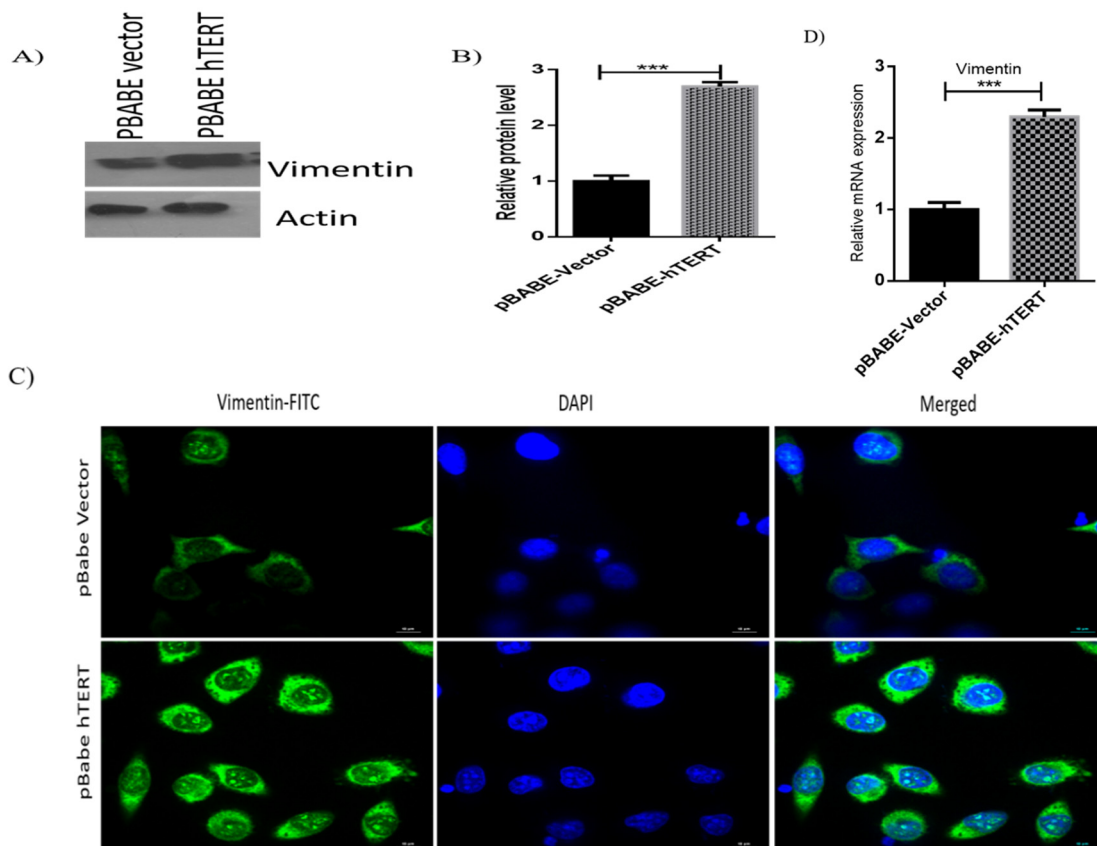
Figure 14: hTERT overexpression resulted in increased expression of vimentin in HeLa cells. Vimentin is a mesenchymal and frequently used by researchers to check whether EMT is induced or not. We have also checked its expression in HeLa cells ectopically expressing hTERT. To confirm the upregulation we performed western blotting (A-B), immuno-fluorescence (C) and qRT-PCR (D).
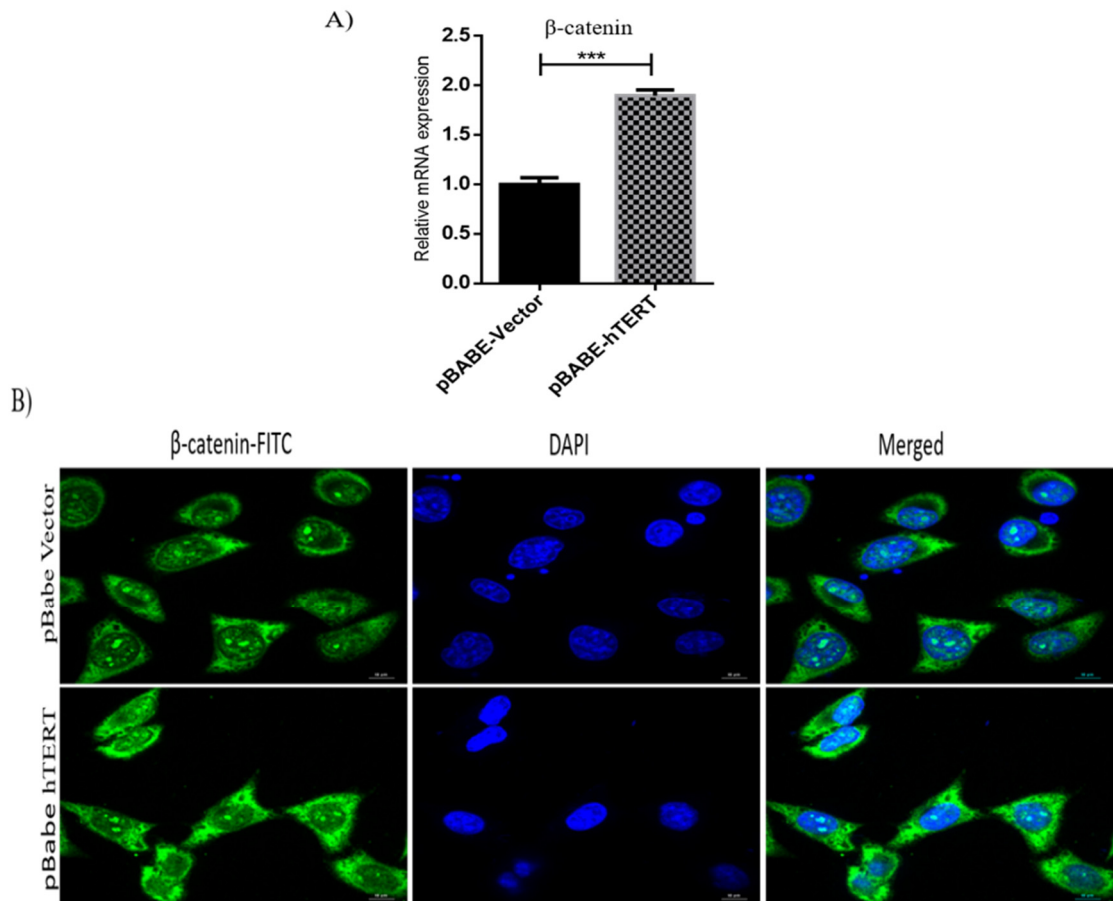
Figure 15: hTERT overexpression increased the expression of the mesenchymal marker β-catenin in HeLa cells. Expression of β-catenin was also checked by (A) qRT-PCR and (B) immunofluorescence in HeLa cells ectopically expressing hTERT. Similar to vimentin, expression level of β-catenin was also upregulated.

4.1.1.5.2 hTERT overexpression results in the downregualtion of epithelial markers in HeLa cells:

E-cadherin in the cell is required for tight association with neighboring cells. It is also frequently used as an epithelial marker. An assay of E-cadherin expression in hTERT expressing HeLa cells showed reduced expression of E-cadherin to <50% [Fig16A-B].
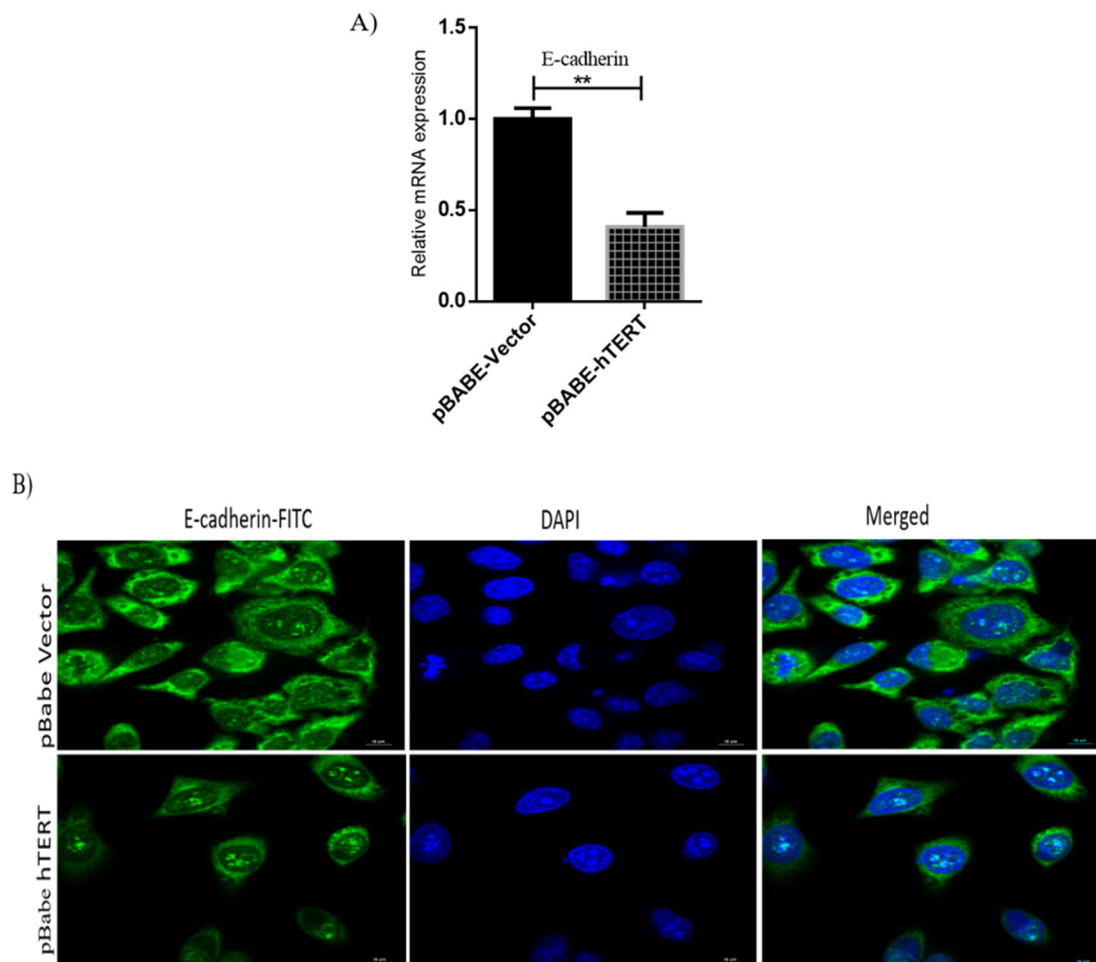
Figure 16: hTERT overexpression reduced the expression of epithelial marker in HeLa cells. The hTERT mediated downregulation of E-cadherin was confirmed by (A) (qRT-PCR) and (B) immunofluorescence.

4.1.1.5.3 hTERT overexpression increased the expression of mesenchymal markers in U2OS cells:

Similar to HeLa cells we have also assayed expression of mesenchymal markers in hTERT expressing U2OS cells. It was observed that hTERT overexpression in U2OS cells increased the expression of mesenchymal markers vimentin [Fig17A-D] and β-catenin [Fig18A-B].
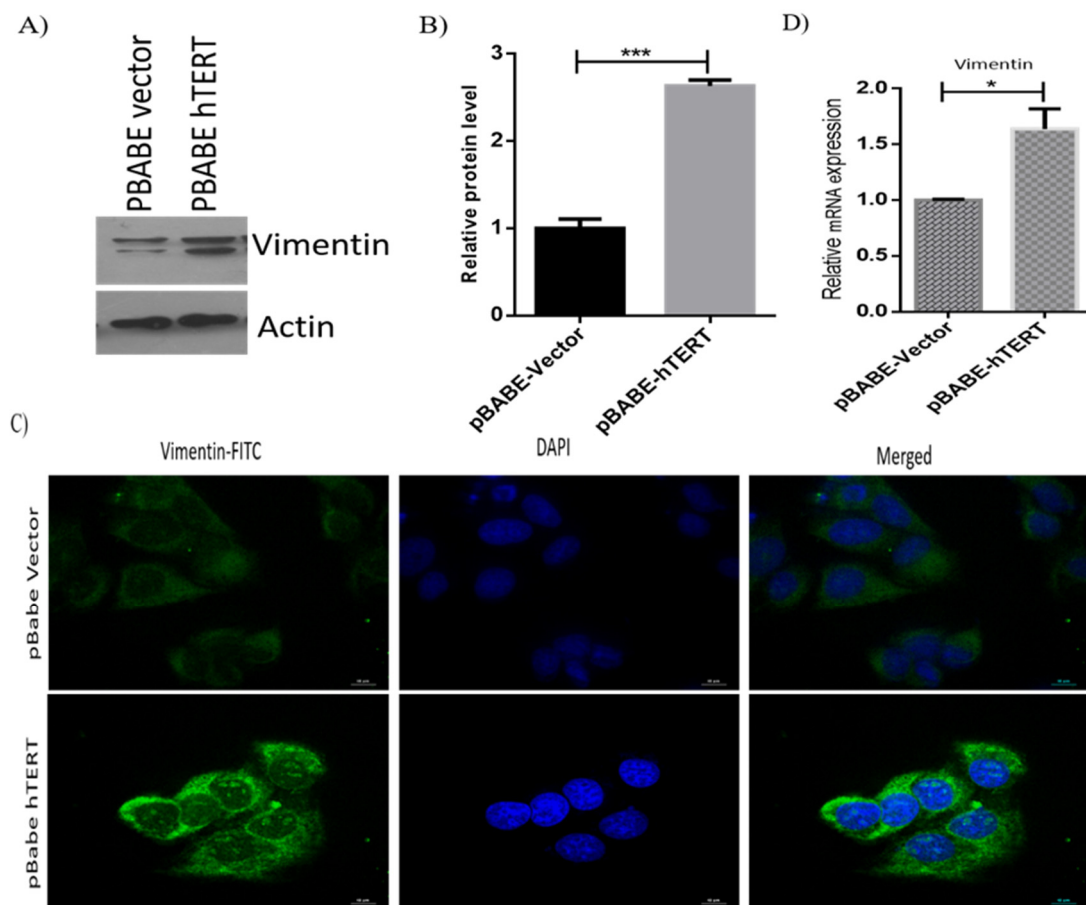
Figure 17: hTERT overexpression led to increased expression of vimentin in U2OS cells. Vimentin is a mesenchymal marker reflecting on EMT. We have also checked its expression in U2OS cells ectopically expressing hTERT. To confirm the upregulation we performed western blotting (A-B), immunofluorescence (C) and qRT-PCR (D).

A)

B)
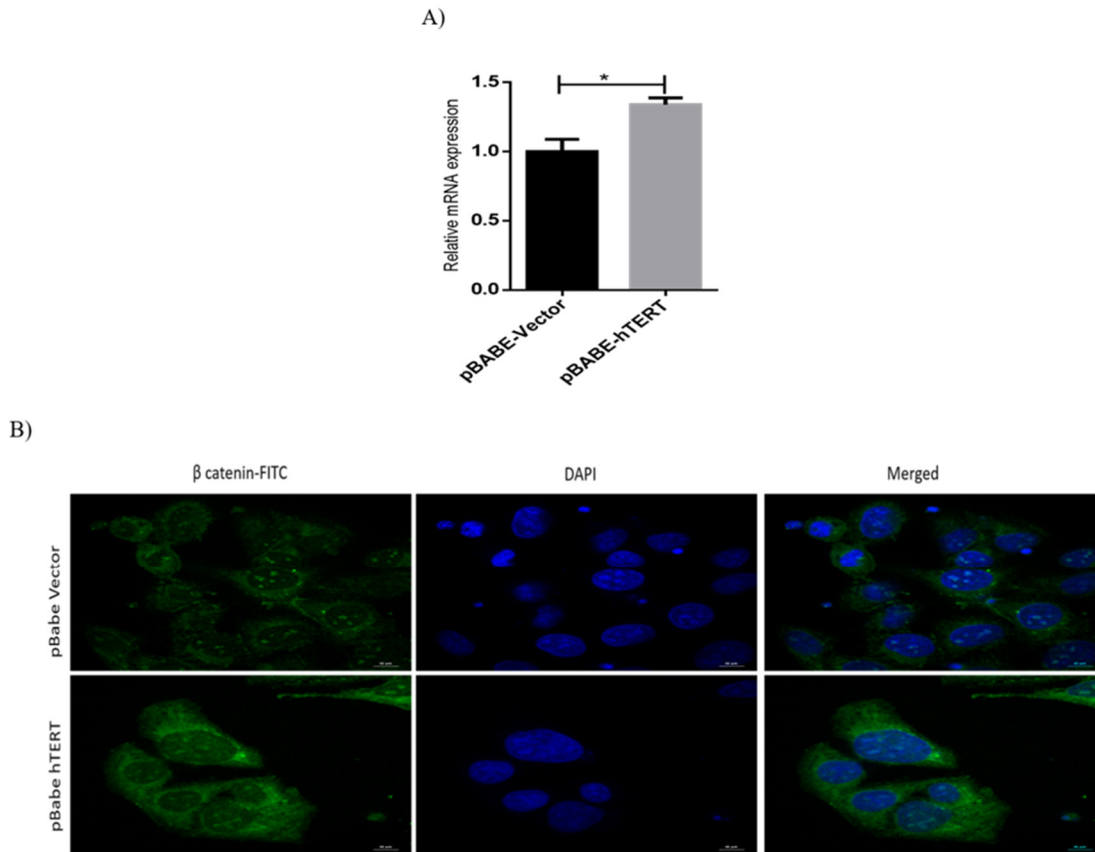
Figure 18: hTERT overexpression increased the expression of β-catenin in U2OS cells. β-catenin expression level was also determined by (A) qRT-PCR and (B) immunofluorescence. Similar to vimentin, expression level of β-catenin was also found upregulated in U2OS cells ectopically expressing hTERT.

4.1.1.5.4 hTERT overexpression leads to the downregulation of epithelial markers in U2OS cells:

Similar to HeLa cells we have also determined E-cadherin expression level in U2OS cells having overexpression of hTERT. We have performed qRT-PCR and immunofluorescence and found that overexpression of hTERT drastically reduced the expression (~10% in terms of mRNA)) of E-cadherin as compared to control [Fig19A-B].
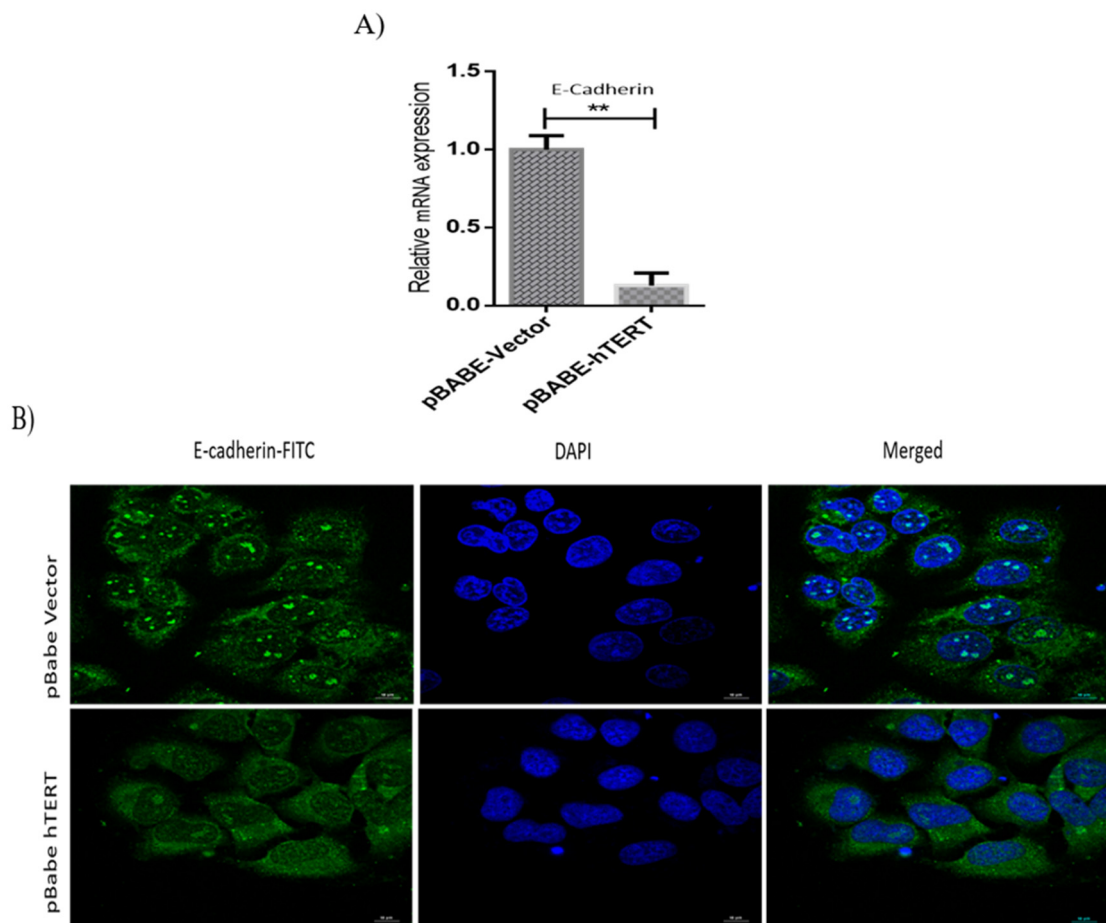
Figure 19: hTERT overexpression reduced the expression of E-cadherin in U2OS cells. (A) qRT-PCR and (B) immunofluorescence revealed that overexpression of hTERT resulted in decreased expression of E-cadherin in U2OS cells.

4.1.1.6 Knocking down of hTERT in HeLa cell line:

To confirm the association of EMT markers with hTERT, as revealed by overexpressing the latter in cells, we repeated reciprocal inquiry about the effect of dowregulating hTERT on expression of uPA and uPAR. Two micrograms each of pMKO.1-puro empty vector and pMKO.1-shRNA- hTERT were transfected in to HeLa cells by using lipofectamine 3000 (Invitrogen). After 48hrs, transfected cells were selected by using 2μg/ml of puromycin and finally maintained in 1μg/ml of puromycin. Western blotting and qRT-PCR were performed to check knockdown of hTERT [Fig20A-C].
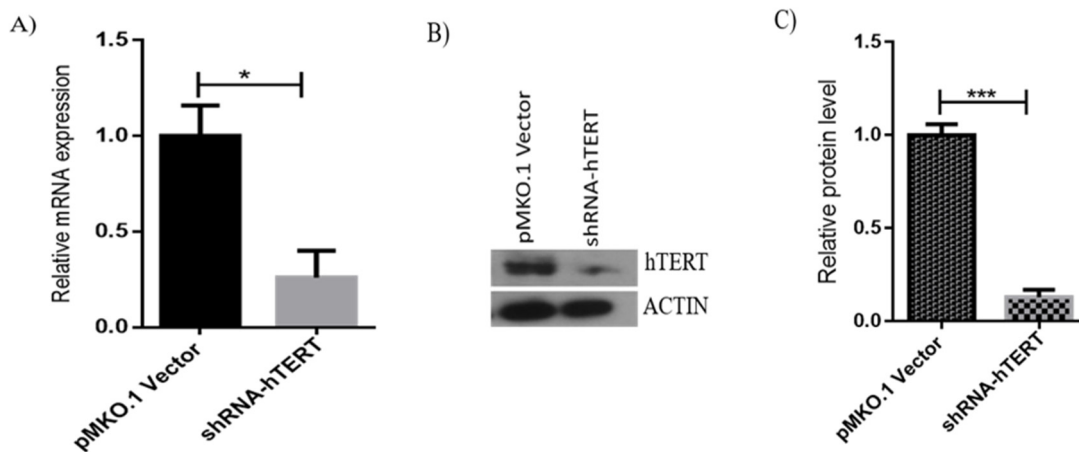
Figure 20: Validation of hTERT knockdown in HeLa cells. Knockdown of hTERT in shRNA-hTERT transfected Hela cells was confirmed by (A) qRT-PCR which showed around 70% knockdown of hTERT, (B) Western blotting also confirmed hTERT knockdown. (C) Histogram representing quantification of western blot.

4.1.1.6.1 hTERT knockdown led to repress of uPA expression in cancer cells:

A positive association between hTERT and uPA was revealed following over expression of hTERT as presented in preceding sections. So to further confirm this we checked uPA expression in cells knocked down for hTERT by cognate shRNA. hTERT knockdown concurrently reduced the uPA expression at protein level as confirmed by western blot and immunofluorescence [Fig21A-C] while anomalously we found enhanced expression of uPA at mRNA level in hTERT knockdown HeLa cells [Fig21D].
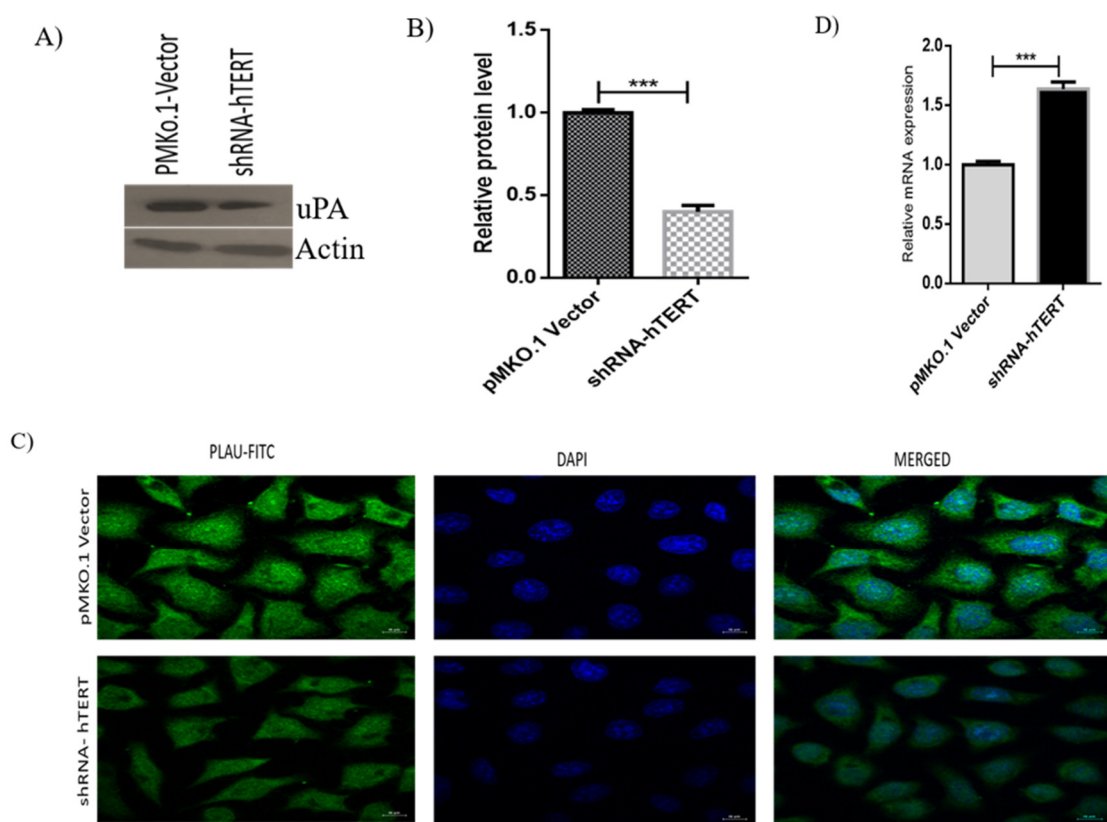
Figure 21: Knockingdown of the hTERT leads to downregulation uPA in cancer cells. (A-B) Western blotting showed reduced expression of uPA at protein level in hTERT knockdown cells. (C) Immunofluorescence also confirmed downregulation of uPA (D) at mRNA level (assayed by realtime PCR) we found enhanced expression of uPA in hTERT knockdown cells.

4.1.1.6.2 hTERT knockdown also reduced the uPAR expression in cancer cells:

The uPAR expression level was also assayed in hTERT knockdown cells. We found decreased expression of uPAR in hTERT knockdown cells in terms of protein as well as mRNA (Fig22A-D].
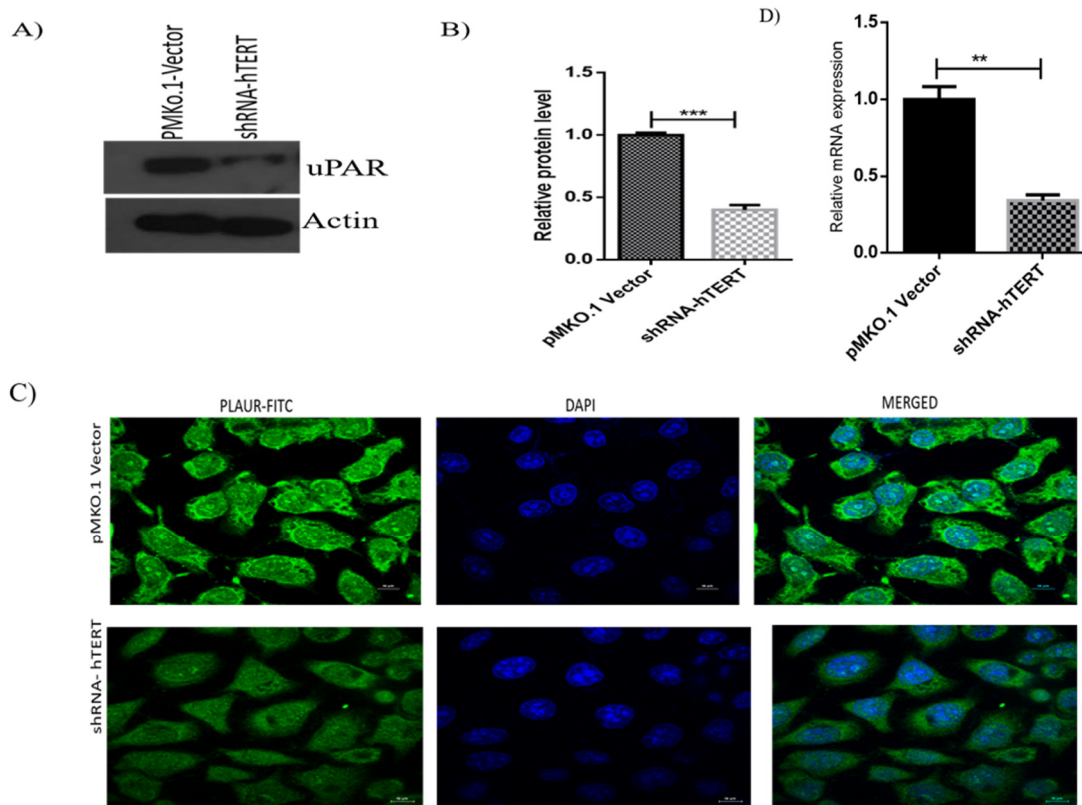
Figure 22: Knockingdown of the hTERT leads to downregulation uPAR in cancer cells. (A-B) Western blotting showed repressed expression of uPAR at protein level in hTERT knockdown cells. (C) Immunofluorescence and (D) qRT-PCR also confirmed downregulation of uPAR in cells in hTERT knockdown cells.

4.1.1.6.3 hTERT knockdown reduces the expression of Vimentin and enhances the expression of E-cadherin in HeLa cells:

Expression of epithelial and mesenchymal markers were also checked in HeLa cells knocked down for hTERT. hTERT knock down led to decreased expression of msenchymal markers in cancer cell with the level of vimentin going down whereas expression of E-cadherin, an epithelial marker, increased [Fig23A-C].
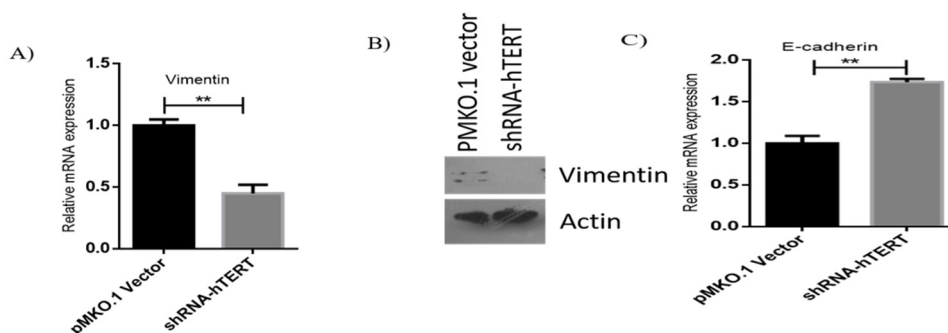
Figure 23: hTERT downregulation led to reduced expression of vimentin and enhanced expression of E-cadherin. (A-B) qRT-PCR and western blot were performed to demonstrate the level of vimentin in hTERT knockdown cells. (C) E-cadherin expression was also checked by qRT-PCR.

4.1.1.7 uPA knockdown in HeLa cells:

pLKO.1-puro empty vector, pLKO.1-puro-uPA-shRNA, pSPAX and pMD2G were transfected in to 293T cells with the help of lipofectamine 3000 (Invitrogen). After 48hrs, cells were selected for resistance to 2μg/ml of puromycin and after 7 days virus particles were collected. 600 μl of this virus particle were again transfected in HeLa cells and after 48hrs, cells were selected for resistance to puromycin. Real-time PCR and western blotting confirmed up to 70% knock down of uPA [Fig 24A-C].
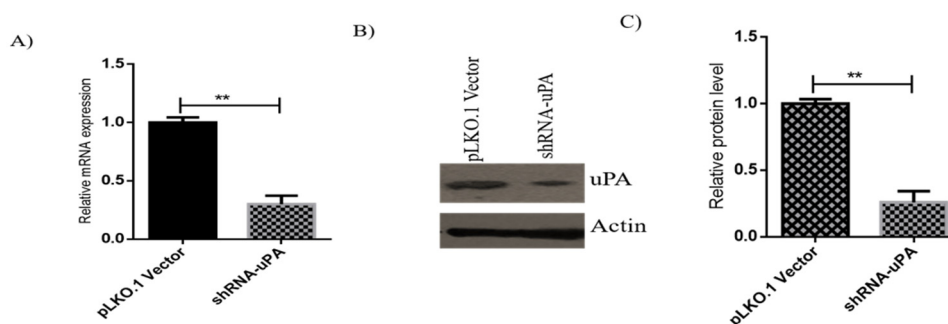
Figure 24: Validation of uPA knockdown in HeLa cells. To validate the knockdown of uPA in shRNA-uPA transfected Hela cells we performed (A) qRT-PCR which showed around 70% knockdown of uPA. Results of western blotting shown in B & C also confirmed uPA knockdown in HeLa cells.

4.1.1.7.1 Knocking down of urokinase plasminogen activator leads to the downregulation of hTERT in HeLa cell line: When studying the effect of knocking down uPA on the expression level of hTERT, we found there is substantial (>70%) downregulation of hTERT both in terms of protein as well as RNA. This clearly suggested that there is positive association of expression of these two molecules in HeLa cells [Fig 25 A-C].
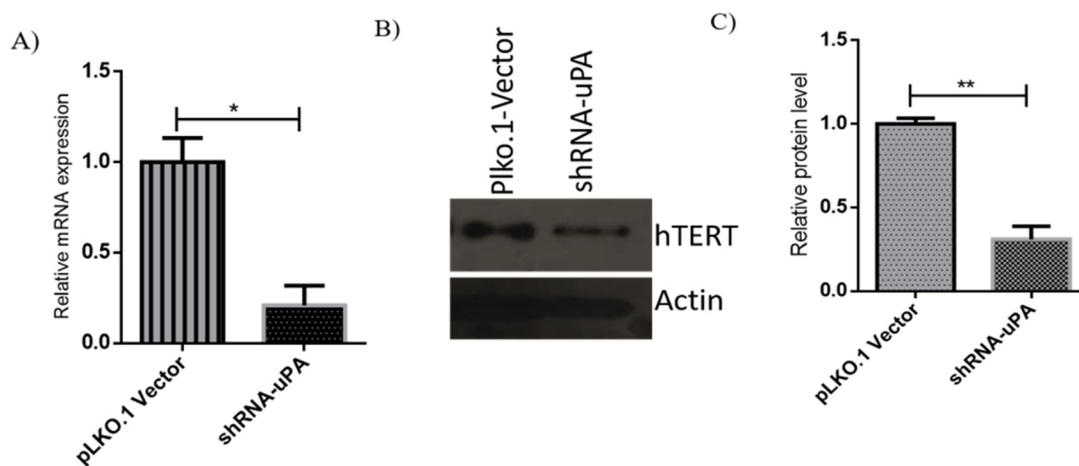
Figure 25: Downregulation of uPA causes reduced expression of hTERT. (A) qRT-PCR was performed to assay mRNA of hTERT in uPA knockdown cells. (B-C) Western blot also confirmed the uPA knockdown lowers the expression of hTERT.

4.1.1.8 Urokinase type plasminogen activator and TGF-Beta:

Till now we found that hTERT regulates uPA expression in cancer cells but the molecular mechanism by which hTERT regulates the expression of plasminogen activator was not known. TGF-β is a well known inducer of EMT while hTERT enhances EMT via TGF-β regulated Wnt/ β-catenin pathway. TGF-β treatment to the cells leads to the binding of β-catenin and hTERT and they further bind with other transcription factors like BRG1. The β-catenin/hTERT/BRG1 complex moves into the nucleus where they bind and stimulate β-catenin responsive genes. There are many β-catenin target genes e.g., Axin 2, LEF1, WNT4 and some EMT markers like vimentin and snail. There are many extracellular proteases which are secreted at the time of cancer metastasis, and are significantly involved in cancer invasion. Matrix metalloproteinase-2 (MMP-2) and urokinase-type plasminogen activator (u-PA) are the

key proteolytic enzymes with a crucial role in invasion and angiogenesis of tumor cells. TGF-beta treatment leads to the upregulation of uPA in cells. The uPA is also a target of wnt/β-catenin pathway. So on the basis of this, we hypothesize that hTERT upregulates the expression uPA through TGF-beta mediated pathway.

4.1.1.8.1 TGF-beta treatment leads to mesenchymal like morphology of cancer cells:

TGF-β is an inducer of EMT. When EMT is induced, epithelial morphology changed into elongated shape, fibro-blastoid morphological features in cells. We treated A549 cells with 5ng/ml of TGF-β and after 24 hours we examined cells under microscope. We found that as compared to control TGF-β treated cell were more elongated and displaying fibroblast-like appearance [Fig 26].
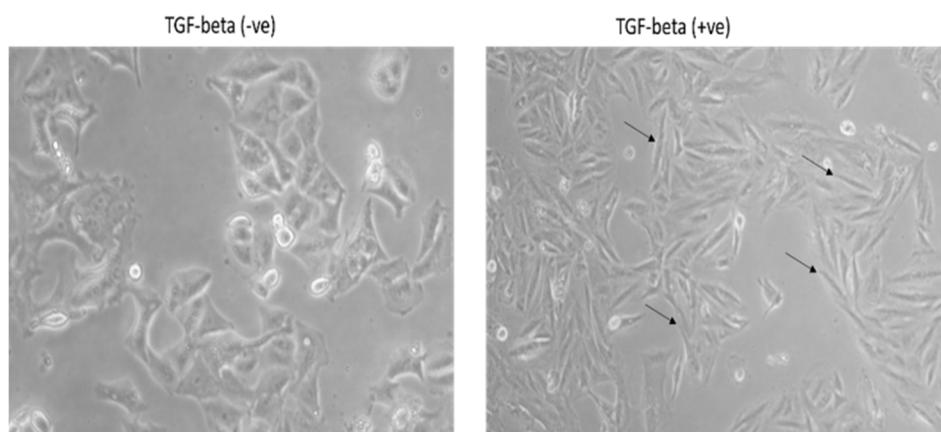
Figure 26: TGF-β treatment induces EMT in cancer cells. Phase contrast images of A549 cells with and without TGF-β treatment. A549 cells under TGF-β treatment gain a spindle-type morphology.

4.1.1.8.2 uPA is a TGF-β inducible protein:

A549 cells were treated with 5ng/ml of TGF-β for different time periods and expression of uPA was assayed. It was found that TGF-β treatment enhanced the expression of uPA in A549 cells. After TGF-β induction uPA expression pattern was similar to that of vimentin which is a very well established early mesenchymal marker [Fig 27A-E]. Furthermore, in our lab it is established that the effect of TGF-β peaks at 24 hrs. We checked uPA expression after TGF-β treatment at different time point and found similar

result [Fig 27A-E]. Expression of uPA after TGF-β treatment was also checked by immunofluorescence.
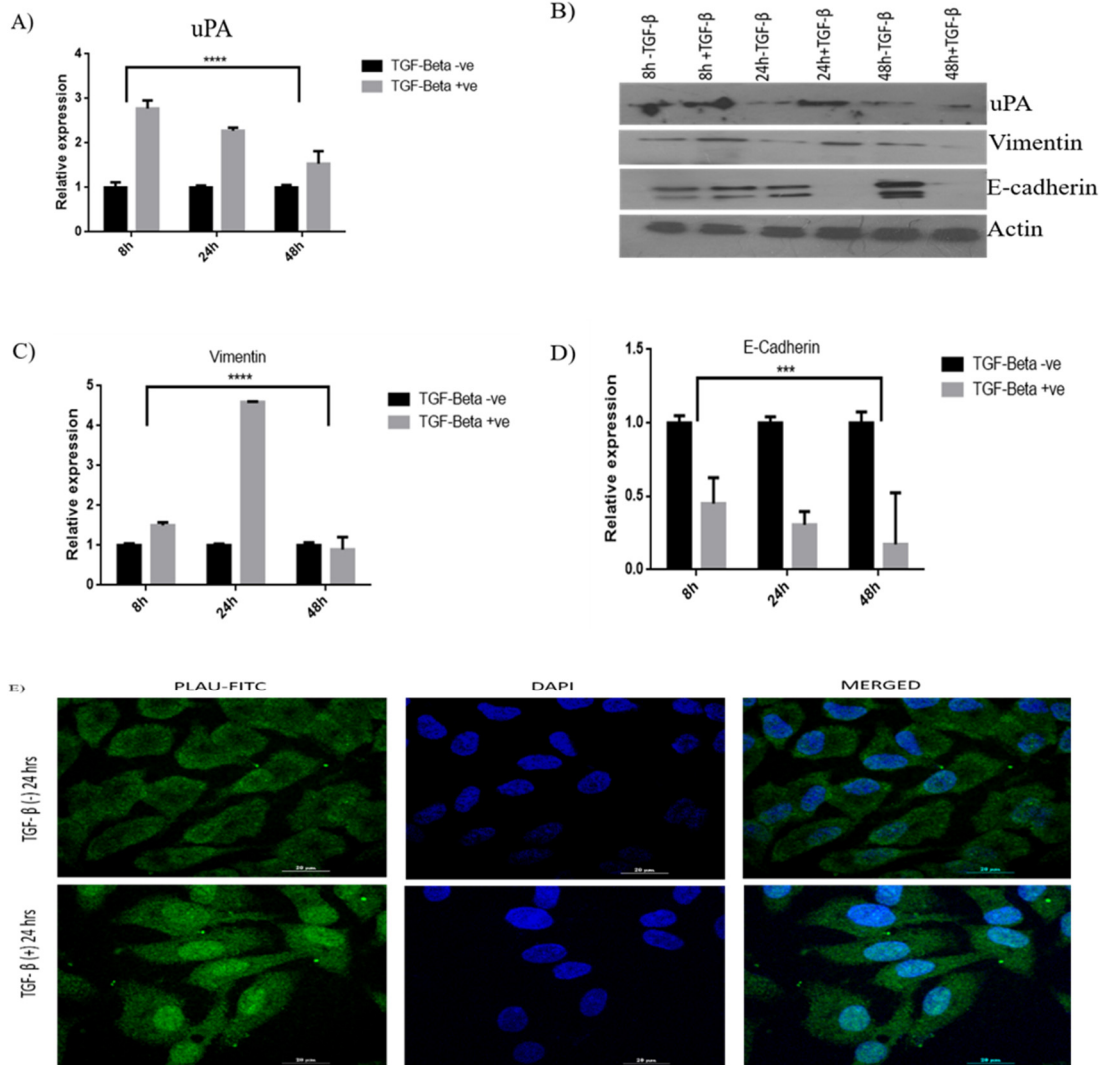
Figure 27: TGF-β treatment leads to the upregulation of uPA. (A) qRT-PCR confirmed upregulation of uPA after TGF-β treatment at different time points. (B) Western blotting was performed to check the expression of uPA, vimentin and E-cadherin. After TGF-β treatment uPA expression pattern was similar to that of vimentin which is a very well established early mesenchymal marker while E-cadherin level went down indicating reduction in epithelial property. (C-D) qRT-PCR was also performed to check the expression of vimentin and E-cadherin at mRNA level. (E) Immunofluorescence also confirmed upregulation of uPA after TGF-β treatment.

4.1.1.8.3 TGF-β induced uPA expression is hTERT mediated:

We were now interested to see whether TGF-β induced uPA expression is hTERT dependent. We treated hTERT knockdown cells with 5ng/ml of TGF-β. Compared to vector transfected cells hTERT knockdown cells are unable to induce uPA expression after TGF-β treatment. Vimentin is an early mesenchymal marker and TGF-β treatment enhanced its expression in cancer cells. Earlier it was found that after TGF-β induction uPA expression pattern was similar to that of vimentin. When hTERT was knocked down both uPA and vimentin expression went down. We have also observed upregulation of E-cadherin indicating EMT is reversed [Fig 28 A-D].
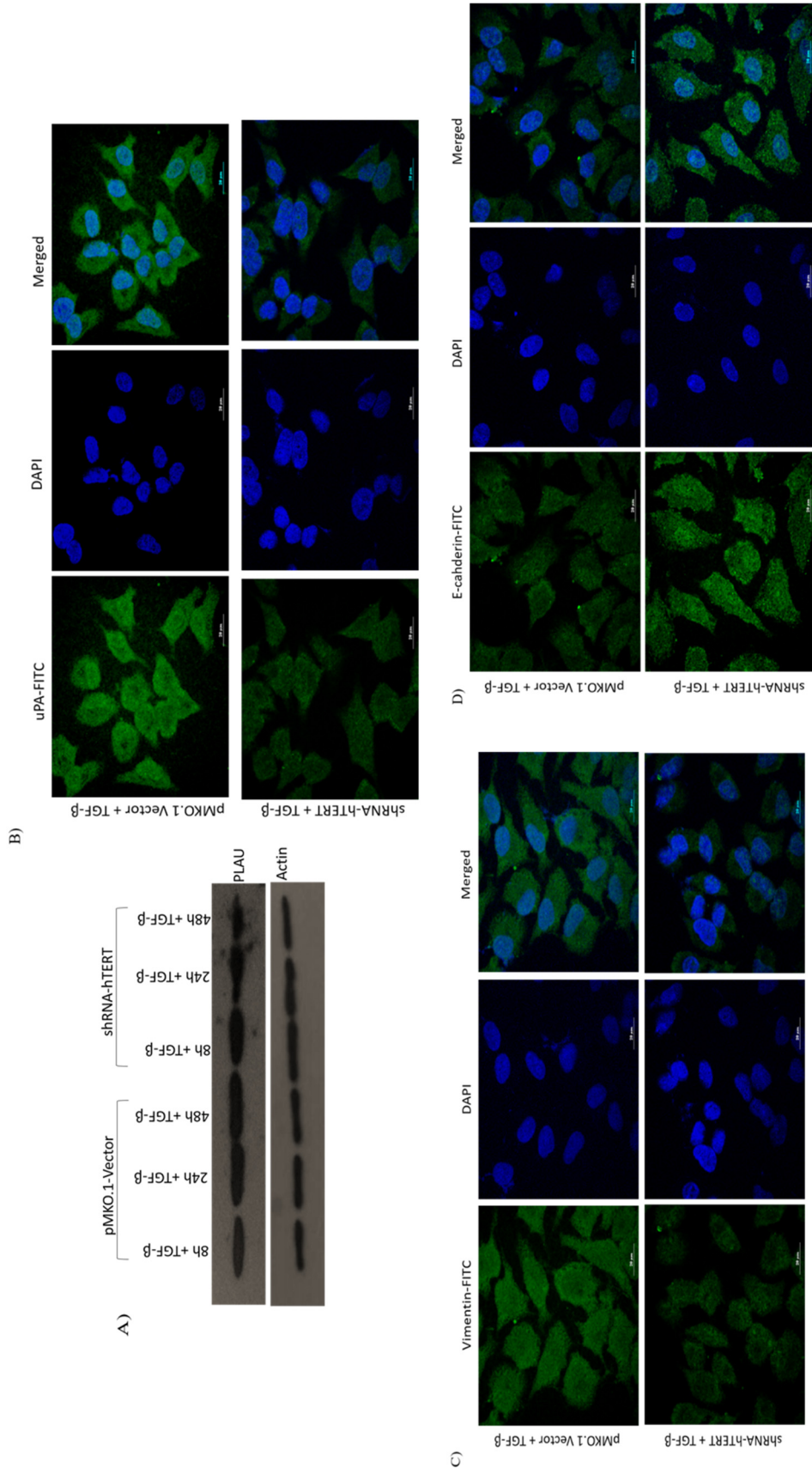
Figure 28: TGF-β induced uPA expression seems to dependent on the status of hTERT (A) Vector transfected and hTERT knockdown A549 cells were treated with 5ng/ml of TGF-β and western blotting was performed to check uPA expression. TGF-β was unable to induce uPA expression in hTERT knockdown cells. (B) Immunofluorescence also confirmed the above result that TGF-β mediated induction of uPA was also repressed by knocking down hTERT. (C) Interestingly expression level of vimentin was also suppressed after TGF-β treatment in hTERT knockdown cells. (D) Enhanced expression of E-cadherin was observed in TGF-β treated hTERT knockdown cells.

4.1.1.8.4 Cell migration property of hTERT is uPA dependent:

The uPA is a protease and its role in cell migration is very well established. As shown above, TGF-β mediated upregulation of uPA seems to be hTERT dependent. Hence and it was a good idea to check migratory properties of hTERT knockdown cells treated or not treated with TGF-β. We performed wound healing assay in A549 cells, A549 cells treated with TGF-β, hTERT knockdown cells and hTERT knockdown cells treated with TGF-β. We checked the effect of TGF-β on cell migration by treating A549 cells with 5ng/ml of TGF-β. We found that A549 cells treated with TGF-β have higher migration potential in comparison to A549 cells only. We also treated hTERT knockdown A549 cells with TGF-β and found that TGF-β treatment in hTERT knockdown A549 cells does not enhance migration [Fig 29].
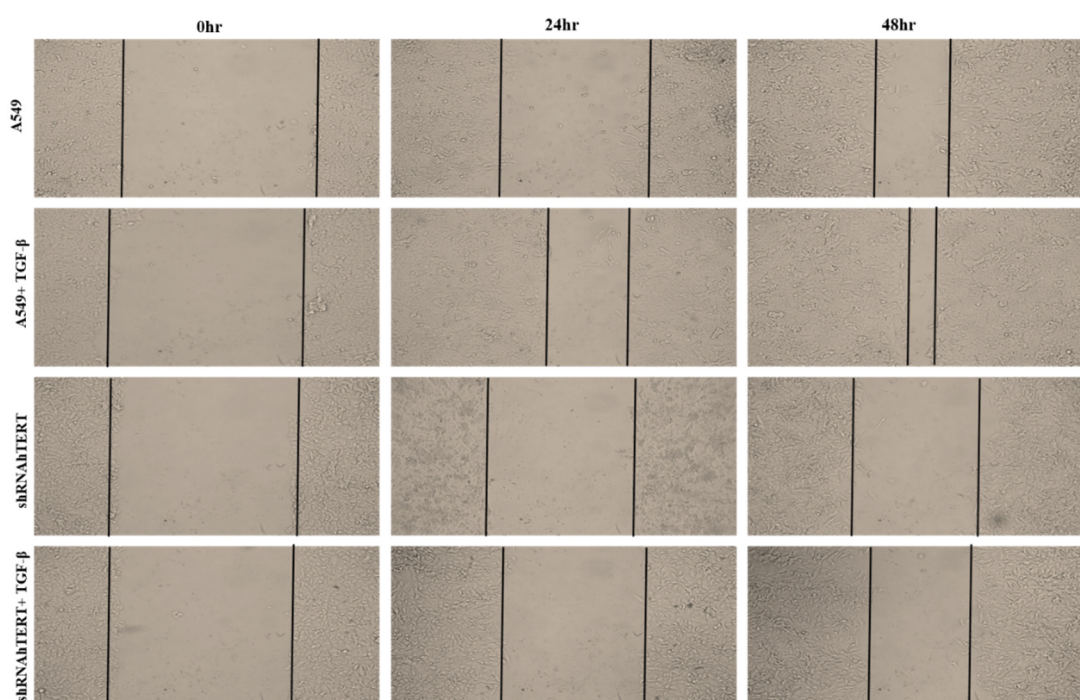
Figure 29: The uPA helps in cell migration. To check the involvement of uPA in hTERT mediated cell migration wound healing assay was performed in A549 cells, A549 cells treated with TGF-β, hTERT knockdown A549 cells and hTERT knockdown cells treated with TGF-β. TGF- β does not induce mobility in hTERT knockdown cells.

4.1.1.9 Discussion: hTERT regulate the expression of plasminogen activators in cancer cells:

Intactness of telomeres is a necessary basis for licensing a cell to proceed with genome replication and cell division. Thus telomerase and other telomere-associated proteins

occupy pivotal position in qualifying the cells for proceeding along the cell cycle andperform other cellular functions. It is only expected that such molecules would beeffective in regulating cellular phenomena. Lieu et al. (2012) reported a potential roleof hTERT in EMT. They have shown that hTERT promotes EMT and stem cell liketraits in gastric cancer cells when hTERT is ectopically expressed in these cells [4].TGF-β is a well-known inducer of EMT, but down-regulation of hTERT by siRNAinhibited the TGF-β and β-actenin mediated EMT [4]. The role of uPA in EMT is alsovery well established [363]. We have already published our transcriptomics datashowing regulation of uPA by hTERT [6]. But the molecular mechanism behind thiswas not elucidated. Therefore here we tried to explore the exact association betweenhTERT and uPA system as well as molecular mechanism by which hTERT regulatesuPA expression. The hTERT overexpression in HeLa cells enhanced the expression ofuPA at protein level but reduced uPA expression at RNA level [Fig 10A-D].Interestingly uPAR expression at both protein and RNA level was enhanced in HeLacells ectopically expressing hTERT [Fig 12A-D]. Moreover, uPA and uPAR expressionwere also upregulated in U2OS (a telomerase negative cells) cells [Fig11A-D & 13A-D]. The hTERT knockdown repressed the uPA and uPAR expression in HeLa cells [Fig21A-D & 22A-D]. Interestingly uPA knock down reduced the hTERT expression in HeLa cells. Collectively the results confirmed a positive association between hTERT and uPA system. Both hTERT and uPA are pro-survival for cancer cells. Moreover, we have already mentioned about TGF-β/wnt-β-catenin mediated EMT is hTERT dependent and uPA is a TGF-β/ wnt-β-catenin responsive gene. Here these two pro-survival proteins were correlated. We observed upregulation of uPA after TGF-β treatment in A549 cells [Fig27A-D). However uPA upregulation was repressed when hTERT was knocked down indicating that TGF-β mediated upregulation of uPA is hTERT dependent (Fig28A-D). Vimentin and E-cadherin are the EMT markers frequently used by many researchers to check whether EMT is induced or repressed [138]. The expression of vimentin and uPA in cancer cells undergoing EMT remains high. We found uPA expression pattern was similar to vimentin which was expected. E-cadherin expression repressed when A549 cells treated with TGF-β while hTERT knockdown reversed TGF-β effect on E-cadherin expression. The role of uPA in cell migration and invasion are very well demonstrated [8]. It is a protease which degrades ECM and increases migration of the cancer cells. TGF-β enhanced cell migration in

cancer cells [422]. We found that TGF-β treatment to A549 cells enhanced its migration in comparison to untreated A549 cells [Fig29]. We also observed that hTERT knockdown reduced the migration potential of the cells [Fig29]. But when we repressed hTERT expression in A549 cells TGF-β treatment becomes ineffective in enhancing cell moveability migration. This may be due to lower uPA expression when hTERT was knocked down in cancer cells.

Our findings revealed a unique adaptation of cancer cells where both hTERT and uPA expression remain high for cancer cell progression. We have also shown that hTERT regulates the expression of urokinase type plasminogen activator and its receptor in both telomerase positive and telomerase negative cell lines. We have also established that TGF-β mediated induction of Urokinase type plasminogen activator is hTERTdependent.

Objective 2: Identification of molecules regulated by hTERT.

4.2 Background:

Cancer cells have unlimited proliferation potential. One way of acquiring this involves reactivation of a specialized reverse transcriptase called telomerase which solves the end replication problem by adding telomeric repeats on to the 3' ends of template strands so as to minimize on attrition of the lagging strands at their terminal 5' ends. Telomerase activity is found to be high in nearly 90% of cancerous cells as compared to normal differentiated somatic cells which do not have detectable telomerase activity. The telomerase basically consists of six main subunits viz. hTERT (human telomerase reverse transcriptase), dyskerin, p23, Hsp90, hTERC (human telomerase RNA component) and TEP1 (telomerase-associated protein 1) [3]. Out of these six subunits, hTERT and hTERC can reconstitute the classical telomere lengthening in vitro and also perform many extracurricular functions of regulatory nature in vivo [2]. Stabilization of telomere length of fibroblast and other cell types is achieved by ectopic expression of hTERT in these cell lines which thus acquire infinite replicative potential [82]. Immortalization of both cancer cells and normal stem cells can be achieved by overexpression of telomerase [423–425]. Moreover, knowing the main roles of telomerase in cancer cells would be helpful in the development of exact therapeutic strategies on the basis of telomerase inhibition [426,427]. Here, we have generated proteomic profile of cells following hTERT knockdown and overexpression in HeLa, a cervical cancer cell line that has its own telomerase activity. We have also viewed the effect of expressing hTERT in a telomerase negative cell line viz., the human osteosarcoma cell line U2OS.

Results:

4.2.1 Overexpression of hTERT in U2OS and HeLa cell line: Overexpression of hTERT in HeLa and U2OS cells was confirmed by qRT-PCR and western blotting. Remarkably, hTERT mRNA expression was upregulated to approximately 180 fold in U2OS cells transfected with pBABE-puro-hTERT in comparison to vector transfected cells [Fig 30A] while in HeLa it is upregulated to 36 fold [Fig 30C). Overexpression of hTERT in U2OS and HeLa cell lines was further confirmed by western blotting [Fig 30B-D).
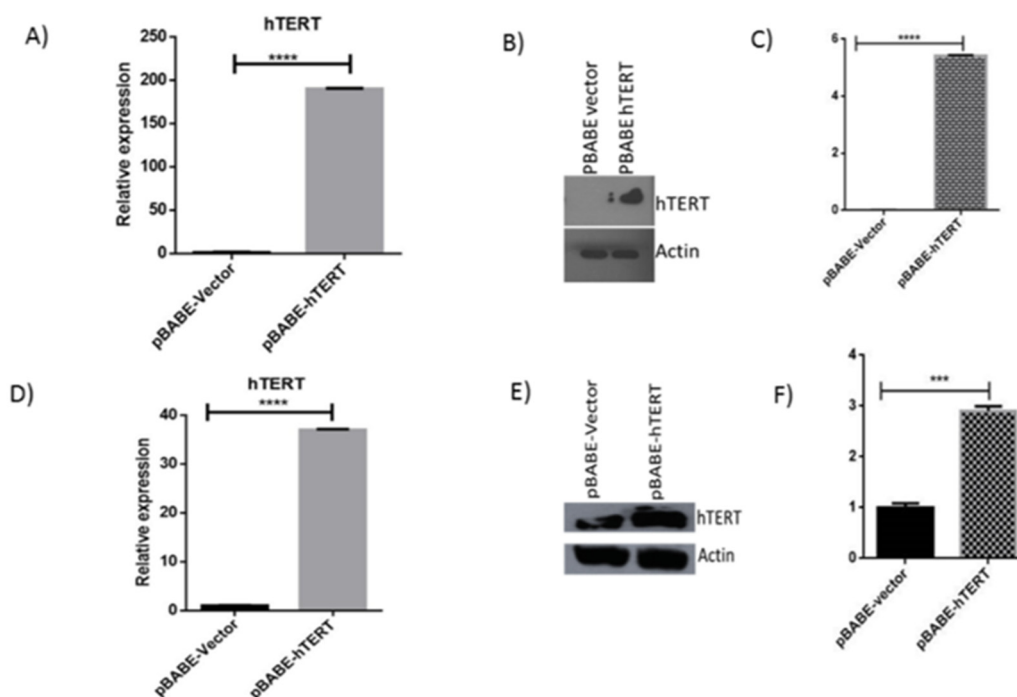
Figure 30: hTERT overexpression in U2OS and HeLa cell lines. The hTERT is overexpressed in U2OS and HeLa cell lines. (A & D) mRNA level of hTERT in U2OS & HeLa cell lines was determined by quantitative real-time PCR. (B & E) Western blotting confirms overexpression of hTERT in U2OS and HeLa cell line. (C & F) Histograms depict densitometric quantification of the hTERT overexpression of three corresponding independent Western blot experiments in U2OS and HeLa cell lines.

4.2.2 hTERT overexpression alters the proteomic profile of human cervical cancer and human osteosarcoma cells:

U2OS is a telomerase negative cell line and it has an ALT pathway to maintain the telomere length and thus it offers a clean baseline for observing any alteration in protein expression after hTERT overexpression in this cell line. After staining and analysis of gels by ImageMaster 2D Platinum v7.0 gel analysis software-(GE Healthcare Life Sciences), 28 spots (Table 12) showed differentially expressed proteins in U2OS cells out of which 23 were upregulated and 5 were down regulated [Fig 31A]. In HeLa cells we excised 23 spots (Table 13) representing differentially expressed proteins out of which 21 were up regulated and 2 were downregulated [Fig 31B] proteins. Most of these differentially expressed proteins seemed to be functionally associated with tumorigenesis. We found proteins involved in intermediate filament formation, glycolysis, antioxidant activity, heat shock proteins, apoptosis, nucleotide-sugar

biosynthesis, metastasis, xenobiotic metabolism, ubiquitination and glycosylation [Table 12 and Table 13].
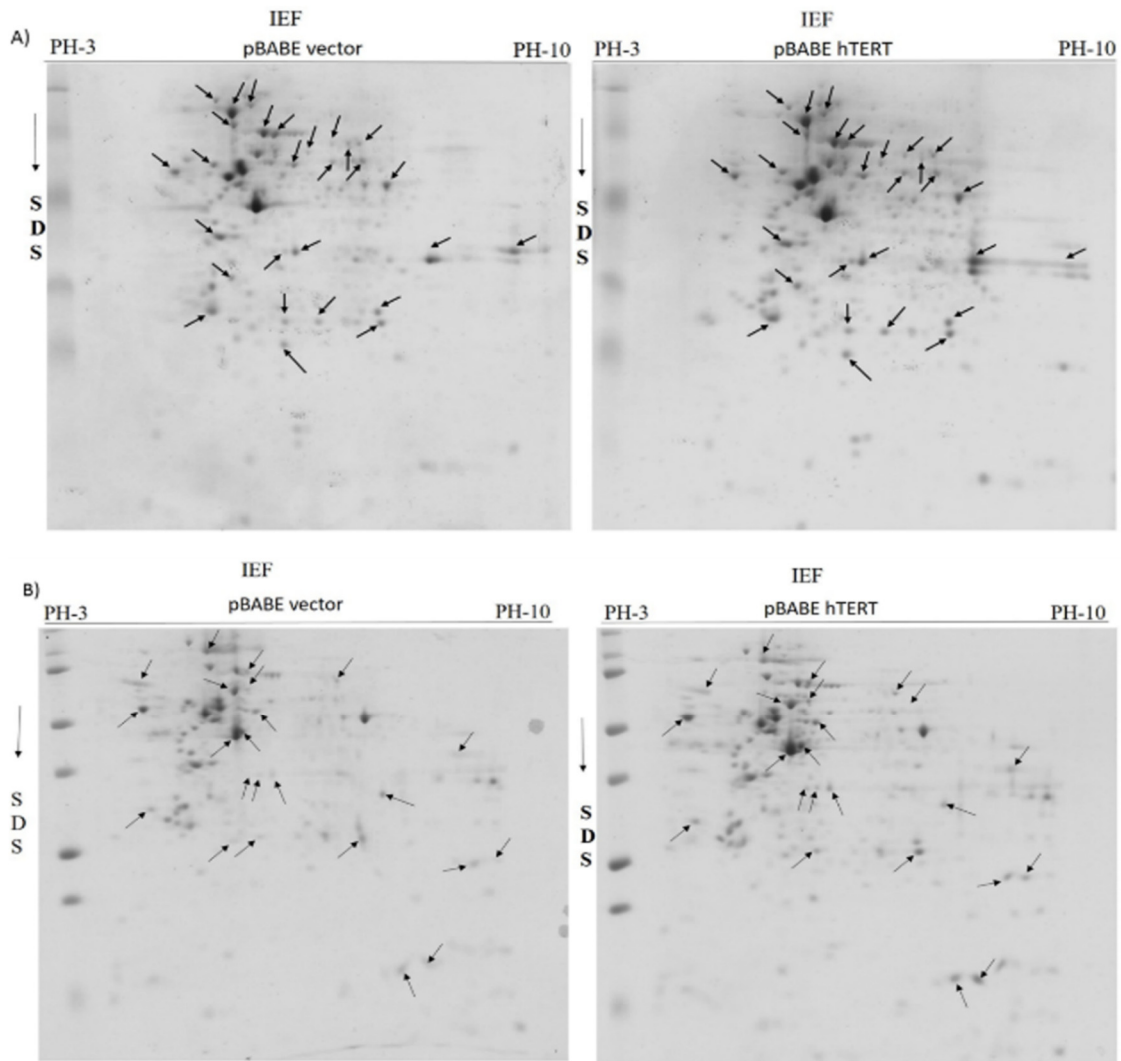
Figure 31: Two dimensional gel electrophoresis of hTERT overexpressing (A) U2OS and (B) HeLa cell line. Total proteins were extracted from hTERT overexpressing U2OS and HeLa cells and separated non-linearly on IPG strip of pH 3-10, followed by electrophoresis through 12% polyacrylamide gels. The gels were further stained and analyzed by image master 2D platinum software.

Table 12: A list of proteins differentially expressed following hTERT overexpression in U2OS cells.

Spot no.	Protein Identification	Sequence Coverage (%)	pI	Mass (kDa)	Expression level	Score
1	Immunoglobulin gamma heavy chain variable region, partial	56.1%	10	109	Downregulation	51
2	UDP-glucuronic acid decarboxylase 1 isoform 3	21.8%	9.6	28	Upregulation	47
3	Glyceraldehyde-3-phosphate dehydrogenase isoform 2	23.9%	7.9	32	Upregulation	68
4	Heat shock protein HSP 90-beta isoform c	21.1%	4.8	83	Upregulation	99
5	Heat shock 70kDa protein 8 isoform 1 variant, partial	33.7%	5.2	71	Upregulation	152
6	hCG2038865, partial	31.9%	10.3	102	Upregulation	46
7	L-lactate dehydrogenase B chain isoform LDHB	11.4%	5.7	37	Upregulation	44
8	Unnamed protein product	21.0%	4.6	51	Upregulation	100
9	dnaJ homolog subfamily C member 12 isoform X1	30.2%	5.9	137	Upregulation	40
10	Alternative protein CDH6	49.4%	11.9	101	Downregulation	43
11	Alpha-enolase isoform X1	14.1%	6.7	47	Upregulation	49
12	PDZ and LIM domain 2 (mystique), isoform CRA_c, partial	19.9%	13	178	Upregulation	53
13	Heat shock 60kDa protein 1 (chaperonin)	12.4%	9.1	60	Upregulation	72
14	hCG1789535	15.6%	9.3	50	Downregulation	15
15	hCG2040343, partial	55.6%	10.8	79	Upregulation	31
16	Enolase 1 variant, partial	21.0%	7.7	47	Upregulation	73
17	Mucin, partial	66.7%	12.8	27	Upregulation	38
18	Immunoglobulin M heavy chain, partial	100.0%	4.2	28	Upregulation	36
19	Glutathione S-transferase P	17.6%	4.1	23	Upregulation	81
20	BiP protein, partial	15.2%	5.1	71	downregulation	67
21	Ubiquitin carboxy-terminal hydrolase L1, partial	19.2%	5.2	23	downregulation	40
22	hCG2045028	35.9%	6	44	Upregulation	36
23	Unnamed protein product	10.4%	6.8	26	Upregulation	36
24	Unknown, partial	61.5%	5	45	Upregulation	35
25	RAMP2	13.7%	5.4	19	Upregulation	41
26	Annexin A5	45.6%	4.8	35	Upregulation	155
27	T-complex protein 1 subunit beta isoform 1	20.7%	6	58	Upregulation	89
28	StAR-related lipid transfer protein 7, mitochondrial precursor	12.4%	9.5	43	Upregulation	41

Results and discussion

Table 13: A list of proteins differentially expressed following hTERT overexpression in HeLa cells.

Spot no.	Protein Identification	Sequence Coverage (%)	pI	Mass (kDa)	Expression level	Score
1	Keratin 18	29.3%	5.3	48	Upregulation	68
2	Chain A, Crystal Structure Of The Globular Domain Of Human Calreticulin	32.8%	4.6	60	Upregulation	53
3	Chain A, Structural Basis Of Human Triosephosphate Isomerase Deficiency. Mutation E104d And Correlation To Solvent Perturbation.	47.2%	6.5	27	Upregulation	88
4	Glyceraldehyde-3-phosphate dehydrogenase isoform 2	31.4%	7.9	32	Upregulation	75
5	Peroxiredoxin 1, isoform CRA_b, partial	34.6%	6.5	21	Upregulation	47
6	Peroxiredoxin-1	62.3%	9.2	22	Upregulation	130
7	Chain K, Acetyl-Cypa:cyclosporine Complex	36.4%	7.8	18	Upregulation	73
8	Calcium-activated chloride channel regulator family member 3	29.4%	9.2	30	Upregulation	47
9	Chain A, Human Heart L-lactate Dehydrogenase H Chain, Ternary Complex With Nadh And Oxamate	27.3%	5.7	37	Upregulation	52
10	Mitochondrial ribosomal protein L46, isoform CRA_d	29.5%	9.1	19	Upregulation	34
11	F-actin-capping protein subunit alpha-1	33.2%	5.4	33	Upregulation	47
12	Chain A, Structural Basis For The Interaction Of Human β-defensin 6 And Its Putative Chemokine Receptor Ccr2 A nd Breast Cancer Microvesicles	59.2%	10.2	57	Upregulation	45
13	Heat shock 70kDa protein	14.0%	6	73	Upregulation	69
14	Galactose-1-phosphate uridyl transferase	100.0%	9.5	32	Upregulation	40
15	T cell receptor alpha, partial	46.5%	9.6	78	Upregulation	40
16	PR domain containing 8, isoform CRA_b	39.8%	7.6	19	Upregulation	44
17	T cell receptor alpha chain V-J-region, partial	9.6%	9.5	12	Upregulation	39

18	Unnamed protein product	19.3%	5.4	59	Upregulation	36
20	Chaperonin (HSP60)	29.4%	5.5	60	Upregulation	79
21	Chromosome 14 open reading frame 68, isoform CRA_a	60.8%	12.1	78	Upregulation	41
22	Cofilin 1 (non-muscle), isoform CRA_c, partial	38.7%	9.4	158	Downregulation	49
23	Unnamed protein product	10.4%	6.8	126	Downregulation	36

4.2.3 Upregulation of Hsp90, Hsp70 and Hsp60 in cells over-expressing hTERT:

We found significant over-expression of heat shock proteins Hsp70 and Hsp60 in hTERT overexpressing U2OS and HeLa cells. Hsp90 was upregulated in only U2OS cells. Hsp90 is an important subunit of telomerase and it helps in stabilizing a functional telomerase structure and in primer loading and extension[428]. Hsp70 is overexpressed in most of the cancer cells though its expression in cancer cells is typically a poor marker for prognosis [429]. Heat shock protein 60 (HSP60) plays a crucial role in malignant cell survival [430]. To confirm regulation of Hsp60 and Hsp70 by hTERT, we performed qRT-PCR [Fig 32A-B] and western blotting [Fig 32C-D] to check their expression in U2OS and HeLa cells carrying pBABE-Vector and pBABE-hTERT expression construct. QRT-PCR and Western blotting confirmed the upregulation of Hsp90 at transcript as well as protein level in U2OS cells [Fig 32E-F]. Along with the higher expression, we have also observed two bands in the case of Hsp90 in hTERT overexpressing U2OS cells which may be a splice variant of Hsp90.

4.2.4 Upregulation of GAPDH:

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is basically a glycolytic enzyme and a well-known housekeeping marker and commonly used as an endogenous control to assess cancer related gene expression. However, reports indicate involvement of GAPDH in other diverse functions independently of its role in energy metabolism. Deregulation in the expression level of GAPDH is found in many cancer cells [431]. Expression of GAPDH was enhanced in both U2OS and HeLa cells overexpressing hTERT. To confirm the upregulation of GAPDH by hTERT we performed qRT-PCR [Fig 33A-B) and western blotting [Fig 33C-D) in both U2OS and HeLa cell line. We found hTERT causes upregulation of GAPDH at transcriptional level only, there being no conspicuous change at protein level in either cell line.
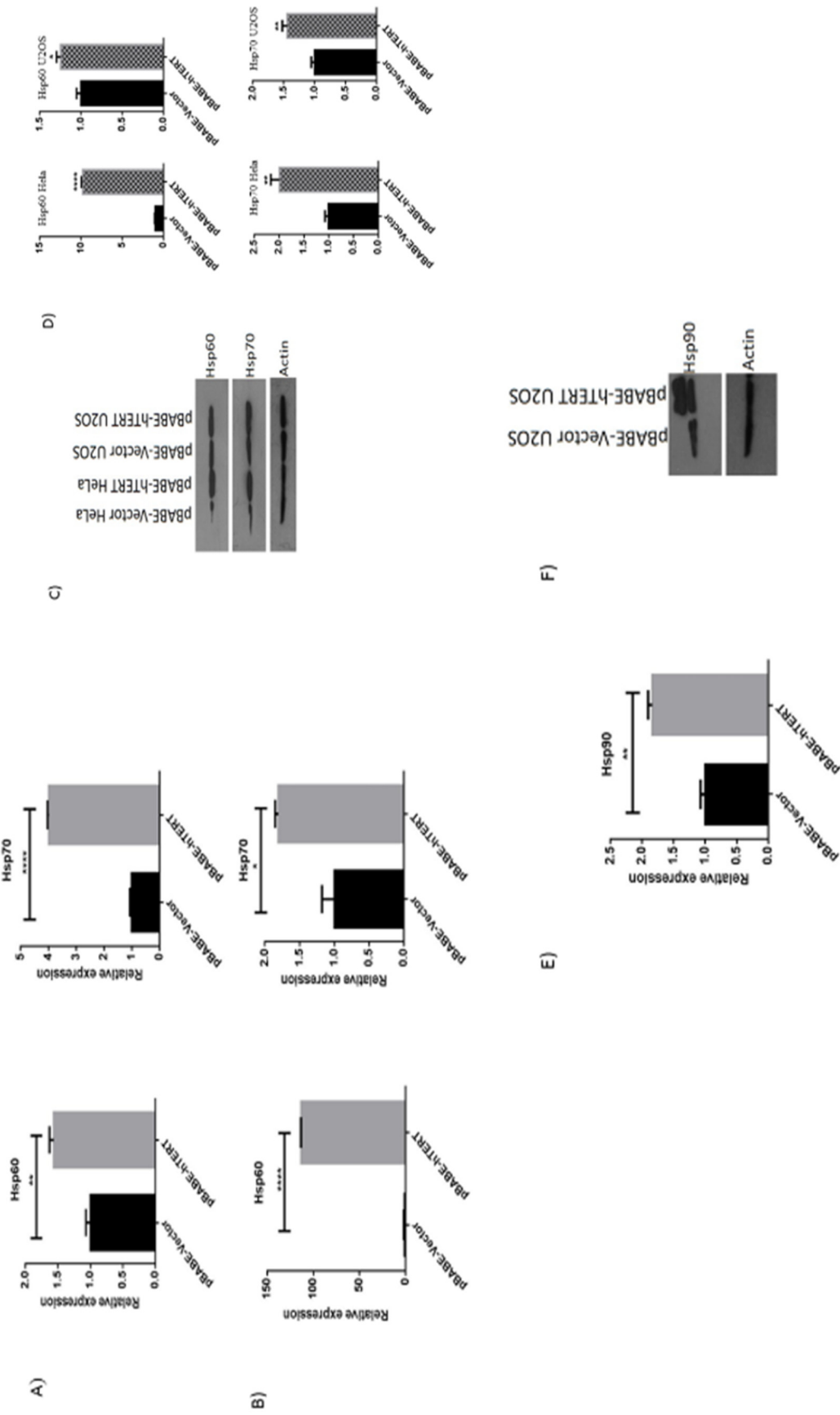
Figure 32: Validation of upregulation of heat shock proteins in U2OS and HeLa cell lines. The hTERT induced upregulation of Hsp60 and hsp70 were confirmed by qRT-PCR in (A) U2OS and (B) HeLa cell lines respectively. C) Western blotting showing upregulation of Hsp60 and Hsp70 at protein level. D) Histogram shows the results by applying ImageJ software. D) To confirm the upregulation of Hsp90 in U2OS cell line E) QRT-PCR and F) western blotting is performed.
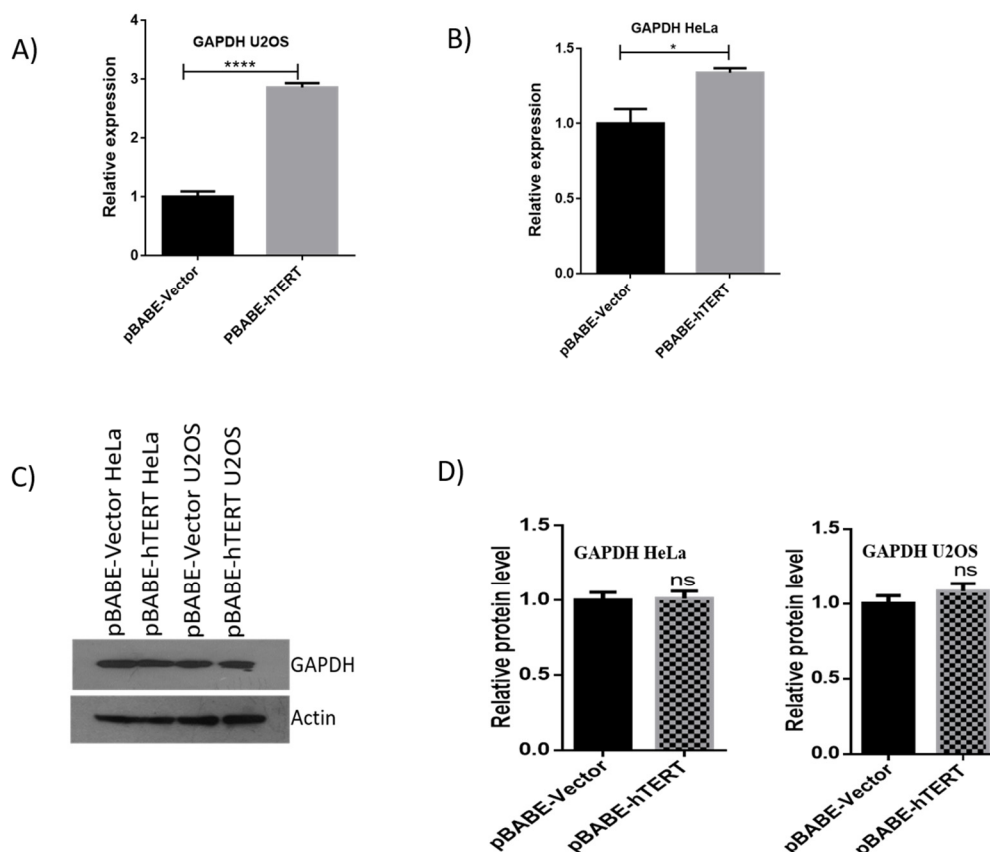
Figure 33: Validation of GAPDH in HeLa and U2OS cell lines. QRT-PCR was performed to check the hTERT induced upregulation of GAPDH in (A) U2OS and (B) HeLa cell lines. C) Represents results of Western blotting to check the up-regulation of GAPDH at protein level while (D) represents histograms of respective blots showing no conspicuous difference in expression of GAPDH at protein level.

4.2.5 Discussion: Proteomic identification of proteins differentially expressed following overexpression of hTERT (human telomerase reverse transcriptase) in cancer cells.

Telomerase components perform many functions apart from their canonical role in telomere lengthening. One such extracurricular function of hTERT is the regulation of cells signaling pathways. Many types of cancer do not express telomerase and being telomerase negative cannot manifest extracurricular activity of telomerase. They have ALT pathway to maintain telomere length. Remarkably, hTERT could be expressed at very high levels even in cells with a basal endogenous level of expression and allowed normal viability. Telomerase negative cells nevertheless offer a good experimental system to study the effect of hTERT overexpression on the expression of other genes. Human osteosarcoma is a primary malignant tumor of the bone and the U2OS cell line

derived from it is telomerase negative. In the present study, we document, for the first time significant effect of hTERT overexpression on the proteomic profile of U2OS cells. Here, we found Hsp90, Hsp70 and Hsp60 upregulated after hTERT overexpression in this cell line. Heat shock proteins usually act as molecular chaperones and are expressed at high levels in many cancers, although Hsp overexpression is only a poor prognosis in terms of survival and response to therapy in specific cancer types [432]. Heat shock protein 90 (Hsp90) is an abundant molecular chaperone that helps in conformational maturation and stabilization of various oncogenic proteins leading to tumor cell survival and disease progression [433]. Hsp90 interacts with a variety of intracellular proteins and is involved in differentiation, survival and cell growth [428]. It is demonstrated that inhibiting Hsp90 in osteosarcoma cells induces apoptosis [433]. Jennifer McCleese (2009) reported that inhibition of Hsp90 results in loss of cell viability, induction of apoptosis, and inhibition of cell proliferation in osteosarcoma cells [434]. It has also been shown that blocking HSP90 addiction inhibits tumor cell proliferation, metastasis and development [435]. Moreover, Hsp90 is also a subunit of telomerase complex and it stabilizes telomerase and helps in loading of telomerase complex to telomere [428]. Though there is no hTERT in U2OS cells, its overexpression upregulated Hsp90 (Fig 32D) suggesting intactness of the circuitry of intercommunication between hTERT and Hsps in telomerase negative cells.

Similar to Hsp90, the role of Hsp70 in cancer is very well studied. Being a molecular chaperone, Hsp70 is an important part of cellular networks, involving signaling, membrane, transcriptional and organelles functions [436]. In many cancer increased expression of Hsp70 is correlated with poor prognosis [436]. Lei Zhao et al. reported that viability of osteosarcoma cells was adversely affected after knocking down of Hsp70 [437]. It is also demonstrated that Hsp70 expression prevents apoptosis in osteosarcoma cells [438]. The role of Hsp70 in invasion and metastasis of cancer cells are also very well studied [439]. Moreover, Hsp70 inhibitor in cervical cancer also inhibits cancer cell proliferation [440]. R Ralhan et al. (1995) found that Hsp70 overexpression can be correlated with elevated proliferation and tumor size in uterine cervical cancer [441]. Importantly, Hsp70 is a potent buffering system for cellular stress either from extrinsic (physiological, viral and environmental) or intrinsic (replicative or oncogenic) stimuli. For survival, cancer cells depend heavily on this buffering system [429]. Moreover, reports have also shown association of hTERT with Hsp70.

Hsp70 binds with hTERT when hTR is absent and it gets dissociated when telomerase is folded into its active state [442] suggesting a transient association of Hsp70 with telomerase. We found upregulation of Hsp70 both at mRNA and protein level [Fig 32A & C]. Another heat shock protein showing differential expression in association with hTERT is Hsp60 which has been reported to interact with hTERT in mitochondria [116]. Initially, Hsp60 was found only in mitochondria but since last few year, studies confirmed its presence the cytosol, the cell surface and in the extracellular space [443]. Inside the mitochondria it was reported that it binds mainly with Hsp10. However, in addition to its association with Hsp10, other interacting molecules have also been identified for Hsp60 in recent years [443]. Similar to Hsp70, the role of Hsp60 in cancer proliferation, tumor cell survival and metastasis are very well demonstrated in both osteosarcoma and cervical cancer cells [444,445].

We also found upregulation of Hsp60 and Hsp70 in HeLa cells which, unlike U2OS, are telomerase positive [Fig 32B & C]. In the present study we have determined that hTERT may be an important modulator of heat shock proteins in these cells. However, it will be necessary to scan a wider range of cells for their responses in order to arrive at any generalization.

Yet another protein which was differentially overexpressed is GAPDH which is product of a housekeeping gene and is commonly used as internal control in different experimental condition. This enzyme is mainly used during glycolysis but it also has many diverse functions independent of its role in glycolysis [446]. Recent findings including our own show that expression level of GAPDH is highly regulated in various cancer cells [431]

There are signs of involvement of GAPDH in cancer progression and it may serve as a new marker or even a therapeutic target [431]. Moreover, it has been already shown that GAPDH mediates many oxidative stress responses, including nuclear translocation of GAPDH and induction of cell death. Craig Nicholls et al.(2012) reported that GAPDH interacts with telomerase RNA component hTR and inhibits telomerase activity which leads to telomere shortening and senescence in breast cancer cells [447]. Here we show that hTERT overexpression leads to the upregulation of GAPDH in both U2OS and HeLa cell lines suggesting that GAPDH may also interact with hTERT but in a positive manner. We do not find any cell death after hTERT overexpression. Apart

from heat shock proteins and GAPDH there are many other proteins differentially regulated. Keratin 18 is an intermediate filament protein and can be used for detection of proliferating fractions in the breast cancer [448]. This protein is found upregulated in HeLa cells following hTERT overexpression suggesting that hTERT modulates the expression of cytoskeletal proteins in cancer cells. Another upregulated protein we found is peroxiredoxin 1. This is an antioxidant enzyme associated with telomeres and protects it from oxidative damage and preserves telomeres for extension by telomerase [449]. Upregulation of peroxiredoxin in hTERT overexpressing HeLa cells indicates that hTERT enhances the expression of peroxiredoxin 1 to protect telomeres from any oxidative damage. It is well known that hTERT promotes EMT and during EMT there is change in expression of some epithelial and mesenchymal markers; level of mesenchymal markers goes up while that of epithelial markers goes down. CDH6 which is a type 2 cadherin and an epithelial marker drives EMT during embryonic development and it is aberrantly re-activated in cancer [450]. We found downregulation of CDH6 in U2OS cells when hTERT is overexpressed showing that hTERT promotes mesenchymal character in these cells. Another downregulated protein we found is ubiquitin carboxyl-terminal hydrolase L1 (UCHL1). It is a cysteine protease that belongs to the UCH proteases family and has also acquired E3 ubiquitin-protein ligase activity and stabilizes ubiquitin monomers in vivo [451]. This protein has heterogeneous expression in cancer cells and performs both tumor inhibition and promoting functions [452]. Downregulation of this protein in U2OS cells after hTERT overexpression indicates that hTERT modulates the expression of this deubiquitinating enzyme to avoid proteasomal degradation of itself. Furthermore, apart from GAPDH another glycolytic enzyme upregulated is alpha-enolase which is now used as a potential cancer prognostic marker and promotes invasion in cancer cells [453] indicating promotion of invasion by hTERT via regulation of these glycolytic enzymes. We also found that hTERT expression led to the increase in migration rate of both U2OS and HeLa cells indicating that higher level of hTERT is linked with high invasive tumor phenotype.

In conclusion, this study shows that hTERT expression alters the proteomic profile of osteosarcoma and cervical cancer cells. Moreover, heat shock proteins are an important subset of cellular proteins regulated by hTERT. GAPDH is also influenced by hTERT expression in both the cell lines. The findings make it pertinent to further investigate

the relevance of telomerase associated molecules like peroxiredoxin-1 and alpha-enolase, as markers or therapeutic target.

4.3 Knockingdown of hTERT in HeLa cell line:

After overexpression part, we have done protein profiling of hTERT knockdown HeLa cells. Two micrograms each of pMKO.1-puro empty vector and pMKO.1-shRNA-hTERT were transfected into HeLa cells by using lipofectamine 3000 (Invitrogen). After 48hrs, transfected cells were selected by using 2μg/ml of puromycin and finally maintained in 1μg/ml of puromycin. Western blotting and qRT-PCR were performed to check knockdown of hTERT [Fig 34A-C].
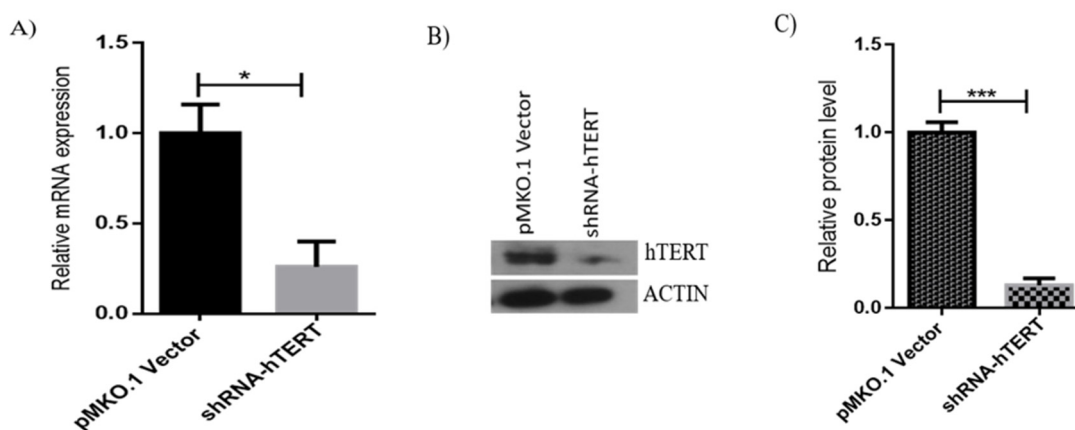
Figure 34: Validation of hTERT knockdown in HeLa cells. To validate the knockdown of hTERT in shRNA-hTERT transfected Hela cells (A) qRT-PCR was performed which showed around 70% knockdown of hTERT (B). Western blotting also confirmed hTERT knockdown. (C) Histogram represents quantification of western blot.

4.3.1 hTERT knockdown alters the proteomic profile of human cervical cancer cells: Protein profiling of hTERT knockdown HeLa cells were done by two-dimensional gel electrophoresis. After staining gels were analyzed by ImageMaster 2D Platinum v7.0 gel analysis software-(GE Healthcare Life Sciences). 22 differentially expressed spots were picked and submitted for MALDI-TOF [Fig35].
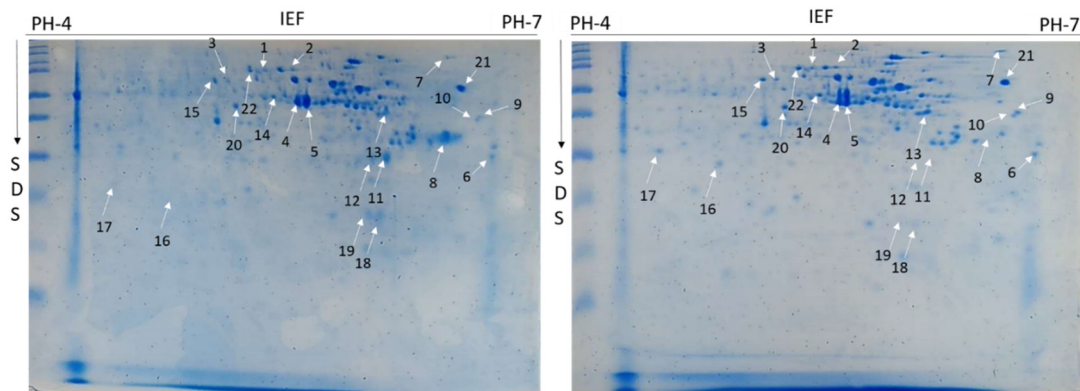
Figure 35: Two-dimensional gel electrophoresis of hTERT knockdown HeLa cell line. Total proteins were extracted from hTERT knockdown HeLa cells and separated non-linearly on IPG strip of pH 4-7, followed by electrophoresis through 12% polyacrylamide gels. The gels were further stained and analyzed by image master 2D platinum software.

4.4 Global gene expression profiling of a telomerase negative cell line (U2OS) following ectopic expression of hTERT in it:

Telomerase is a well-known enzyme that maintains the length of the telomere and the physical ends of the eukaryotic chromosome in embryonic stem cells and cancer cells. Telomerase activation extends the lifespan of cells in culture by maintaining the length of the telomere. It has now become clear that the role of telomerase is much more complex than just telomere lengthening. Compared to normal somatic differentiated cells, telomerase activity remains high in around 90% of cancer cells. In fibroblast and some other cell types, telomere length was stabilized following ectopic expression of hTERT in them giving them infinite replicative potential [82]. In both cancer cells and normal stem cells, immortalization can be achieved by overexpression of telomerase [423,424]. Furthermore, for the development of exact therapeutic strategies on the basis of telomerase inhibition it is necessary to know the main role of telomerase in cancer cells [6]. Telomerase is not present in every cancer cells and around 10% cancer cells are telomerase negative. They have ALT pathway to maintain telomere length. Many studies have already been done to explore gene profiling of a telomerase positive cell by knocking down of hTERT in it [6,454]. We therefore performed global gene profiling of a telomerase negative cell by ectopically expressing hTERT in it. Here we have studied changes in the global gene expression patterns in telomerase negative cells by overexpressing hTERT in it.

Results:

4.4.1 Overexpression of hTERT in U2OS cell line:

Overexpression of hTERT U2OS cells was confirmed by qRT-PCR and western blotting. Remarkably, hTERT mRNA expression was upregulated to approximately 180 fold in U2OS cells transfected with pBABE-puro-hTERT in comparison to vector transfected cells (Fig 1A) Overexpression of hTERT in U2OS cell lines was further confirmed by western blotting [Fig 36B & C].
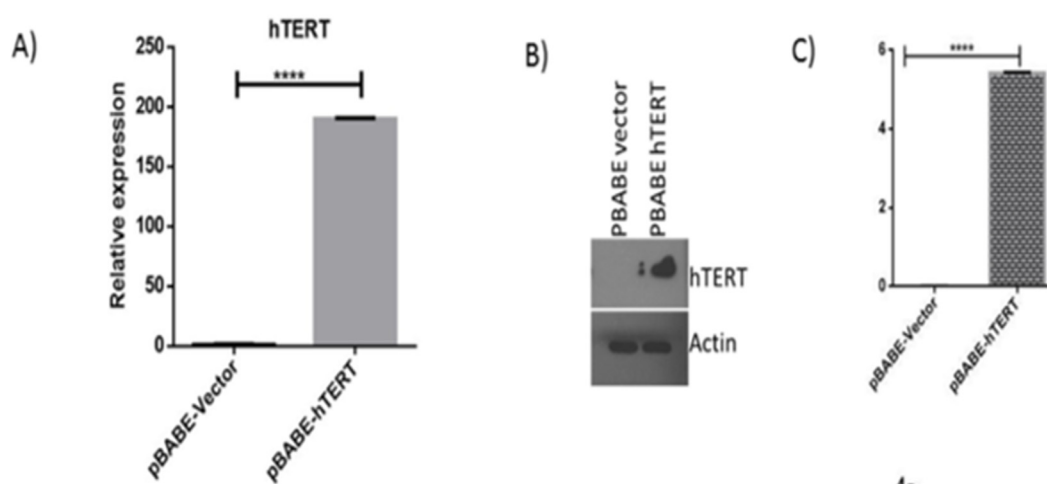
Figure 36: hTERT overexpression in telomerase-negative U2OS cell lines. (A) mRNA level of hTERT in U2OS cell line was determined by quantitative real-time PCR. (B) Western blotting confirms overexpression of hTERT in U2OS cell line. (C) Histograms depict densitometric quantification of the hTERT overexpression of three independent Western blot experiments in U2OS cell lines.

4.4.2 Genome-wide profiling of differentially expressed genes following over-expression of hTERT in U2OS cells:

In addition to telomere maintenance, telomerase also regulates gene expression by different unknown mechanisms. Being a telomerase negative cell line U2OS offers a clean baseline for performing global gene expression profiling after hTERT overexpression. This will give a list of differentially expressed genes whose expression is influenced by telomerase. For large scale gene expression measurement, there are a number of microarray technologies available. Among them, oligonucleotide arrays and cDNA arrays are most commonly used approaches. We have performed cDNA

expression microarray analysis in U2OS cells stably expressing hTERT. Total RNA was isolated at different passages and RNA purity was assessed by ND-1000 spectrophotometer and RNA integrity number were evaluated by Agilent 2100 bioanalyzer. Isolated RNA was reverse-transcribed to cDNA using a T7 oligo-dT primer. To characterize the transcriptomic profiles of the stable cells, quantile normalization of log2 (probes) values of two treatments in duplicates were plotted with respect to the basal expression range and the distribution of the total number of genes/probes analyzed. Pair-wise comparison was performed to analyze differential gene expression. Total number of probes used were 47320 and we found 76 differentially expressed genes having fold change ≥1.5 and $p<0.05$ [Table14, Fig 37A-B]. Out of 76 differentially regulated genes, 38 were upregulated and 36 were downregulated [Table14, Fig 37C]. We performed microarray in duplicates and that's why LPE test was used to analyze the data. This test is especially useful when the number of replicates is small.
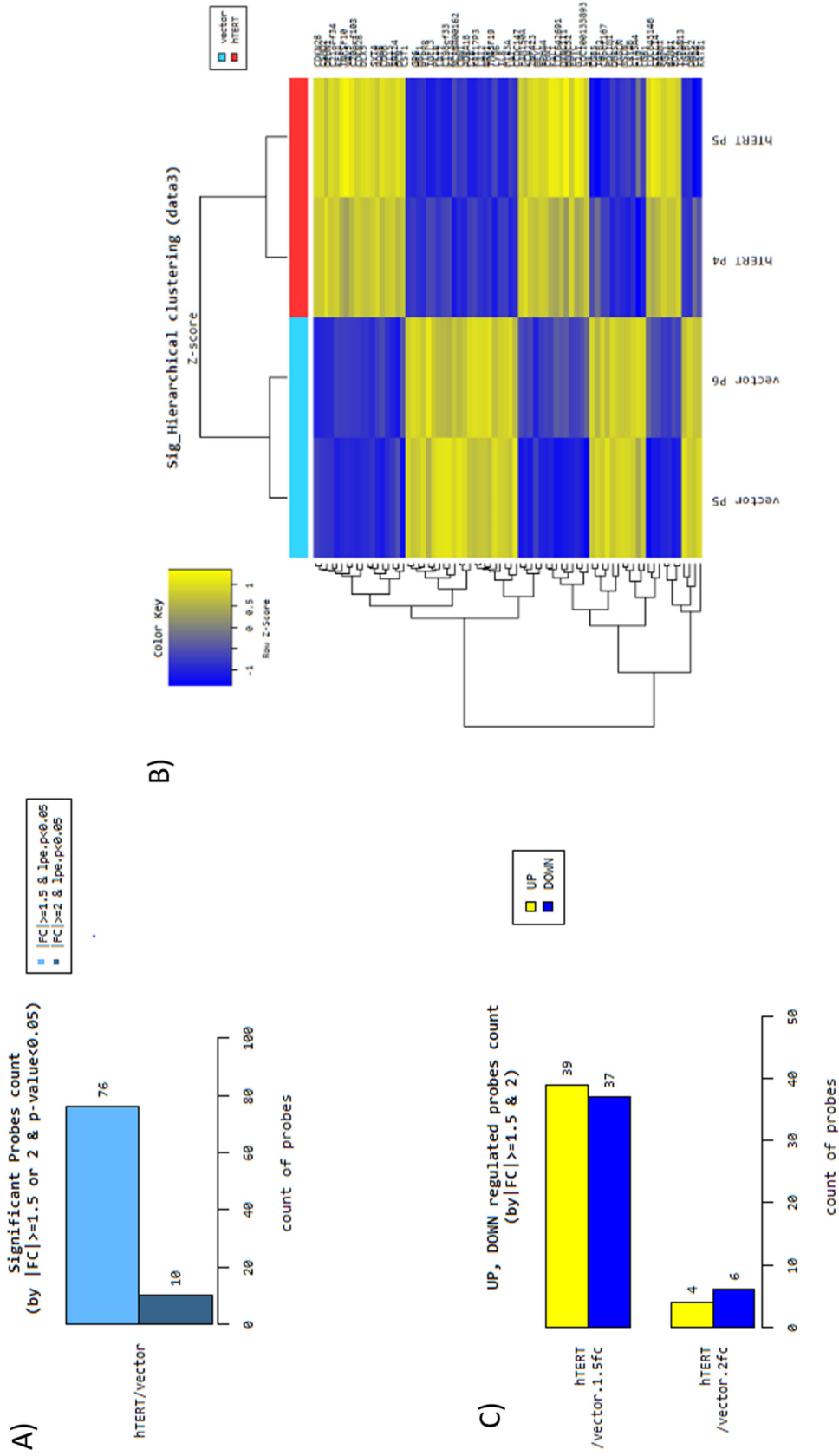
Figure 37: Genome wide expression profiling of U2OS cells following hTERT overexpression (A) Total number of differentially regulated genes. (B) Heat map analysis of microarray data representing hierarchical clustering of 76 differentially expressed genes regulated in the hTERT overexpressing cells compared with the vector transfected cells. (C) Yellow color represents up-regulated genes while blue color indicates downregulated genes (FC>1.5).

Table14: List of differentially expressed genes and their role in cancer.

S.No.	Gene	Full name	Function in context to cancer	References
Genes up regulated following Overexpression of TERT in U2OS cells				
1	HEYL	Hes Related Family BHLH Transcription Factor With YRPW Motif-Like.	Downstream effector of Notch signaling which may be required for cardiovascular development (By similarity).	[455]
2	PARM1	Prostate Androgen Regulated Mucin-Like Protein 1	May regulate TLP1 expression and telomerase activity, thus enabling certain prostatic cells to resist apoptosis.	[456]
3	PI15	Peptidase Inhibitor	PI15 might jointly participate in the regulation of drug resistance in ovarian cancer and serve as potential targets in targeted therapies	[457]
4	TSPAN13	Tetraspanin	Inhibit apoptosis of cancer cells.	[459]
5	EPHA4	Tyrosine-Protein Kinase Receptor SEK	Involved in cancer cell motility and invasion.	[460]
6	FOXF2	Forkhead-Related Transcription Factor 2	Inhibit invasion and metastasis.	[461]
7	EPYC	Dermatan Sulfate Proteoglycan 3	Increased EPYC mRNA expression has been demonstrated in patients with invasive pancreatic ductal adenocarcinoma, compared with normal pancreatic tissues.	[462]

8	CHN1	Chimerin 1 Rho GTPase-Activating Protein 2	CHN1 and TNFAIP3 are candidate biomarkers for ESCC to aid in the diagnosis of dysplasia and carcinoma	[463]
9	DYNC1I1	Dynein, Cytoplasmic, Intermediate Polypeptide 1	Has high expression in cancer cells but function is unknown	[464]
10	MIR99AHG	Mir-99a-Let-7c Cluster Host Gene	Unknown	
11	BAMBI	BMP And Activin Membrane Bound Inhibitor	BAMBI is a novel modulator of TGF-beta signaling and found overexpressed in ovarian cancer	[465]
12	C20orf103	Lysosomal Associated Membrane Protein Family Member 5	It express on the surface of cancerous tumors, mainly in cells of highly metastatic cancer.	[466]
13	GJA1	Gap Junction Protein Alpha 1	It involved in cell migration and provide cancer cell polarity. TGF-β enhanced its expression via the Smad and ERK1/2 signaling pathway.	[468]
14	KERA	Keratan Sulfate Proteoglycan Keratocan	It is involved in corneal transparency. Defects in this gene leads to autosomal recessive cornea plana 2 (CNA2).	[469]
15	RORB	RAR Related Orphan Receptor B	RORB is a transcriptional enhancer of inhibitor HBP1 of the Wnt pathway.	[470]
16	CXCL14	C-X-C Motif Chemokine Ligand 14	Play anticancer role in cancer cells.	[471]
17	KAL1	Kallmann Syndrome Interval Gene 1	Involved in neural cell migration.	[472]
18	CST1	Cystatin SN	Increase expression of CST1 promote breast cancer progression.	[473]

19	FAM150A	Family With Sequence Similarity 150 Member A	Act as a ligand for anaplastic lymphoma kinase (ALK).	[474]
20	DLX5	Distal-Less Homeobox 5	It promotes cancer progression.	[475]
21	CDH6	Cadherin 6, Type 2, K-Cadherin	Elevated expression of CDH6 promote cancer cell EMT and metastasis.	[476]
22	Ar	Androgen Receptor	Blocking of androgen receptor by drug inhibit cancer cell growth.	[477]
23	SYT4	Synaptotagmin 4	It is involve in dendrite formation by melanocytes.	[478]
24	CSMD2	CUB And Sushi Multiple Domains 2	Its loss in cancer cell contributes to poor prognosis.	[479]
25	KCNJ2	Potassium Voltage-Gated Channel Subfamily J Member 2	Silencing of KCNJ2 enhance cisplatin induced cell death of cancer cells.	[480]
26	SNX24	Sorting Nexin 24	It is upregulated in cancer cells.	[481]
27	LOC642691	Uncharacterized	Uncharacterized	
28	TNFSF10	Tumor Necrosis Factor Superfamily Member 10	It is p53-transcriptional target gene by which p53 suppress tumor.	[482]
29	HMGCS2	3-Hydroxy-3-Methylglutaryl-CoA Synthase 2	It enhances invasion and metastasis of cancer cells.	[483]
30	ZNF423	Zinc Finger Protein 423	It act as a critical transcriptional modulators in cancer cells.	[484]
31	DCN	Decorin	It is a potential biomarker in cancer.	[485]
32	VAV3	Vav Guanine Nucleotide Exchange Factor 3	It plays role in cancer cell growth by regulating PI3K-AKT signaling pathway.	[486]
33	LOC100133893	MANSC domain-containing protein	Uncharacterized	
34	LOC645146	Uncharacterized	Uncharacterized	
35	SGK1	Serum/Glucocorticoid Regulated Kinase 1	It enhances cancer cell growth and progression.	[487]
Genes downregulated following Overexpression of TERT in U2OS cells				

1	HBE1	Hemoglobin Subunit Epsilon 1)	Involved in protein heterooligomerization, associated with anemia and sickle cell anemia.	[488]
2	TGFBI	Transforming Growth Factor Beta Induced	Transforming growth factor-beta-induced (TGFBI) act as a linker protein and involved in the cell proliferation, migration, adhesion, activation of morphogenesis, inflammation and differentiation.	[458]
3	PTPRR	Protein Tyrosine Phosphatase, Receptor Type R	PTPRR inhibit invasion and metastasis by inhibiting MAPK-signaling	[489]
4	ADRA1B	Adrenergic, Alpha-1B-, Receptor	ADRA1B involved in the development and progression of gastric cancer.	[490]
5	IGFL3	Insulin Growth Factor-Like Family Member 3	Has high expression in cancer cells but function is unknown.	[491]
6	NRP1	Neuropilin 1	It is a marker of the progression of cancer.	[492]
7	C19orf33	Chromosome 19 open reading frame 33	Macrophage stimulating 1 (hepatocyte growth factor-like)	[467]
8	LY96	Lymphocyte Antigen 96	Ly96 involve in cancer cell proliferation and migration.	[493]
9	CA9	Carbonic Anhydrase 9	This is most widely studied gene in response to hypoxia. Enhance invasive property of cancer cells.	[494]
10	DHRS2	Dehydrogenase/Reductase 2	Act as a tumor suppressor gene.	[495]
11	CRYAB	Crystallin Alpha B	Act as a tumor suppressor gene.	[496]
12	GP6	Glycoprotein VI Platelet	It act as a mediator of metastasis.	[497]

13	KRT34	Keratin 34	It is found downregualted in cancer cells.	[498]
14	TAGLN	Transgelin	It altered expression of gene involved in cell motility.	[499]
15	CPA4	Carboxypeptidase A4	Its level is diagnostic serum biomarker for pancreatic cancer	[500]
16	PDLIM1	Carboxyl Terminal LIM Domain Protein 1	It prevents EMT by stabilizing E-cadherin/β-catenin complex.	[501]
17	KRT81	Keratin 81	It is found downregulated in cancer cells.	[502]
18	LAT2	Linker For Activation Of T-Cells Family Member 2	It is involved in the recruitment of multiple adapter proteins and downstream signaling molecules into multimolecular signaling complexes which is situated near the site of TCR engagement	[503]
19	CNN1	Calponin 1	It inhibits metastatic cell motility	[504]
20	TGFB2	Transforming Growth Factor Beta 2	It act as both tumor suppressor as well as tumor inducer	[422]
21	C9orf167	Chromosome 9 Open Reading Frame	It is found downregulated in cancer cells.	[505]
22	CGB1	Chorionic Gonadotropin Beta Subunit 1	It is found downregulated cells.	[506]
23	CCDC147	Coiled-Coil Domain-Containing Protein 147	Uncharacterized	
24	CT45A4	Cancer/Testis Antigen Family 45 Member A3	It is a protein coding gene.	
25	CGB5	Chorionic Gonadotropin Beta Subunit 5	It involves in trophoblastic tumor.	[507]
26	IL8	Interleukin 8	It enhance cancer cells progression.	[508]
27	PKIB	Protein Kinase (CAMP-Dependent, Catalytic) Inhibitor Beta	It promotes prostate cancer aggressiveness	[509]

			by linking Akt and PKA pathways.	
28	MSLN	Mesothelin	It promotes EMT and tumorigenicity of cancer cells.	[510]
29	HTR3A	5-Hydroxytryptamine (Serotonin) Receptor 3A, Ionotropic	It involves in postoperative vomiting.	[511]
30	LTB	Lymphotoxin Beta	It enhances cancer cell invasion and metastasis.	[512]
31	TM4SF19	Transmembrane 4 L Six Family Member 19	It regulates breast cancer cell migration and apoptosis via PI3K/AKT/mTOR pahway.	[513]
32	KRT17P3	Keratin 17 Pseudogene 3	It has reduced expression in cancer cells.	[514]
33	NCRNA00162	Uncharacterized	Uncharacterized	

4.4.3 Functional analysis of differentially expressed genes by in-silico method:

Enrichment test which was based on gene ontology (GO,http://geneontology.org/) DB was conducted with significant gene list. Table 14 summarizes the result of enrichment.Pathway maps were built by using gene expression data and genes were found to playrole in different pathways such as TGF-β signaling pathway, NF-kB signaling pathway,Foxo signaling pathway, cytokine-cytokine receptor interaction, natural killer cellmediated cytotoxicity, Fc epsilon RI signaling pathway, chemokine signaling pathway,pathways in cancer, rheumatoid arthritis, HTLV-1 infection and malaria [Fig 38A]. Wefound 39 upregulated and 37 downregulated genes and most of them play significantrole in cancer [Fig 38B]. The detailed functional role of some of these upregulated anddownregulated genes are shown in Table1. Out of these 76 genes, we have randomlypicked 3 upregulated genes viz., TSPAN13, DLX5, HMGCS2 and 3 downregulatedgenes viz., DHRS2, CRYAB and PDLIM1 and validated their expression by qRT-PCR

[Fig 39A-B]. Our qRT-PCR results were in concordance with microarray data. Out of these six genes role of TSPAN13 in cancer progression has been studied recently. It is reported that it inhibits the apoptosis of the cancer cells and helps in cancer progression. We observed that hTERT overexpression enhances its expression in cancer cells which

suggests that hTERT regulates expression of genes which help in cancer progression. Further study is needed to explore the pathways by which hTERT regulates TSPAN13 expression.
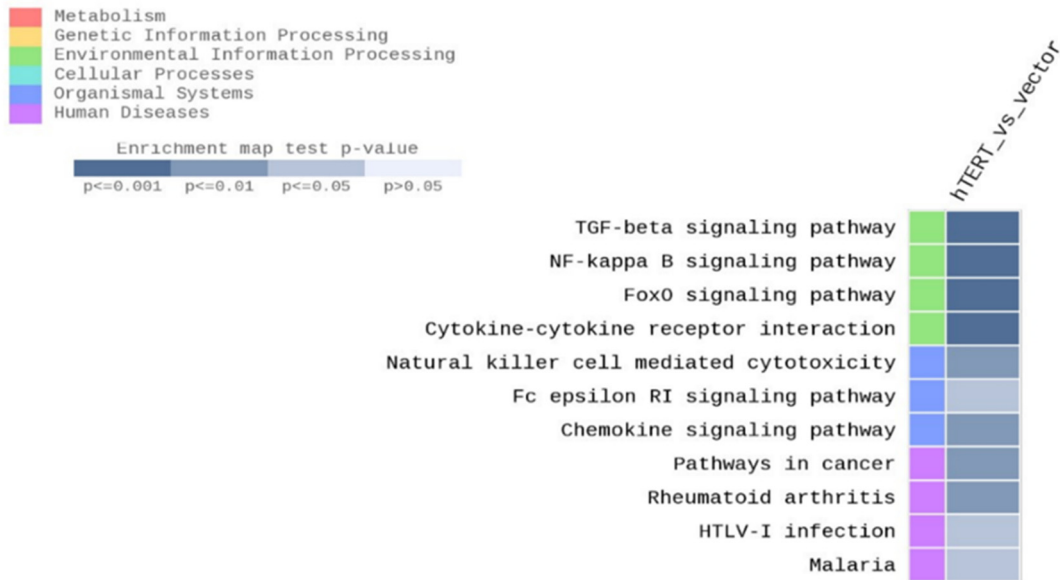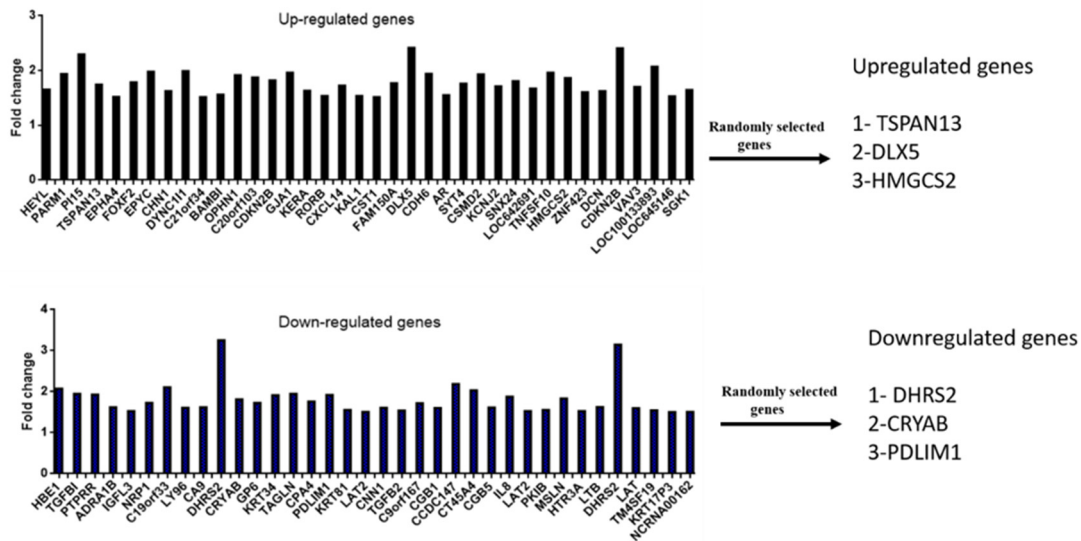
Figure 38: (A) KEGG Enrichment heatmap showing pathways in which differentially expressed genes are involved. (B) An overview of differentially expressed genes following hTERT overexpression in U2OS cells. Fold change are shown by the histogram. Out of total 76 differentially expressed genes, 38 are upregulated and 36 were downregulated.
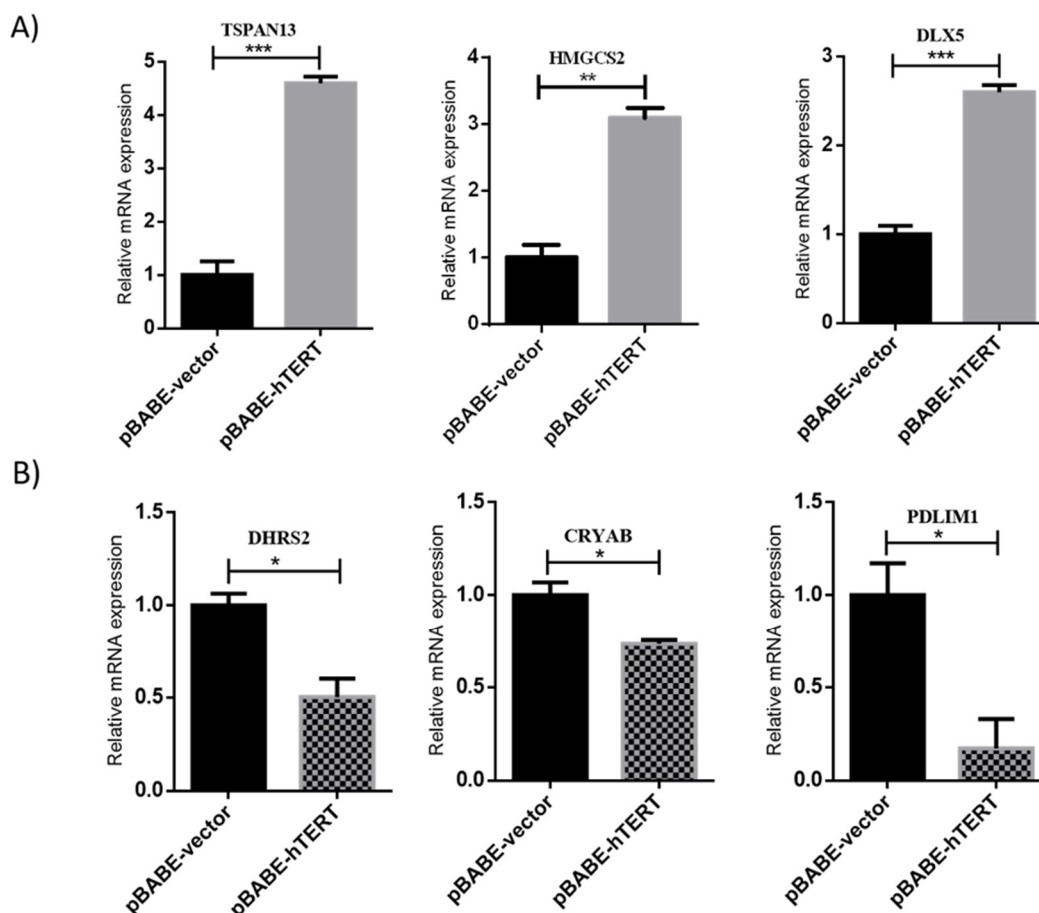
Figure 39: qRT-PCR validation of chosen differentially expressed genes. (A) Out of 39 upregulated genes three genes i.e. TSPAN13, HMGCS2 and DLX5 were randomly chosen and validated by real-time PCR. (B) Three genes i.e. DHRS2, CRYAB and PDLIM1 were also chosen from 37 downregulated genes and validated by real-time PCR. The data presented are inconformity with the results of micro-array analysis.

4.4.4 Discussion: Genome-wide profiling of a telomerase negative cell line (U2OS) following overexpression of hTERT in it:

Accumulating evidence suggests that telomerase performs various functions independently of its classical function of telomere maintenance [2,5]. Reports suggest that the role of hTERT in cell signaling are increasing day by day. Liu et al. showed a potential role of hTERT in EMT mediated by wnt/β-catenin mediated pathway [4]. Okamoto N et al. reported that hTERT interacts with nucleolar GTP-binding protein nucleostemin/GNL3L and BRG1 and forms a complex to modulate transcriptional programs necessary for maintenance of the state of tumor-initiating cells or of cancer stem cells [145]. Qin Y et al. reported that hTERT/ZEB1 complex directly regulates

E-cadherin to promote epithelial-to-mesenchymal transition (EMT) in colorectal cancer [146]. Collectively this shows that hTERT regulates many signaling molecules and hence in order to arrive at a comprehensive list of signaling molecules affected by telomerase, it is important to perform a genome-wide expression profiling by either inhibiting telomerase expression in a telomerase positive cells or by ectopically expressing telomerase in telomerase negative cells. Nevertheless, telomerase negative cells offer a good experimental system to study the effect of hTERT overexpression on the expression of other genes. Here we have performed microarray in a telomerase negative cell line U2OS which is a primary malignant tumor of the bone. In this study, we have identified genes that are regulated by expression of hTERT in a telomerase negative cell line. We found 76 differentially regulated genes following hTERT overexpression in U2OS cells. Out of these 76 genes, 37 were upregulated and 36 were downregulated. As we have discussed earlier, telomerase function is not limited to maintenance of telomere length. Maintaining telomere length only provides immortality to the cells and to completely transform a cell requires the involvement of other factors. It has been shown that overexpression of TERT in mice leads to invasive mammary carcinoma, suggesting involvement of telomerase in other pathways which help in cancer progression [5]. In our case, we found most of the upregulated genes are involved in cancer progression indicating hTERT may enhance expression of these genes in cancer cells to promote cancer progression. We have randomly chosen TSPAN13, HMGCS2 and DLX5 and validated their expression by real-time PCR. DLX5 (distal-less homeobox 5) is a transcription factor involved in jaw formation and appendage differentiation during embryonic development. Yinfei Tan et al. reported that DLX5 upregulation promotes ovarian cancer cell proliferation by stimulating IRS-2-AKT signaling [475]. Jinfei Xu et al. reported enhanced tumor cell proliferation by DLX5 via transcriptional regulation of MYC [515]. We observed upregulation of DLX5 following hTERT overexpression suggesting hTERT may promote cancer cell proliferation by regulating IRS-2-AKT and MYC pathways by enhancing expression of DLX5. Another upregulated gene chosen by us was HMGCS2. HMGCS2 (Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase) is an enzyme mainly involved in ketogenesis. Its role in cancer progression is also well studied. Shih-WenChen et al. reported the role of HMGCS2 in cancer invasion and metastasis [483]. Thismay be another pathway by which hTERT enhances cancer cell invasion and

metastasis. TSPAN13 (tetraspanin13) inhibits cancer cell apoptosis by downregulating the expression of antiapoptotic genes and its upregulation after hTERT expression suggests that hTERT activates TSPAN13 expression to inhibit apoptosis of cancer cells [516].

We also validated expression of three downregulated genes DHRS2, CRYAB and PDLIM1 by real-time PCR. *DHRS2* (Dehydrogenase/Reductase 2) is basically a tumor suppressor gene [495]. Overexpression of hTERT reduces expression of this gene indicating that hTERT expression in cancer cells downregulates expression of tumor suppressor genes to facilitate cancer progression. Similar to DHRS2, CRYAB (Alpha B-crystallin) is also a tumor suppressor gene and its downregulation is significantly associated with tumor progression [496]. Another important downregulated gene is PDLIM1 (PDZ and LIM domain protein 1) and it is involved in inhibition of EMT. Hai-Ning Chen et al. reported that PDLMI1 inhibits EMT and metastasis by stabilizing E-cadherin/β-catenin complex indicating another way hTERT might be promoting EMT [501]. Overall we can conclude that hTERT is a modulator of genes which enhance and suppress the expression of genes concomitant with cancer progression.

Objective 3-Phenotypic consequences of knocking down and overexpressing the above molecules.

4.5 hTERT overexpression enhances the migratory potential and colony formation efficiency of cancer cells:

It is already observed by many researchers that hTERT enhances the migratory potential and colony formation efficiency of cancer cells [517]. We have confirmed this by performing wound healing and colony formation assay in HeLa and U2OS cells.

4.5.1 Wound healing assay following hTERT overexpression in HeLa and U2OS cells: Wound healing assay has been done at different time points to check the migratory behaviour of the cells after hTERT overexpression in HeLa cells. Briefly HeLa cells were seeded in 2 wells of a 6 well plate and cultured until confluency. Then by using a pipette tip, we made a straight scratch, simulating a wound. The plates were washed gently and fresh DMEM replaced with supplements (serum, antibiotics). The cells were observed by phase contrast microscopy. We found that in comparison to vector control there is increased migration in hTERT overexpressing HeLa and U2OS cells [Fig 40A-D].
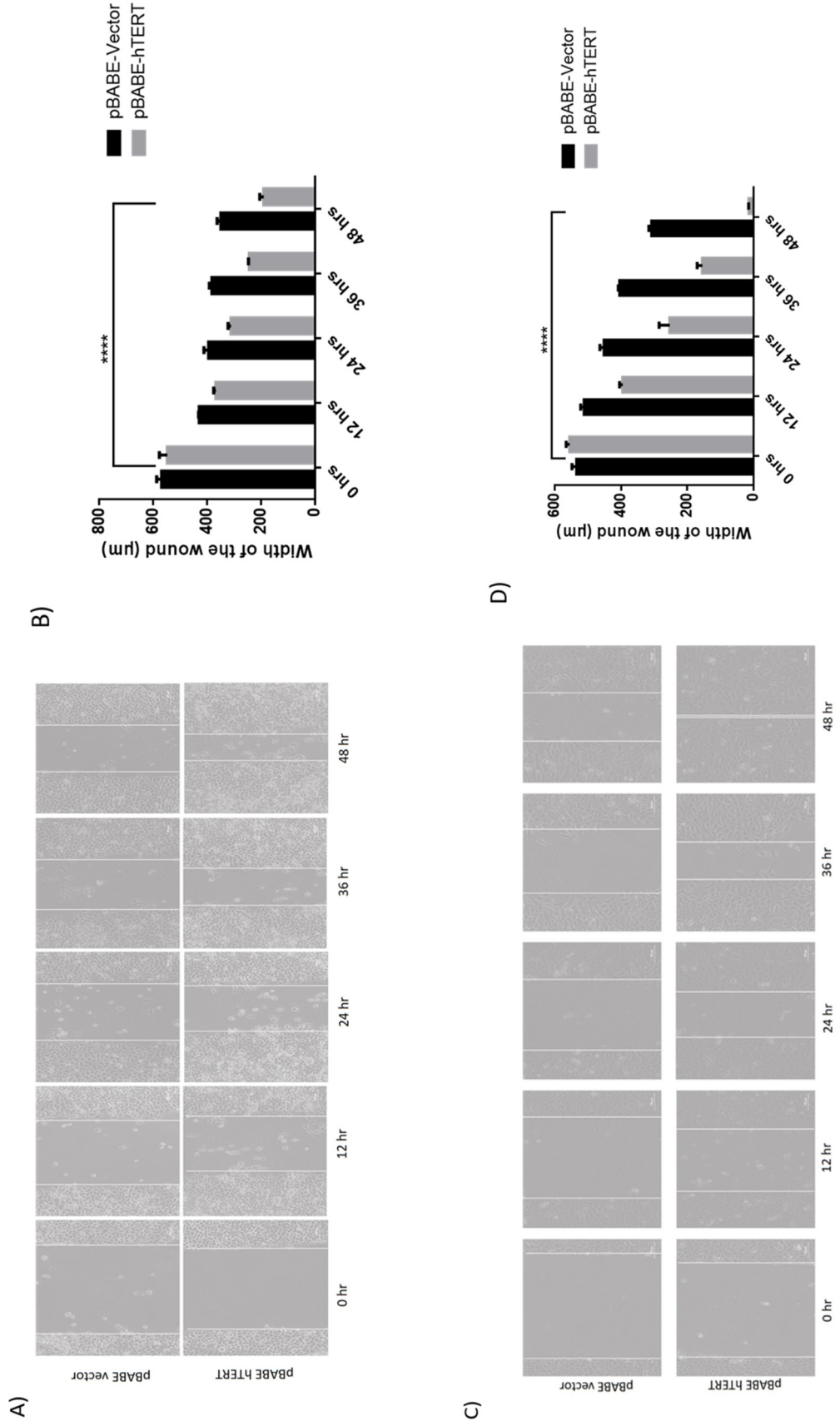
Figure 40: Migratory behaviour of HeLa and U2OS cells following ectopic expression of hTERT. (A & C) Microscopic images of in vitro wound healing at 0, 12, 24, 36 h and 48 h after the creation of wounds in HeLa and U2OS cells respectively. (B & D) Histograms represent quantification of the hTERT overexpression effect on cell motility (% migration) in HeLa and U2OS cells respectively.

4.5.2 Colony formation assay shows hTERT overexpression elevate the colony formation efficiency of HeLa and U2OS cells:

Colony formation assay was also performed to check the effect of hTERT on colony formation efficiency in HeLa Na U2OS cells. Briefly, hTERT overexpressing HeLa and U2OS cells were trypsinized, counted, and seeded for colony formation assay in 6-well plates at 500/well. During colony growth, the culture medium was replaced every 3 days. On the 10th day after seeding, the cells were fixed and then stained with crystal violet, and the number of colonies was counted. We have found that colony formation potential of hTERT overexpressing HeLa and U2OS cells were higher than vector transfected cells [Fig 41A-B].
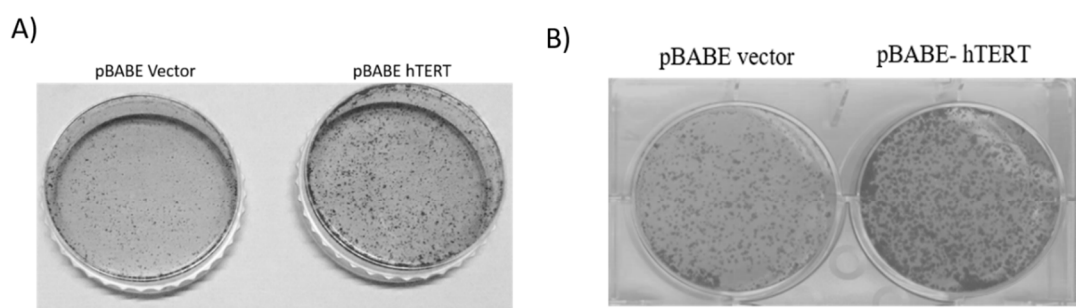
Figure 41: Colony formation assay in HeLa and U2OS cell line. (A) Representative images of crystal violet stained colonies in HeLa cells and (B) U2OS cells. We observed that hTERT overexpression increase the colony formation efficiency in both cell lines.

4.5.3 hTERT downregulation reduces the migratory potential and colony formation efficiency of cancer cells:

We have also checked migratory potential and colony formation efficiency after hTERT knockdown in HeLa cells. We find that hTERT knockdown suppresses the migratory potential and colony formation efficiency in HeLa cells [Fig 42A-C].
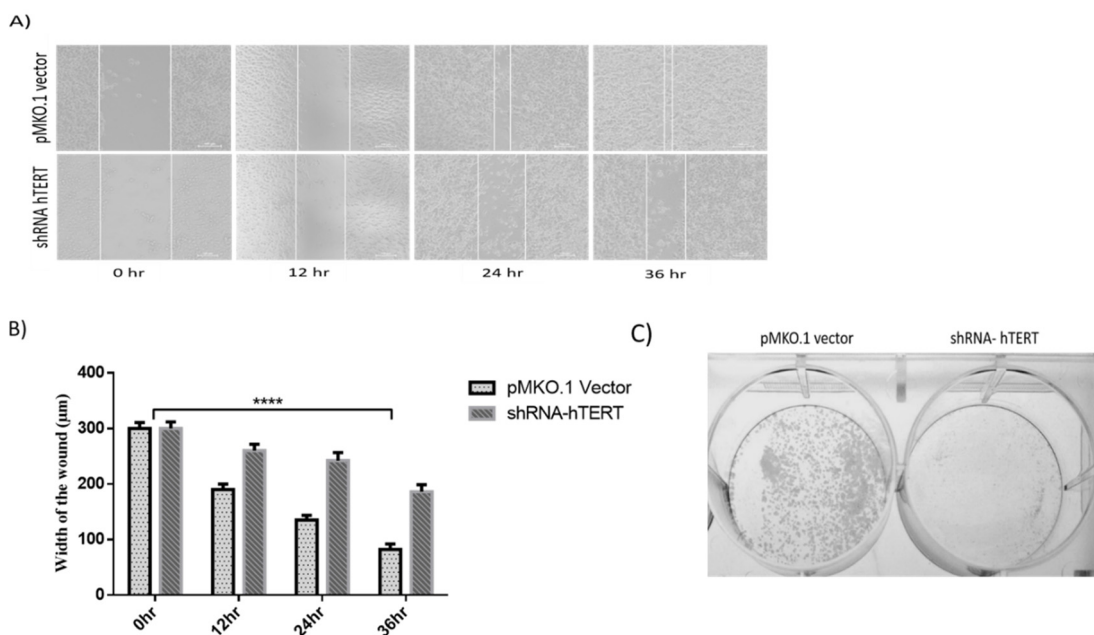
Figure 42: Migratory behaviour and colony formation ability of HeLa cells following hTERT knockdown. (A) Microscopic images of in vitro wound healing at 0, 12, 24 and 36 h after the creation of wounds in hTERT knockdown HeLa cells. (B) Histograms represent the effect of hTERT knockdown on cell motility (% migration) in HeLa cells. (C) Colony formation ability of hTERT knockdown cells was found less than vector transfected cells.

4.5.4 uPA downregulation reduces the migratory potential and colony formation efficiency of cancer cells:

We have also checked migratory potential and colony formation efficiency of HeLa cells after uPA knockdown. We have revealed that uPA knockdown suppresses the migratory potential and colony formation efficiency in HeLa cells [Fig 43A-B].
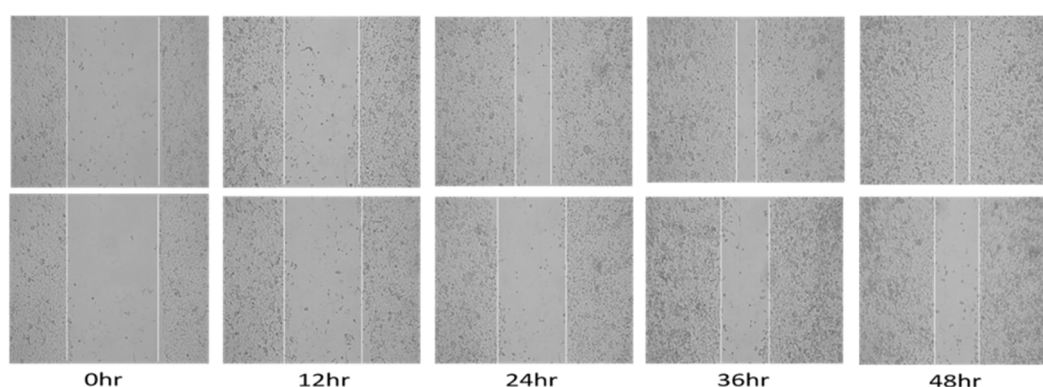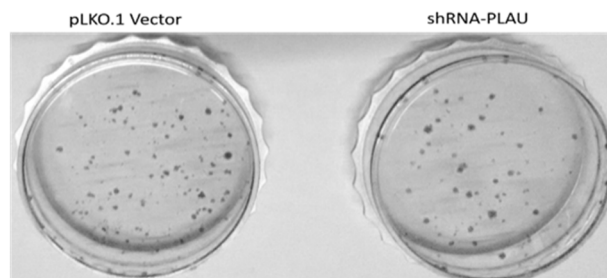
Figure 43: Migratory behaviour and colony formation ability of HeLa cells after uPA knockdown. (A) Microscopic images of in vitro wound healing at 0, 12, 24, 36 and 48h after the creation of wounds in uPA knockdown HeLa cells. (B) Colony formation ability of uPA knockdown cells was found less than vector transfected cells.

4.5.5 Discussion: Phenotypic consquences after overexpression and knock down of hTERT and uPA.

Cancer cells have a broad spectrum of invasion and migration mechanisms. These consist both collective and individual cell migration strategies [518]. It is reported that hTERT promotes EMT in cancer cells [4]. One of the most important parts of EMT is cancer cell migration. To check this we performed wound healing assay after hTERT

overexpression in HeLa and U2OS cells. We found that hTERT enhances cell migratory potential in both the cell lines. We have also performed migratory assay after hTERT knock down and observed that hTERT downregulation reduces the migratory potential of cancer cells. Similarly, we have also done colony formation assay in hTERT knockdown and overexpressing cells. We observe increased colony formation efficiency following hTERT overexpression in the cancer cells while its knockdown reduces cancer cell efficiency to form colonies. The urokinase like plasminogen activator (uPA) is another important molecule we have studied and its role in cancer cell migration is very well studied [8]. Wound healing and colony formation assay in uPA knockdown HeLa cells dmonstrate that uPA knockdown suppressed both migratory potential and colony formation ability of cancer cells.

Summary

Chapter 5: Summary

Cancer refers to a large group of diseases involving deregulated cell growth. Cancer cells divide and grow uncontrollably and in many cases form malignant tumors, and invade nearby and distant parts of the body. Cancer cells acquire unlimited proliferation potential. One of the ways of acquiring this involves reactivation of a specialized reverse transcriptase called telomerase which solves the end replication problem by adding telomeric repeats on to the ends of newly replicated chromosomes. Telomerase performs many other functions as well apart from maintaining telomere length such as gene regulation, apoptosis and DNA repair. Recently hTERT has also been shown to play role in cancer cell EMT. Lieu et al. (2012) reported a potential role of hTERT in EMT. They have shown that hTERT promotes EMT and stem cell like traits in gastric cancer cells when hTERT is ectopically expressed in these cells [4]. TGF-β is a well-known inducer of EMT, but down-regulation of hTERT by siRNA inhibited the TGF-β and β-catenin mediated EMT [4]. The role of uPA in EMT is also very well established [363]. We have already published our transcriptomics data showing regulation of uPA by hTERT [6]. But the molecular mechanism behind this was not elucidated. Therefore here we tried to explore the exact association between hTERT and uPA system as well as molecular mechanism by which hTERT regulates uPA expression. The hTERT overexpression in HeLa cells enhanced the expression of uPA at protein level but reduced uPA expression at RNA level [Fig 10A-D]. Interestingly uPAR expression at both protein and RNA level was enhanced in HeLa cells ectopically expressing hTERT [Fig 12A-D]. Moreover, uPA and uPAR expression were also upregulated in U2OS (a telomerase negative cells) cells [Fig11A-D & 13A-D]. The hTERT knockdown repressed the uPA and uPAR expression in HeLa cells [Fig21A-D & 22A-D]. Interestingly uPA knock down reduced the hTERT expression in HeLa cells. Collectively the results confirmed a positive association between hTERT and uPA system. Both hTERT and uPA are pro-survival for cancer cells. Moreover, we have already mentioned about TGF-β/wnt-β-catenin mediated EMT is hTERT dependent and uPA is a TGF-β/ wnt-β-catenin responsive gene. Here these two pro-survival proteins were correlated. We observed upregulation of uPA after TGF-β treatment in A549 cells [Fig27A-D). However uPA upregulation was repressed when hTERT was knocked down indicating that TGF-β mediated upregulation of uPA is

Summary

hTERT dependent (Fig28A-D). Vimentin and E-cadherin are the EMT markers frequently used by many researchers to check whether EMT is induced or repressed [138]. The expression of vimentin and uPA in cancer cells undergoing EMT remains high. We found uPA expression pattern was similar to vimentin which was expected. E-cadherin expression repressed when A549 cells treated with TGF-β while hTERT knockdown reversed TGF-β effect on E-cadherin expression. The role of uPA in cell migration and invasion are very well demonstrated [8]. It is a protease which degrades ECM and increases migration of the cancer cells. TGF-β enhanced cell migration in cancer cells [422]. We found that TGF-β treatment to A549 cells enhanced its migration in comparison to untreated A549 cells [Fig29]. We also observed that hTERT knockdown reduced the migration potential of the cells [Fig29]. But when we repressed hTERT expression in A549 cells TGF-β treatment becomes ineffective in enhancing cell movability migration. This may be due to lower uPA expression when hTERT was knocked down in cancer cells.

Our findings revealed a unique adaptation of cancer cells where both hTERT and uPA expression remain high for cancer cell progression. We have also shown that hTERT regulates the expression of urokinase type plasminogen activator and its receptor in both telomerase positive and telomerase negative cell lines. We have also established that TGF-β mediated induction of Urokinase type plasminogen activator is hTERTdependent.

We have also generated proteomic profile of hTERT overexpressing HeLa which is a telomerase positive cells and U2OS which is natural telomerase negative cells. Telomerase negative cells nevertheless offer a good experimental system to study the effect of hTERT overexpression on expression of other genes. In the present study, we document, for the first time significant effect of hTERT overexpression on the proteomic profile of U2OS cells. Here, we found Hsp90, Hsp70 and Hsp60 upregulated after hTERT overexpression in this cell line. Among several differentially regulated proteins we found some proteins such as Hsp60, Hsp70, and GAPDH disabide upregulated in both the cell lines whereas Hsp90 was upregulated only in U2OS cells. We have validated expression of these proteins by qRT-PCR and western blotting. In conclusion, this study shows that hTERT expression alters the proteomic profile of osteosarcoma and cervical cancer cells. Moreover, heat shock proteins are an important

subset of cellular proteins regulated by hTERT. GAPDH is also influenced by hTERT expression in both the cell lines. The findings make it pertinent to further investigate the relevance of telomerase associated molecules like peroxiredoxin-1 and alpha-enolase, as markers or therapeutic target.

We have also screened for genome-wide expression patterns by microarray following hTERT overexpression in U2OS cells. A list of 76 differentially regulated genes has been generated out of which 37 genes were upregulated and 36 genes were downregulated. We have randomly chosen three upregulated genes (TSPAN13, DLX5 and HMGCS2) and three downregulated genes (DHRS2, CRYAB and PDLIM1). Expression of these differentially expressed genes was validated by real-time PCR. Among these genes, TSPAN13 is very promising for future study as it inhibits cancer cell apoptosis. We found higher expression of this gene after hTERT overexpression which suggests that this may be an intermediate in the pathway by which telomerase inhibit apoptosis of cancer cells.

We have also performed wound healing and colony formation assay to know the metastatic nature of hTERT and uPA in cancer cells. Both hTERT and uPA enhance migratory potential and colony formation ability of cancer cells.

To sum up, our results demonstrate several extra-curricular function, reflect on its possible role as a transcription regulator, and retains its memory for these functions even in hTERT negative cells.

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Publications

Proteomic identification of proteins differentially expressed following overexpression of hTERT (human telomerase reverse transcriptase) in cancer cells

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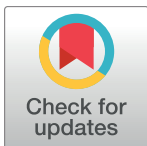
Abstract

Reverse transcriptase activity of telomerase adds telomeric repeat sequences at extreme ends of the newly replicated chromosome in actively dividing cells. Telomerase expression is not detected in terminally differentiated cells but is noticeable in 90% of the cancer cells. hTERT (human telomerase reverse transcriptase) expression seems to promote invasiveness of cancer cells. We here present proteomic profiles of cells overexpressing or knocked down for hTERT. This study also attempts to find out the potential interacting partners of hTERT in cancer cell lines. Two-dimensional gel electrophoresis (2-DE) of two different cell lines U2OS (a naturally hTERT negative cell line) and HeLa revealed differential expression of proteins in hTERT over-expressing cells. In U2OS cell line 28 spots were picked among which 23 spots represented upregulated and 5 represented down regulated proteins. In HeLa cells 21 were upregulated and 2 were down regulated out of 23 selected spots under otherwise identical experimental conditions. Some heat shock proteins viz. Hsp60 and Hsp70 and GAPDH, which is a housekeeping gene, were found similarly upregulated in both the cell lines. The upregulation of these proteins were further confirmed at RNA and protein level by real-time PCR and western blotting respectively.

Introduction

Cancer cells have unlimited proliferation potential. One way of acquiring this involves reactivation of a specialized reverse transcriptase called telomerase which solves the end replication problem by adding telomeric repeats on to the 3' ends of template strands so as to minimize on attrition of the lagging strands at their terminal 5' ends. Telomerase activity is found to be high in nearly 90% of cancerous cells as compared to normal differentiated somatic cells which do not have detectable telomerase activity. The telomerase basically consists of six main subunits viz. hTERT (human telomerase reverse transcriptase), dyskerin, p23, Hsp90, hTERC (human telomerase RNA component) and TEP1 (telomerase-associated

protein 1) [1]. Out of these six subunits, hTERT and hTERC can reconstitute the classical telomere lengthening in vitro and also perform many extracurricular functions of regulatory nature in vivo [2]. Stabilization of telomere length of fibroblast and other cell types is achieved by ectopic expression of hTERT in these cell lines which thus acquire infinite replicative potential [3]. Immortalization of both cancer cells and normal stem cells can be achieved by overexpression of telomerase [4–6]. Moreover, knowing the main roles of telo-merase in cancer cells would be helpful in the development of exact therapeutic strategies on the basis of telomerase inhibition [7,8]. Here, we have studied proteomic profile of cells fol-lowing hTERT overexpression in two different cell lines viz., the human osteosarcoma cell line U2OS, which is telomerase negative and HeLa, a cervical cancer cell line that has its own telomerase activity.

Materials and methods

Cell culture

Two cell lines Viz., 1) U2OS (an hTERT negative human osteosarcoma cell line) and 2) HeLa cells (an hTERT expressing cervical cancer cell line) were obtained from National Centre for Cell Science, Pune and grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone, South Logan Utah,) with 10% fetal bovine serum (FBS) (Himedia). Cells were maintained at 37˚C and 5% CO2 in a humidified CO2 incubator.

Transfection of cells and establishment of stable cell lines overexpressing hTERT

One day before transfection HeLa and U2OS cells were seeded in 6 well plates and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS and antibiotics (penicil-lin/ streptomycin). Two micrograms each of pBABE-puro empty vector and pBABE-puro hTERT obtained from Addgene and 2 μg of each were transfected in to HeLa cells and U2OS cells by using lipofectamine 3000 (Invitrogen). After 48hrs, transfected cells were selected by using 2μg/ml of puromycin and finally maintained in 1μg/ml of puromycin. Total protein was extracted at passage number 5 for two-dimensional gel electrophoresis.

Wound healing assay

Cell migration required for healing artificially created wound was assayed at 0, 12, 24, 36 and 48 hrs for hTERT overexpressing HeLa and U2OS cells. Briefly both HeLa and U2OS cells were separately seeded in 2 wells of a 6 well plate and cultured until confluency. Then by using a pipette tip we made a straight scratch, simulating a wound. The plates were washed gently and fresh DMEM replaced with supplements (serum, antibiotics). The cells were observed by phase contrast microscopy.

Two dimensional gel electrophpresis

We profiled cellular proteins after the overexpression of hTERT in HeLa and U2OS cells. 2D-Gel electrophoresis was performed as reported by Diao S et al [9]. Briefly, isoelectric focus-ing (IEF) was performed using an Ettan IPGphore 3 apparatus (GE healthcare) and using the nonlinear IPG strips of 13 cm in the pH range of 3.0–10.0. A total of 250 μg protein was diluted to 250 μl in a rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 1% DTT, 0.5% IPG buffer and some traces of bromophenol blue) and the rehydration step was continued for 16 h at room temperature. IEF was run following a step-wise voltage increase procedure in the

following order, 500 V for 5 hrs, 1000 V for 1 h and 8000 V for 3.5 h. After IEF, the IPG gel strips were placed in an equilibration buffer (5M urea, 10% SDS, 10% glycerol, 1.5 M Tris-HCL, pH 8.8 and some traces of bromophenol blue) 1 and 2 for 15 min each and then kept in SDS-page running buffer for 5 min. Equilibration buffer 1 contained 1% DTT while equilibration buffer 2 contained 2.5% iodoacetamide. Separation in the second dimension was performed by SDS-polyacrylamide (12%) gel elctrophoresis at constant voltage of 120 volt till the bromophenol blue dye front reached the lower end of the gels. Gels were fixed for 1 h in fixing solution (50% methanol and 10% acetic acid) and stained in colloidal Coomassie G-250 stain (that is compatible with downstream MS analysis, as peviously described [10] for 3 h, and then destained with 10% acetic acid. The images were scanned with a scanner (Ettan IPGphor3). Images were analysed by using ImageMaster 2D Platinum v7.0 gel analysis software-(GE Healthcare Life Sciences). After analysis of spots and normalizing for the background we excised 28 spots of our interest from the U2OS and 23 from HeLa and submitted them for mass analysis.

In-gel protein digestion and MALDI-TOF-TOF/MS analysis

Trypsin digestion of the excised bands were done according to Shevchenko et al. [11]. Briefly, after distaining, the gel was washed twice with mili-Q water and the spots of interest excised from the gel and cut into 1mm cubes. These small 1mm cubes of gels were transferred to a 1.5 ml microcentrifuge tube pre-rinsed with 100% acetonitrile. Gel particles were further washed with a solution of 100 mM ammonium bicarbonate in 100% acetonitrile and water. After 15 minute of incubation on a rotatory shaker supernatant was discarded and this step was repeated till completion of destaining. After destaining all the remaining liquids were removed and enough acetonitrile was added to cover the gel particles which let the particles to shrink together. Acetonitrile was removed completely and gel particles were dried down in a vacuum centrifuge at room temperature. Further gel particles were swelled in a solution of 50 μl each of 10 mM DTT and 100 mM ammonium bicarbonate and incubated for 45 minute at 56˚C. After this incubation, tubes were cooled at room temperature and excess liquid removed and replaced quickly by same volume as above of freshly prepared solution of 55 mM iodoacetamide in 100 mM ammonium bicarbonate and further incubated for 30 min at room temperature. The gel was washed again with a solution of acetonitrile and ammonium bicarbonate and dried down in a vacuum centrifuge. Enough sequencing grade modified trypsin was added in the tube and incubated at 37˚C for 30 minutes. 5μl of 25 mM ammonium bicarbonate was added to keep the gel moistened. Trypsin added tubes were further incubated at 37˚C overnight. Next day supernatants were collected in new microfuge tubes and 10 μl of 1% TFA and 10 μl of 100% acetonitrile were added to the gel and the mix sonicated for 20 min at room temperature. Supernatants were taken and pooled and further dried in Speed vac and submitted for mass analysis.

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from U2OS and HeLa cells using TRIzol reagent (Sigma). cDNA was synthesized by using reverse transcription kit (Thermofisher) according to the manufacturer's protocol. 1 μg cDNA was used as template for PCR reaction using gene specific primers. The real-time primers sequences are given in Table 1. Real Time PCR conditions were: 15 sec at 95˚C for denaturation, 1 min at 60˚C for both annealing and elongation over 40 cycles in Applied Biosystems 7500. Data were normalized with reference to actin used as endogenous control.

Table 1. Primers used for PCR-based assays.

Gene name	Real-time primers sequences (5'-3')
HSP60	Forward primer- `TGCCAATGCTCACCGTAAG`
	Reverse primer- `ACTGCCACAACCTGAAGAC`
HSP70	Forward primer- `ACCAAGCAGACGCAGATCTTC`
	Reverse primer- `CGCCCTCGTACACCTGGAT`
HSP90	Forward primer- `ACTACACATCTGCCTCTGGTGATGA`
	Reverse primers- `TGTTTCCGAAGACGTTCCACAA`
hTERT	Forward primer- `CGGCGACATGGAGAACAAG`
	Reverse primers- `CCAACAAGAAATCATCCACCAAA`
GAPDH	Forward primer- `GTCTTCACCACCATGGAGAAGGCT`
	Reverse primers- `CATGCCAGTGAGCTTCCCGTTCA`
ACTIN	Forward primer- `GGCACCCAGCACAATGAAG`
	Reverse primers- `GCCGATCCACACGGAGTACT`

https://doi.org/10.1371/journal.pone.0181027.t001

Western blotting

Western blotting was performed as previously described [12]. Stable U2OS and HeLa cells carrying pBABE-puro empty vector and pBABE-hTERT were lysed in RIPA buffer, (GCC biotech). Cell lysates were quantified by Bradford assay and 40 μg of total protein was separated by SDS polyacrylamide gel electrophoresis. Proteins resolved on SDS-PAGE gels were further transferred by making sandwich of (-ve pole) transfer pads-two Whatman filter paper-gel-Polyvinylidene difluoride (PVDF) membrane (Millipore)-two Whatman filter paper-transfer pads (+ve pole) clamped between transfer sheets. The whole assembly was fitted in the transfer apparatus in a way that the membrane should be on the positive pole while the gel should be on negative pole which facilitates the migration of negatively charged proteins towards positive pole. Whole assembly is run at 80 volts of constant voltage at 4 degree for 2 hrs. The blots were kept in blocking buffer (5% skimmed milk in 1xPBST) for two hours on a reciprocating shaker. After blocking, blots were incubated with primary antibody against hTERT (Santa Cruz, USA, Sc-393013, lot# F0716), β-actin (Santa Cruz, USA, Sc-47778, Lot# 12208), HSP90 (Enzo-Life sciences, ADI-SPA-844-F), HSp70 (Enzo-Life sciences, ADI-SPA-757-F), GAPDH and HSP 60 (Enzo-Life sciences, ADI-SPA-806-F), followed by incubation with secondary antibodies i.e., Goat anti-rabbit immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked antibody (dilution, 1:5,000, Bangalore Genei, 114038001A) and Goat anti-mouse immunoglobulin G (IgG), horseradish peroxidase (HRP)-linked antibody (dilution, 1:5,000, Bangalore Genei, 114068001A). Luminata™ Forte western HRP substrate was used for band visualization according to the manufacturer's protocol. β-actin was used as an internal control for protein expression. Quantification of protein expression was done by ImageJ software. (Primary data related to methodology and results presented in this paper may be viewed in S1 File)

Results

Overexpression of hTERT in U2OS and HeLa cell line

Overexpression of hTERT in HeLa and U2OS cells was confirmed by qRT-PCR and western blotting. Remarkably, hTERT mRNA expression was upregulated to approximately 180 fold in U2OS cells transfected with pBABE-puro-hTERT in comparison to vector transfected HeLa
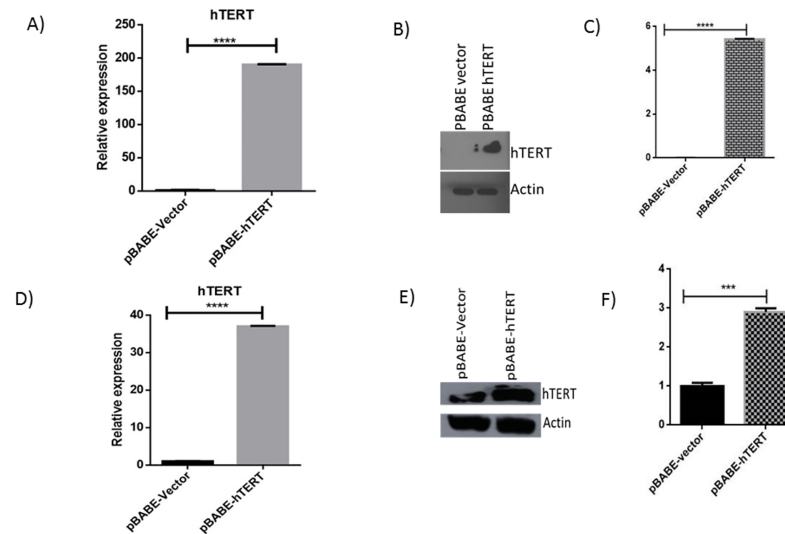
Fig 1. hTERT overexpression in HeLa and U2OS cell lines. hTERT is overexpressed in U2OS and HeLa cell lines. (A & D) mRNA level of hTERT in U2OS & HeLa cell lines was determined by quantitative real-time PCR. (B & E) Western blotting confirms overexpression of hTERT in U2OS and HeLa cell line. (C & F) Histograms depict densitometric quantification of the hTERT overexpression of three corresponding independent Western blot experiments in U2OS and HeLa cell lines.

cells (Fig 1A) while in HeLa it is upregulated to 36 fold (Fig 1C). Overexpression of hTERT in U2OS and HeLa cell lines was futher confirmed by western blotting (Fig 1B and 1D).

Overexpression of hTERT in U2OS and HeLa cell line enhances the migration rate of these cells

The biological consequence of hTERT overexpression in HeLa and U2OS cells was studied employing wound healing assay which revealed increased migration rate of hTERT overexpressing U2OS cells (Fig 2A and 2B) and HeLa cells (Fig 2C and 2D) in comparison to vector transfected cells.

hTERT overexpression alters the proteomic profile of human cervical cancer and human osteosarcoma cells

U2OS is a telomerase negative cell line and it has an ALT pathway to maintain the telomere length and thus it offers a clean baseline for observing any alteration in protein expression after hTERT overexpression in this cell line. After staining and analysis of gels by ImageMaster 2D Platinum v7.0 gel analysis software-(GE Healthcare Life Sciences), 28 spots (Table 2) showed differentially expressed proteins in U2OS cells out of which 23 were upregulated and 5 were down regulated (Fig 3A). In HeLa cells we excised 23 spots (Table 3) representing differentially expressed proteins out of which 21 were up regulated and 2 were downregulated (Fig 3B) proteins. Most of these differentially expressed proteins seemed to be functionally associated with tumorigenesis. We found proteins involved in intermediate filament formation, glycolysis, antioxidant activity, heat shock proteins, apoptosis, nucleotide-sugar biosynthesis, metastasis, xenobiotic metabolism, ubiquitination and glycosylation [Tables 2 and 3]
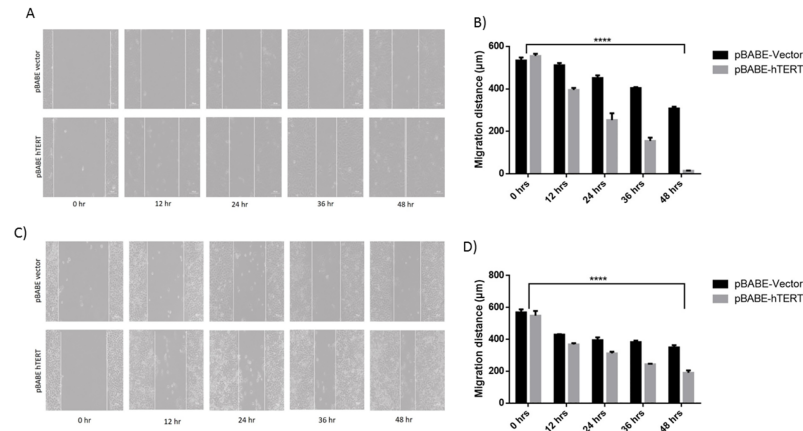
Fig 2. hTERT overexpression enhances the migration of cancer cells. A) Microscopic images of in vitro wound healing at 0, 12, 24, 36 and 48 h after the creation of wounds in U2OS cells. B) Histogram represents quantification of the effect of hTERT overexpression on cell mobility (% migration) in U2OS cell line. C) Microscopic images of in vitro wound healing at 0, 12, 24, 36 and 48 h after the creation of wounds in HeLa cells. D) Histogram representing quantification of the effect of hTERT overexpression on cell mobility (% migration) in HeLa cell line.

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Table 2. List of proteins differentially expressed following hTERT overexpression in U2OS cells.

| Spot no.	Protein Identification	Sequence Coverage (%)	pI	Mass (kDa)	Expression level	Score
1	Immunoglobulin gamma heavy chain variable region, partial	56.1%	10	109	Downregulation	51
2	UDP-glucuronic acid decarboxylase 1 isoform 3	21.8%	9.6	28	Upregulation	47
3	Glyceraldehyde-3-phosphate dehydrogenase isoform 2	23.9%	7.9	32	Upregulation	68
4	Heat shock protein HSP 90-beta isoform c	21.1%	4.8	83	Upregulation	99
5	Heat shock 70kDa protein 8 isoform 1 variant, partial	33.7%	5.2	71	Upregulation	152
6	hCG2038865, partial	31.9%	10.3	102	Upregulation	46
7	L-lactate dehydrogenase B chain isoform LDHB	11.4%	5.7	37	Upregulation	44
8	Unnamed protein product	21.0%	4.6	51	Upregulation	100
9	dnaJ homolog subfamily C member 12 isoform X1	30.2%	5.9	137	Upregulation	40
10	Alternative protein CDH6	49.4%	11.9	101	Downregulation	43
11	Alpha-enolase isoform X1	14.1%	6.7	47	Upregulation	49
12	PDZ and LIM domain 2 (mystique), isoform CRA_c, partial	19.9%	13	178	Upregulation	53
13	Heat shock 60kDa protein 1 (chaperonin)	12.4%	9.1	60	Upregulation	72
14	hCG1789535	15.6%	9.3	50	Downregulation	15
15	hCG2040343, partial	55.6%	10.8	79	Upregulation	31
16	Enolase 1 variant, partial	21.0%	7.7	47	Upregulation	73
17	Mucin, partial	66.7%	12.8	27	Upregulation	38
18	Immunoglobulin M heavy chain, partial	100.0%	4.2	28	Upregulation	36
19	Glutathione S-transferase P	17.6%	4.1	23	Upregulation	81
20	BiP protein, partial	15.2%	5.1	71	downregulation	67
21	Ubiquitin carboxy-terminal hydrolase L1, partial	19.2%	5.2	23	downregulation	40
22	hCG2045028	35.9%	6	44	Upregulation	36
23	Unnamed protein product	10.4%	6.8	26	Upregulation	36
24	Unknown, partial	61.5%	5	45	Upregulation	35
25	RAMP2	13.7%	5.4	19	Upregulation	41
26	Annexin A5	45.6%	4.8	35	Upregulation	155
27	T-complex protein 1 subunit beta isoform 1	20.7%	6	58	Upregulation	89
28	StAR-related lipid transfer protein 7, mitochondrial precursor	12.4%	9.5	43	Upregulation	41

https://doi.org/10.1371/journal.pone.0181027.t002

Fig 3. Two dimensional gel electrophoresis of hTERT overexpressing (A) U2OS and (B) HeLa cell line.
Total proteins were extracted from hTERT overexpressing U2OS and HeLa cells and separated non-linearly on IPG strip of PH 3–10, followed by electrophoresis through 12% polyacrylamide gels. The gels were further stained and analyzed by image master 2D platinum software.

https://doi.org/10.1371/journal.pone.0181027.g003

Upregulation of Hsp90, Hsp70 and Hsp60

We found significant over-expression of heat shock proteins Hsp70 and Hsp60 in hTERT overexpressing U2OS and HeLa cells. Hsp90 was upregulated in only U2OS cells. Hsp90 is an important subunit of telomerase and it helps in stabilizing a functional telomerase structure and in primer loading and extension [13]. Hsp70 is overexpressed in most of the cancer cells though its expression in cancer cells is typically a poor marker for prognosis [14]. Heat shock protein 60 (HSP60) plays a crucial role in malignant cell survival [15]. To confirm regulation of Hsp60 and Hsp70 by hTERT, we performed qRT-PCR (Fig 4A and 4B) and western blotting (Fig 4C & 4D) to check their expression in U2OS and HeLa cells carrying pBABE-Vector and pBABE-hTERT expression construct. QRT-PCR and Western blotting confirmed the upregulation of Hsp90 at transcript as well as protein level in U2OS cells (Fig 4E).

Upregulation of GAPDH

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is basically a glycolytic enzyme and a well-known housekeeping marker and commonly used as an endogenous control to assess cancer related gene expression. However, reports indicate implication of GAPDH in other diverse functions independently of its role in energy metabolism. Deregulation in the expression level of GAPDH are found in many cancer cells [16]. Expression of GAPDH was enhanced in both U2OS and HeLa cells overexpressing hTERT. To confirm the upregulation

Table 3. List of proteins differentially expressed following hTERT overexpression in HeLa cells.

Spot no.	Protein Identification	Sequence Coverage (%)	pI	Mass (kDa)	Expression level	Score
1	Keratin 18	29.3%	5.3	48	Upregulation	68
2	Chain A, Crystal Structure Of The Globular Domain Of Human Calreticulin	32.8%	4.6	60	Upregulation	53
3	Chain A, Structural Basis Of Human Triosephosphate Isomerase Deficiency. Mutation E104d And Correlation To Solvent Perturbation.	47.2%	6.5	27	Upregulation	88
4	Glyceraldehyde-3-phosphate dehydrogenase isoform 2	31.4%	7.9	32	Upregulation	75
5	Peroxiredoxin 1, isoform CRA_b, partial	34.6%	6.5	21	Upregulation	47
6	Peroxiredoxin-1	62.3%	9.2	22	Upregulation	130
7	Chain K, Acetyl-Cypa:cyclosporine Complex	36.4%	7.8	18	Upregulation	73
8	Calcium-activated chloride channel regulator family member 3	29.4%	9.2	30	Upregulation	47
9	Chain A, Human Heart L-lactate Dehydrogenase H Chain, Ternary Complex With Nadh And Oxamate	27.3%	5.7	37	Upregulation	52
10	Mitochondrial ribosomal protein L46, isoform CRA_d	29.5%	9.1	19	Upregulation	34
11	F-actin-capping protein subunit alpha-1	33.2%	5.4	33	Upregulation	47
12	Chain A, Structural Basis For The Interaction Of Human β-defensin 6 And Its Putative Chemokine Receptor Ccr2 A nd Breast Cancer Microvesicles	59.2%	10.2	57	Upregulation	45
13	Heat shock 70kDa protein	14.0%	6	73	Upregulation	69
14	Galactose-1-phosphate uridyl transferase	100.0%	9.5	32	Upregulation	40
15	T cell receptor alpha, partial	46.5%	9.6	78	Upregulation	40
16	PR domain containing 8, isoform CRA_b	39.8%	7.6	19	Upregulation	44
17	T cell receptor alpha chain V-J-region, partial	9.6%	9.5	12	Upregulation	39
18	Unnamed protein product	19.3%	5.4	59	Upregulation	36
20	Chaperonin (HSP60)	29.4%	5.5	60	Upregulation	79
21	Chromosome 14 open reading frame 68, isoform CRA_a	60.8%	12.1	78	Upregulation	41
22	Cofilin 1 (non-muscle), isoform CRA_c, partial	38.7%	9.4	158	Downregulation	49
23	Unnamed protein product	10.4%	6.8	126	Downregulation	36

of GAPDH by hTERT we performed qRT-PCR (5A & 5B) and western blotting (Fig 5C and 5D) in both U2OS and HeLa cell line. We found hTERT causes upregulation of GAPDH at transcriptional level only, there being no conspicuous change at protein level in either cell line.

Discussion

Telomerase components perform many functions apart from its canonical role in telomere lengthening. One such extracurricular function of hTERT is the regulation of cell's signaling pathways. Many types of cancer do not express telomerase and being telomerase negative cannot manifest extracurricular activity of telomerase. They have ALT pathway to maintain telomere length. Remarkably, hTERT could be expressed at very high levels even in cells with a basal endogenous level of expression and allowed normal viability. Telomerase negative cells nevertheless offer a good experimental system to study the effect of hTERT overexpression on expression of other genes. Human osteosarcoma is a primary malignant tumor of the bone and the U2OS cell line derived from it is telomerase negative. In the present study, we document, for the first time significant effect of hTERT overexpression on the proteomic profile of U2OS cells. Here, we found Hsp90, Hsp70 and Hsp60 upregulated after hTERT overexpression in this cell line. Heat shock proteins usually act as molecular chaperones and are expressed at high levels in many cancers, although Hsp overexpression is only a poor prognosis in terms of survival and response to therapy in specific cancer types [17]. Heat shock protein 90 (Hsp90) is an abundant molecular chaperone that helps in conformational maturation and

Fig 4. Validation of upregulation of heat shock proteins in U2OS and HeLa cell lines. The hTERT induced upregulation of Hsp60 and hsp70 were confirmed by qRT-PCR in (A) U2OS and (B) HeLa cell lines respectively. C) Western blotting showing upregulation of Hsp60 and Hsp70 at protein level. D) Histogram shows the results by applying ImageJ software. D) To confirm the upregulation of Hsp90 in U2OS cell line E) QRT-PCR and F) western blotting is performed.

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stabilization of various oncogenic proteins leading to tumor cell survival and disease progression [18]. Hsp90 interacts with a variety of intracellular proteins and is involved in differentiation, survival and cell growth [13]. It is demonstrated that inhibiting Hsp90 in osteosarcoma cells induces apoptosis [18]. Jennifer McCleese (2009) reported that inhibitioin of Hsp90 results in loss of cell viability, induction of apoptosis, and inhibition of cell proliferation in osteosarcoma cells [19]. It has also been shown that blocking HSP90 addiction inhibits tumor cell proliferation, metastasis and development [20]. Moreover, Hsp90 is also a subunit of telomerase complex and it stabilizes telomerase and helps in loading of telomerase complex to telomere [13]. Though there is no hTERT in U2OS cells, its overexpression upregulated Hsp90
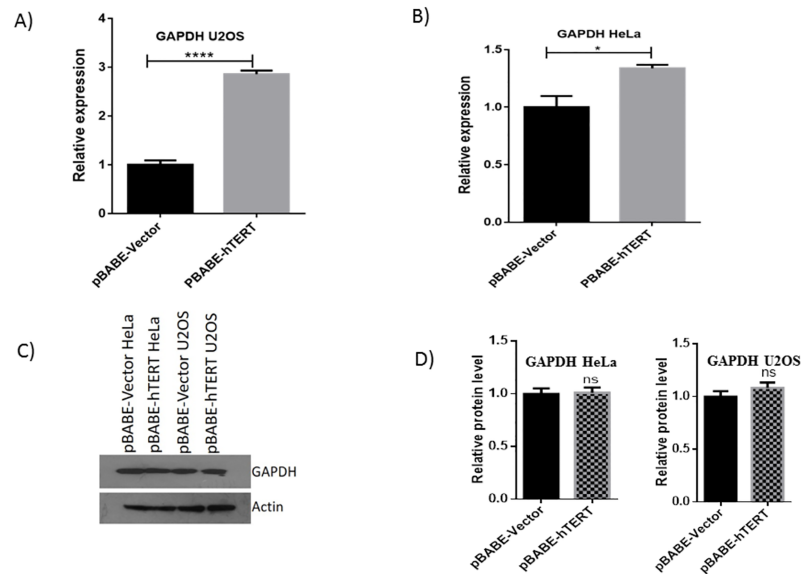
Fig 5. Validation of GAPDH in HeLa and U2OS cell lines. QRT-PCR was performed to check the hTERT induced upregulation of GAPDH in (A) U2OS and (B) HeLa cell lines. C) Represents results of Western blotting to check the up-regulation of GAPDH at protein level while (D) represents histograms of respective blots showing no prominent difference in expression of GAPDH at protein level.

(Fig 4d) suggesting intactness of the circuitry of intercommunication between hTERT and Hsps in telomerase negative cells.

Similar to Hsp90, the role of Hsp70 in cancer is very well studied. Being a molecular chaperone, Hsp70 is an important part of cellular networks, involving signaling, membrane, transcriptional and organelles functions [21]. In many cancer increased expression of Hsp70 is correlated with poor prognosis [21]. Lei Zhao et al. reported that viability of osteosarcoma cells was adversely affected after knocking down of Hsp70 [22]. It is also demonstrated that Hsp70 expression prevents apoptosis in osteosarcoma cells [23]. The role of Hsp70 in invasion and metastasis of cancer cells are also very well studied [24]. Moreover, Hsp70 inhibitor in cervical cancer also inhibits cancer cell proliferation [25]. R Ralhan et al. (1995) found that, Hsp70 overexpression can be correlated with elevated proliferation and tumor size in uterine cervical cancer [26]. Importantly, Hsp70 is a potent buffering system for cellular stress either from extrinsic (physiological, viral and environmental) or intrinsic (replicative or oncogenic) stimuli. For survival, cancer cells depend heavily on this buffering system [14]. Moreover, reports have also shown association of hTERT with Hsp70. Hsp70 binds with hTERT when hTR is absent and it gets dissociated when telomerase is folded into its active state [27] suggesting a transient association of Hsp70 with telomerase. We found upregulation of Hsp70 both at RNA level and protein level (Fig 4A & 4C). Another heat shock protein showing differential expression in association with hTERT is Hsp60 which has been reported to interact with hTERT in mitochondria [28]. Initially, Hsp60 was found only in mitochondria but since last few year, studies confirmed its presence the cytosol, the cell surface and in the extracellular space [29]. Inside the mitochondria it was reported that it binds mainly with Hsp10. However, in addition to its association with Hsp10, other interacting molecules have also been identified for Hsp60 in recent years [29]. Similar to Hsp70, the role of Hsp60 in cancer proliferation, tumor cell survival and metastasis are very well demonstrated in both osteosarcoma and cervical cancer cells [30,31].

We also found upregulation of Hsp60 and Hsp70 in HeLa cells which, unlike U2OS, are telomerase positive (Fig 4B & 4C). In the present study we have determined that hTERT may be an important modulator of heat shock proteins in these cells. However, it will be necessary to scan a wider range of cells for their responses in order to arrive at any generalization.

Yet another protein which was differentially overexpressed is GAPDH which is product of a housekeeping gene and is commonly used as internal control in different experimental condition. This enzyme is mainly used during glycolysis but it also has many diverse functions independent of its role in glycolysis [32]. Recent findings including our own show that expression level of GAPDH is highly regulated in various cancer cells [16].

There are signs of involvement of GAPDH in cancer progression and it may serve as a new marker or even a therapeutic target [16]. Moreover, it has been already shown that GAPDH mediates many oxidative stress responses, including nuclear translocation of GAPDH and induction of cell death. Craig Nicholls et al.(2012) reported that GAPDH interacts with telomerase RNA component hTR and inhibits telomerase activity which leads to telomere shortening and senescence in breast cancer cells [33]. Here we show that hTERT overexpression leads to the upregulation of GAPDH in both U2OS and HeLa cell lines suggesting that GAPDH may also interact with hTERT but in a positive manner. We do not find any cell death after hTERT overexpression. Apart from heat shock proteins and GAPDH there are many other proteins found to be differentially regulated. Keratin 18 is an intermediate filament protein and can be used for detection of proliferating fractions in the breast cancer [34]. This protein is found upregulated in HeLa cells following hTERT overexpression suggesting that hTERT modulates the expression of cytoskeletal proteins in cancer cells. Another upregulated protein we found is peroxiredoxin 1. This is an antioxidant enzyme associates with telomeres and protects it from oxidative damage and preserves telomeres for extension by telomerase [35]. Upregulation of peroxiredoxin in hTERT overexpressing HeLa cells indicates that hTERT enhances the expression of peroxiredoxin 1 to protect telomeres from any oxidative damage. It is well known that hTERT promotes EMT and during EMT there is change in expression of some epithelial and mesenchymal markers; level of mesenchymal markers goes up while that of epithelial markers goes down. CDH6 which is a type 2 cadherin and an epithelial marker drives EMT during embryonic development and it is aberrantly re-activated in cancer [36]. We found downregulation of CDH6 in U2OS cells when hTERT is overexpressed showing that hTERT promotes mesenchymal character in these cells. Another down regulated protein we found is ubiquitin carboxyl-terminal hydrolase L1 (UCHL1). It is a cysteine protease belongs to the UCH proteases family and has also acquired E3 ubiquitin-protein ligase activity and stabilizes ubiquitin monomers in vivo [37]. This protein has heterogeneous expression in cancer cells and performs both tumor inhibition and promoting functions [38]. Down regulation of this protein in U2OS cells after hTERT overexpression indicates that hTERT modulates the expression of this deubiquitinating enzyme to avoid proteosomal degradation of itself. Furthermore, apart from GAPDH another glycolytic enzyme upregulated is alpha-enolase which is now used as a potential cancer prognostic marker and promotes invasion in cancer cells [39] indicating promotion of invasion by hTERT via regulation of these glycolytic enzymes. We also found that hTERT expression led to the increase in migration rate of both U2OS and HeLa cells indicating that higher level of hTERT is linked with high invasive tumor phenotype.

In conclusion, this study shows that hTERT expression alters the proteomic profile of osteosarcoma and cervical cancer cells. Moreover, heat shock proteins are an important subset of cellular proteins regulated by hTERT. GAPDH is also influenced by hTERT expression in both the cell lines. The findings make it pertinent to further investigate the relevance of telomerase associated molecules like peroxiredoxin-1 and alpha-enolase, as markers or therapeutic target.

Supporting information

S1 File. Table A: Real time data of hTERT overexpression in U2OS cells. Fig A: Western blotting of hTERT overexpression in U2OS cells. Table B: Densitometric quantification of western blot. Table C: Real time data of hTERT overexpression in HeLa cells. Fig B: Western blotting of hTERT overexpression in HeLa cells. Table D: Densitometric quantification of western blot. Fig C: Wound healing assay in U2OS cells. Table E: Table 5: Distance migrated (μm) value which is used to make graph in hTERT overexpressing U2OS cells. Fig D: Wound healing assay in HeLa cells. Table F: Distance migrated (μm) value which is used to make graph in hTERT overexpressing HeLa cells. Fig E: Two dimensional gel electrophoresis of vector expressed U2OS cells. Fig F: Two dimensional gel electrophoresis of hTERT overexpressing U2OS cells. Fig G: Two dimensional gel electrophoresis of vector expressed HeLa cells. Fig H: Two dimensional gel electrophoresis of hTERT overexpressing HeLa cells. Table G: Real-time data of Hsp60 in U2OS cells. Table H: Real time data of Hsp70 in U2OS cells. Table I: Real-time data of Hsp60 in HeLa cells. Table J: Real time data of Hsp70 in HeLa cells. Fig I: Western blotting of Hsp60 and Hsp70 in U2OS and HeLa cells following hTERT overexpression. Table K: Distance migrated (μm) value which is used to make graph of Hsp60 in HeLa cells. Table L: Distance migrated (μm) value which is used to make graph of Hsp60 in U2OS cells. Table M: Distance migrated (μm) value which is used to make graph of Hsp70 in HeLa cells. Table N: Distance migrated (μm) value which is used to make graph of Hsp70 in U2OS cells. Table O: Real time data of Hsp90 in U2OS cells. Fig J: Western blotting of Hsp90 in U2OS cells following hTERT overexpression. Table P: Real time data of GAPDH in U2OS cells. Table Q: Real time data of GAPDH in HeLa cells. Fig K: Western blotting of GAPDH in U2OS and HeLa cells following hTERT overexpression. Table R: Densitometric quantification of GAPDH in U2OS cells. Table S: Densitometric quantification of GAPDH in HeLa cells. (DOC)

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Mini review

TELOMERASE AND ITS EXTRACURRICULAR ACTIVITIES

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Abstract: The classical activity of telomerase is to synthesize telomeric repeats and thus maintain telomere length, which in turn ensures chromosome stability and cellular proliferation. However, there is growing evidence that implicates telomerase in many other functions that are independent of TERC being used as its template. Telomerase has an RNA-dependent RNA polymerase (RdRP) activity in the mitochondria. Other than viral RdRPs, it is the only RNA-dependent RNA polymerase that has been identified in mammals. It also plays a role in the Wnt signaling pathway by acting as a transcriptional modulator. Telomerase acts as a reverse transcriptase independent of its core subunit, TERC. Studies indicate that telomerase is also involved in apoptosis and DNA repair.

Key words: Telomerase, Telomere, RNA-dependent RNA polymerase, TERT, TERC, Apoptosis

INTRODUCTION

In evolutionary terms, the multiple linear chromosomes in eukaryotes are at a great selective advantage, because they permit a high rate of recombination involving random reciprocal translocation of chromosomal DNA during sexual

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Abbreviations used: BRG1 – Brahma-related gene 1; PIP1 – POT1-interacting protein 1; PTOP – POT1- and TIN2-organizing protein; RAP1 – repressor and activator protein 1; RdRP – RNA-dependent RNA polymerase; SMARCA4 – SWI/SNF-related matrix-associated actin-dependent regulator of chromatin, subfamily a, member 4; SWI/SNF – SWItch/sucrose non-fermentable; TEP1 – telomerase-associated protein 1; TINT1 – TIN2 Interacting Protein 1; TIN2 or TINF2 – TRF1-interacting nuclear protein 2; TRF1 or TERF1 – telomeric repeat-binding factor 1; TRF2 or TERF2 – telomeric repeat-binding factor 2

reproduction. The presence of linear chromosomes has two negative aspects: the difficulty with replication of the extreme ends; and the need to protect the ends from degradation and fusion with other chromosomes. DNA-dependent DNA polymerase is unable to copy the 3' end of the template, which means that the copy strand shortens with each cycle of DNA replication, while the 3' end of leading strand is continuously synthesized by conventional DNA-dependent DNA polymerase until the end of the template. This means that the terminal DNA gets shorter through successive cycles of replication, which eventually leads to crisis and loss of cell viability [1, 2].

The ends of linear chromosomes are made up of multiple repeats of a short sequence known as the telomere, which is usually G/T-rich and heterochromatic and thus transcriptionally inactive. Telomeres are DNA-protein complexes with repeats that vary widely between species: ciliate *Oxytricha nova* has 4.5 repeats of G_4T_4, while *Saccharomyces cerevisiae* has around 500 bp of telomeric repeats [3].

Human telomeres are made up of 5'-TTAGGG-3' repeat sequences that are bound by a protein complex known as the shelterin complex [3]. Together with telomeric DNA, this protein complex forms a cap-like structure consisting of six proteins: (i) telomeric repeat-binding factor 1 (TRF1 or TERF1); (ii) telomeric repeat-binding factor 2 (TRF2 or TERF2); (iii) TRF1-interacting nuclear protein 2 (TIN2 or TINF2); (iv) TPP1 (also referred to as TINT1, PTOP or PIP1; nomenclature provided by 3 independent research groups that each reported the protein under a different name [4-6]); (v) protection of telomerase 1 (POT1); and (vi) repressor and activator protein 1 (RAP1), which caps the end of the chromosome, protecting it from degradation and chromosomal fusion [7]. Humans have approximately 10-15 kb of telomeric DNA, while mice have 20-50 kb of telomeric repeats. Most of this telomeric DNA is double stranded but also contains a single-stranded region of several hundred base pairs which is always G-rich [8-11]. This single-stranded region basically plays two very important roles: it facilitates extension of the telomere by providing primers to telomerase, and it binds with different components of the shelterin complex to form a cap at the end of the chromosome.

TELOMERASE STRUCTURE AND FUNCTIONS

Cells have evolved a specialized reverse transcriptase called telomerase, which solves the end replication problem by adding telomeric repeats onto the ends of newly replicated chromosomes. Greider and Blackburn were awarded the Nobel Prize in Physiology and Medicine in 2009 for their discovery of telomerase in *Tetrahymena thermophila* [12]. Almost 90% of cancerous cells have telomerase activity, while most normal differentiated somatic cells do not [13].

Telomerase is a ribonucleoprotein complex consisting of at least six subunits: human telomerase RNA component (hTERC); heat shock protein 90 (hsp90); human telomerase reverse transcriptase (hTERT); telomerase-associated protein 1 (TEP1); p23; and dyskerin [14]. hTERC and hTERT are the core subunits and

their *in vitro* reconstitution leads to telomerase activity. hTERT is a rate-limiting component of telomerase. During up- or downregulation of telomerase, only hTERT expression changes proportionately with the level of telomerase activity, while the levels of the rest of the components remain unchanged and high throughout the modulation [14].

TELOMERASE RNA COMPONENT (TERC)

TERC acts as a template for telomeric DNA synthesis [15], and its size varies between species. *T. thermophila* has short TERC (159 nucleotides), while mammals have TERCs of intermediate length (murine TERC has 397 nucleotides and human TERC has 451 nucleotides) [16]. Human TERC is encoded by a single copy gene located on the long arm of chromosome 3 (3q26.3) [17]. TERC is one of the factors influencing the processivity of telomerase. It facilitates several rounds of addition of DNA after only one primer binding step [18, 19]. Telomerase RNA shows divergence of the primary sequences between species, but it also has a remarkably conserved secondary structure in a variety of vertebrate species, which indicates that RNA structure plays a very important role in telomerase activity [20].

The 3' end of TERC in vertebrates differs from that in single-cell eukaryotes – certain sequence motifs are absent in the latter [21]. This region of human TERC contains an H/ACA sequence motif that forms a specific class of non-coding RNA [22, 23] that facilitates the modification of other cellular RNA. On the basis of localization, H/ACA RNA can be divided into two groups. The H/ACA RNA that accumulates in the nucleolus is known as H/ACA small nucleolar RNA (snoRNA) [24]. It takes part in the modification of ribosomal RNA. The H/ACA RNA that accumulates in the Cajal bodies and takes part in the modification of splicing RNA is known as small Cajal body-specific RNA (scaRNA) [25]. Another sequence motif, known as the Cajal body box or CAB box, is responsible for the difference in cellular localization of H/ACA snoRNA and H/ACA scaRNA.

The conserved domain in the hTERC molecule is the binding site for many hTERC-binding proteins that recognize TERC specifically. There are many RNA-binding proteins that interact with hTERC, such as La, hnRNP C1/C1, dyskerin, hStau, L22, hGAR, hNOP10, hTERT and hNHP2 [26, 27]. These RNA protein interactions are involved in hTERC accumulation, stability and maturation, and assembly of the telomerase in a functional form. Accumulation of TERC has been seen in human cancer cells, mainly within the Cajal bodies via RNA fluorescence *in situ* hybridization [28, 29].

Of the TERC-binding proteins, dyskerin is an enzyme – a pseudouridine synthetase. It is required for H/ACA RNA stability, which it achieves by interacting with three small associated proteins: GAR1, NHP2 and NOP10. This enzyme complex is directed to its complementary RNA, i.e., ribosomal RNA and splicing RNA, by H/ACA snoRNA and H/ACA scaRNA, respectively. The

isomerization of uridine to pseudouridine is mediated by the dyskerin complex. These modifications are required for the proper function of these target RNA. The template region is included in the pseudoknot and together makes up the core domain. The core domain and the CR4/CR5 domain independently bind to hTERT. The H/ACA scaRNA domain (including the CR7 domain) binds to the RNP proteins (dyskerin, Gar1, Nop10, and Nhp2) as well as to telomerase Cajal body protein (TCAB1)/WD Repeat domain 79 [30].

TELOMERASE REVERSE TRANSCRIPTASE (TERT)

The TERT subunit of telomerase was initially isolated biochemically as p123 from *Euplotes aediculatus* [31]. TERT, an RNA-dependent DNA polymerase, is also a core subunit of telomerase, and it uses its own RNA template for DNA synthesis (Table 1). The gene that codes TERT is located on chromosome 5 (5p15.33). The cDNA and genomic sequence of hTERT (human telomerase reverse transcriptase) revealed that the hTERT gene spans more than 37 kb and contains 15 introns and 16 exons that code for 1132 amino acid residues [32]. The TERT subunit of

Table 1. Classical and extracurricular activities of telomerase

Functions	Description	Dependence on catalytic activity of TERT	Reference
Nucleus			
Regulation of gene expression	Acts as a transcriptional modulator	-	[61]
Chromatin organization	Regulates the DNA damage response pathway through its action on chromatin structure	-	[47]
Maintenance of telomere	Acts as a reverse transcriptase by using TERC as a template	+	[80]
Enhancement of cell growth	Affects the expression of growth-promoting genes	-	[81]
DNA repair	Associated with primase, a protein involved in DNA repair; increases the expression level of genes involved in DNA damage response and also physically associated with many DNA repair proteins	+	[77-79]
Mitochondria			
In apoptosis	Sensitizes cells to oxidative stress which can cause apoptotis	-	[69]
RNA-dependent RNA polymerase activity	Gene silencing	+	[65]

telomerase is conserved in humans (hTERT), *Schizosaccharomyces pombe* (Trt1), *Saccharomyces cerevisiae* (Est2) and protozoans [33, 34].

The 3D structure of the TERT protein from *Tribolium castaneum* was solved in 2008 by Emmanuel Skordelakes *et al.* at the Wistar institute in Philadelphia [35, 36]. The protein consists of four conserved domains – the RNA-binding domain (TRBD) and the palm, finger and thumb – which organize in a closed-ring tertiary structure with a larger cavity at the center. It is large enough to bind with the primer-template duplex [35].

Structurally and functionally, the TERT protein can be subdivided into three major domains: the reverse transcriptase domain, which contains the finger, palm and thumb subdomains, which may play a role in nucleotide addition and processivity; the telomerase essential N-terminal domain, consisting of 400 amino acid residues; and the TERT RNA-binding domain, which has high affinity for hTERC. The TERT subunit shows significant conservation with the reverse transcriptase enzyme from retroviruses. Inactivation of this catalytic subunit causes loss of telomerase activity.

The expression level of hTERC depends on the telomerase activity in cells and is detectable in all tissues. Cancer cells generally have five times more expression than normal cells, even though only 1 to 5 copies of TERT (mRNA) are found per cancer cell. The level of expression of TERT is low in normal cells and high in immortal cells, showing that TERT rather than TERC characterizes the immortalized cells.

TELOMERE-INDEPENDENT FUNCTION OF TELOMERASE

Telomerase is a well-known enzyme that maintains the length of the telomere and the physical ends of the eukaryotic chromosome in embryonic stem cells and cancer cells. Telomerase activation extends the lifespan of cells in culture by maintaining the length of the telomere. It has now become clear that the role of telomerase is much more complex than just telomere lengthening. Telomerase influences normal cellular physiology, even in cells that contain long telomeres. Due to its key role in telomere lengthening, alteration in telomerase expression is associated with many degenerative diseases, aging and cancer-related functions.

The role of telomerase in cellular immortalization and that of telomere shortening in cellular senescence has been demonstrated by cloning and expression of the TERT gene [37]. Indeed, increased incidence of spontaneous tumors has been found in many independent TERT-transgenic mouse models with constitutive expression of telomerase [38, 39]. With oncogenic stress, the proliferative rate of the cells is increased many fold, and the length of the telomere is a factor which limits cell division capacity. The telomere is shorter in cancer cells than it is in normal cells [40].

Replicative senescence (also known as the Hayflick limit or mortality stage I) is the first cellular response to occur at the time of telomere attrition, and its induction needs the proper action of p53 and RB tumor suppressor pathways [41].

Inactivation of these two tumor suppressor pathways extends the replicative potential of the cell, ultimately leading to continuous telomere erosion and loss of telomere capping. Uncapped telomeres are highly recombinogenic, which leads to the formation of dicentric chromosomes and breakage at the time of cell division. They are also prone to a high degree of genomic instability and loss of cell viability during this period of crisis [42].

Of all the cells that undergo crisis, only the 10^{-7} to 10^{-5} fraction emerges from crisis [43], perhaps accompanying enforced expression of hTERT and activation of telomerase, which helps to avoid both senescence and crisis in primary cultured cells. It also causes transformation of primary human cells by its coexpression with SV40 early genes and H-RAS [44, 45]. Thus, activating telomerase averts the crisis by capping the telomere and reducing the frequency of dicentric and abnormal chromosomes [44]. Without performing telomere lengthening, stabilization of the telomere can also occur in a TERT-mediated, telomere capping-dependent manner, which increases cellular lifespan [44, 46].

Telomerase can play a role in modulation of chromatin structure and response to DNA damage [47]. TERT is also known to induce the expression of pro-proliferative genes and inhibit that of anti-proliferative genes. This promotes cell growth and proliferation independent of telomere elongation [48, 49].

REGULATION OF GENE EXPRESSION BY TELOMERASE

TERT depletion in mouse skin results in a genome-wide transcriptional response in genes involved in signal transduction, epithelial development, the cytoskeleton and adhesion [50 and our unpublished results]. This resembles the transcriptional program regulated by Wnt, a well known player in stem cell maintenance, cellular transformation and proliferation [51-55]. TERT acts as a transcription factor in β-catenin complexes. It is only directly involved in the modulation of the canonical Wnt pathway; non-canonical Wnt patways that do not involve the β-catenin complex are not regulated by TERT. Wnt ligands bind with Wnt receptor(s) (the Frizzled family of transmembrane receptors) [55] and the LRP5/6 coreceptor (a low-density lipoprotein receptor-related protein) (Fig. 1) [56]. The coreceptor facilitates the interaction between the Wnt receptor and its ligand. The interaction leads to activation of a cytoplasmic phosphoprotein, Dishevelled (Dvl), which inhibits the activity of glycogen synthase kinase-3β (GSK-3β), which degrades the β-catenin. Wnt signaling allows accumulation of β-catenin, which then translocates into the nucleus and forms a complex with TERT-BRG1 (Fig. 1). The BRG1 is also known as SMARCA4, a SWI/SNF-related chromatin-remodeling protein that binds to the β-catenin and takes part in Wnt signaling [57]. The β-catenin complex binds at the TCF/LEF site in the promoter/enhancer regions of target genes like Axin2, LEF1, WNT4 and WNT11, and enhances their expression in the canonical pathway (Fig. 1) [58-60].
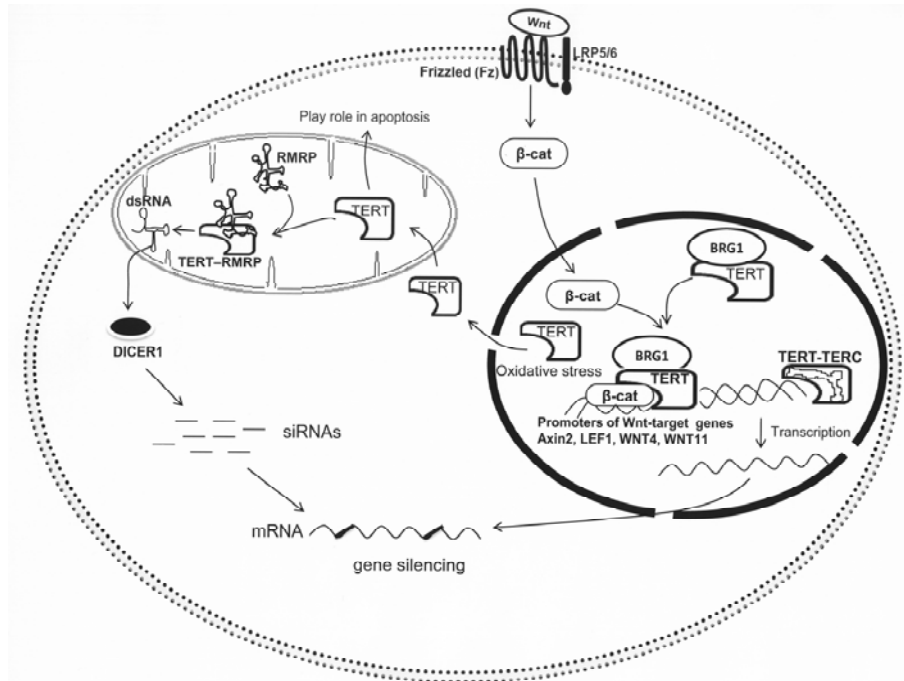
Fig. 1. Multiple functions of telomerase. TERT is directly involved in the modulation of the canonical Wnt pathway, in which it acts as a transcription factor in β-catenin complexes. Stimulation of Wnt receptor(s) after binding with Wnt on the plasma membrane causes binding of TERT with Wnt transcription factor BRG1 and forms a complex, which then binds to the promoters of Wnt-target genes and regulates their expression. TERT also associates with the RNA component of mitochondrial RNA processed into endoribonuclease RMRP. This complex has RdRP activity, which produces a double-stranded RMRP molecule that is further processed in to 22 nucleotide siRNA by dicer and RISC (RNA-induced silencing complex). These siRNA suppress the expression of RMRP. As a result, TERT-RMRP-RDRP regulates the level of RMRP by a negative-feedback control mechanism. These siRNA-mediated suppression pathways mediate control of gene expression by TERT. With oxidative stress, TERT translocates from the nucleus to the mitochondria. Recent results also show that TERT also regulates apoptosis in the mitochondria.

Furthermore, TERT was found to bind with promoters responsive to Wnt signaling and to promoter elements recognized by BRG1 and β-catenin [54, 61]. Studies also indicate a role for TERT in Wnt signaling in collapsing glomerulopathies (characterized by the proliferation of glomerular differentiated epithelial cells, the podocytes) [54, 62]. Further analysis has shown that this effect of TERT on kidney cells is independent of its catalytic activity: it is coupled to its Wnt signaling stimulation, with increased expression and nuclear localization of β-catenin.

However, increasing evidence implies a bidirectional connection between the Wnt pathway and TERT in both embryonic stem cells and cancer [63, 64]. It has

been experimentally shown that embryonic stem cells expressing an activated β-catenin show high telomerase activity and have longer telomeres, while in mice lacking β-catenin, the length of the telomere is short and telomerase activity is also low. Zhang *et al.* found the same results in human cancer cell lines by inducing or repressing β-catenin expression [64]. In fact, in embryonic stem cells, β-catenin binds with Klf4, a transcription factor expressed by pluripotent cells, and regulates the TERT expression, whereas in human cancer cells TERT appears as a direct target of β-catenin/TCF4-mediated transcription. Therefore, during transformation, the Wnt pathway also participates in some carcinogenic processes via the stabilization of the telomere and stimulation of telomerase activity.

RNA-DEPENDENT RNA POLYMERSASE ACTIVITY OF TELOMERASE

TERT is known for its RNA-dependent DNA polymerase activity in association with TERC. Studies also indicate the role of RNA-dependent RNA polymerase activity of TERT in post-transcriptional gene silencing, which is independent of TERC (Table 1). TERT is the only RdRP identified in mammals [65]. This function of TERT depends on a mitochondrial non-coding RNA: mitochondrial RNA-processing endoribonuclease (RMRP). Further analysis shows that TERT is associated with two types of RNA in HeLa cells (which overexpress TERT): TERC and RMRP [66].

RMRP is a non-coding RNA, the mutations of which lead to cartilage-hair hypoplasia, an inherited pleiotropic syndrome that is characterized by premature multi-organ failure, mainly in highly proliferative organs, and that involves stem cell dysfunction [67]. The TERT-RMRP complex has RdRP activity, which produces a double-stranded RMRP molecule [54] that is processed into 21 nucleotide siRNA by dicer and RISC (Fig. 1). These siRNA suppress the expression of RMRP. As a result, TERT-RMRP-RDRP regulates the level of RMRP by a negative-feedback control mechanism. The siRNA-mediated suppression pathway demonstrates control of gene expression by TERT. In the same way, the TERT-RMRP complex may amplify other small non-coding RNA and thereby regulate the expression of other genes by producing specific siRNA.

It has been experimentally shown that TERT has a role in the control of cellular proliferation. TERT is known to increase cellular proliferation by increasing cell division and decreasing apoptosis in TERT-transductant human mammary epithelial cells (HMECs) [68]. Further analysis shows that the effect of TERT on proliferation of cells is connected with alterations in cyclin D1, A2, E2F and pRB, which are all cell cycle regulatory proteins, and requires the catalytic activity of telomerase rather than activation of Wnt signaling by TERT [68]. Mukherjee *et al.* found a reduction in RMRP levels in TERT-transduced HMECs because dicer and RISC process the double-stranded RMRP molecules into 22 nt siRNA that control the level of RMRP. They also showed a connection between the enhancement of cellular proliferation and a decrease in RMRP

levels [68]. Knockdown of RMRP using shRNA (short hairpin RNA) results in proliferation of HMECs, which means that both results are comparable in enhancing cellular proliferation and lowering the RMRP levels. Together, these data indicate that TERT has an RNA-dependent RNA polymerase activity that enhances cellular proliferation via small interfering RNA.

RNA-DEPENDENT DNA POLYMERSAE ACTIVITY OF TELOMERASE

TERT also has RNA-dependent DNA polymerase activity independent of TERC (Table1). TERT is present in the mitochondria and the nucleus (Fig. 2). TERT has an N-terminal mitochondrial targeting signal [69] that helps it to migrate into the mitochondria, probably through the protein complexes known as translocases, which are present on the outer and inner mitochondrial membranes. Translocation of TERT from the nucleus to mitochondria occurs following oxidative stress [54, 69-71] and involves the improvement of mitochondrial function and stress resistance, independent of its telomeric function, which finally leads to the survival of tumor cells. It has been experimentally shown that TERC is not present in the mitochondria, which also supports the idea that TERT reverse transcriptase activity is independent of TERC [72].
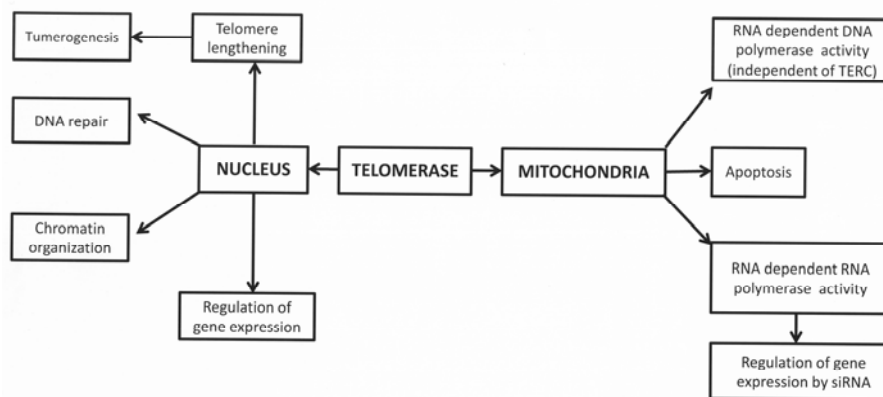

Fig. 2. A schematic representation of the classical and extracurricular activities of telomerase. Telomerase is found to be active in the mitochondria and the nucleus. In the mitochondria, telomerase shows RNA-dependent DNA polymerase activity independently of TERC and uses tRNA as a template. It also shows RNA-dependent RNA polymerase activity, and is the only RNA-dependent RNA polymerase known in mammals. It has been experimentally shown that telomerase plays a role in the regulation of apoptosis. Telomerase sensitizes the DNA of mitochondria to H_2O_2, which causes oxidative damage to mt-DNA perhaps through the modulation of metal homeostasis [69]. In the nucleus, telomerase maintains telomere length. Telomerase is found to be directly involved in the modulation of the canonical Wnt pathway, in which it acts as a transcription factor in β-catenin complexes. Telomerase is also found to be involved in chromatin organization.

In the mitochondria, TERT uses tRNA as a template to synthesize cDNA [72]. Human VA13 cells that do not have TERC and use a recombination-based method of telomere lengthening known as alternative lengthening of telomeres (ALT) were transfected with wild-type TERT. This showed that TERT performs its mitochondrial function. Furthermore, in the same cells, a dominant negative form of the enzyme is inactive [72]. RNA-dependent DNA polymerase activity of TERT based on the TRAP assay has also been shown in rabbit reticulocyte lysates (RRLs), in which translation of TERT in the presence of added TERC results in telomeric-DNA synthesis *in vitro* [72]. Addition of total cellular RNA from TERC-negative VA13 cells or from TERC-positive HeLa cells in the reaction mixture, along with random hexamers to prime the reactions followed by PCR with primers for different mt-tRNA genes demonstrated the synthesis of cDNA in the absence of TERC. It can be concluded that mt-TERT uses tRNA rather than TERC for cDNA synthesis [72]. In the absence of TERT, no products were observed, clearly demonstrating that mt-TERT can act as a reverse transcriptase by using mt-tRNA rather than TERC as a template.

ROLE OF TELOMERASE IN APOPTOSIS

It has been experimentally shown that telomerase plays a role in the regulation of apoptosis (Fig. 2). This role is independent of its conventional function of telomere lengthening. Further analysis has shown that telomerase sensitizes the DNA of mitochondria to H_2O_2, which causes oxidative damage to mt-DNA, perhaps through the modulation of metal homeostasis [69]. The N-terminal leader sequence of TERT contains a mitochondrial localization signal that targets TERT to the mitochondria. Mutation in this region of TERT causes loss of mitochondrial targeting, and cells with mutated hTERT show decreased levels of mt-DNA damage [71]. These observations suggest proapoptotic activity of hTERT in the mitochondria and the roles of TERT in the mitochondria are consistent with reports showing that oxidative stress triggers nuclear export of hTERT [73].

By contrast, it has been shown that hTERT overexpression renders cells resistant to apoptosis. This anti-apoptotic effect of TERT occurs at a pre-mitochondrial step before the release of cytochrome c and apoptosis-inducing factor [74]. The siRNA-mediated downregulation of hTERT triggers the apoptotic pathway devoid of obvious involvement of telomere erosion, but by post-translational activation of BAX, which induces a CD90-independent mitochondrial pathway of apoptosis [75]. In addition, a recent report indicated that TERT enhances cellular and organism viability independently of its telomerase activity. Cultured cells and a transgenic mouse model expressing wild-type TERT were treated with staurosporin and *N*-methyl-*D*-aspartic acid, which both promote apoptosis. Increased resistance against apoptosis was observed in both the cultured cells and the transgenic mice, and this effect of TERT is again independent of telomerase activity [76]. Even though the exact mechanism as to how TERT

regulates apoptosis in mitochondria is unknown, TERT may exhibit discrete functions in apoptosis regulation by promoting apoptosis via alteration of the mitochondrial membrane potential or metal homeostasis in mitochondria.

ROLE OF TELOMERASE IN DNA REPAIR

Telomerase may also play a role in DNA repair independently of its telomere-lengthening function (Fig. 2). It has been experimentally shown that hTERT is also associated with primase [77], a well known protein involved in replication and DNA repair, which indicates a role for telomerase in DNA repair. In addition, studies indicate that ectopic expression of hTERT causes an increase in the expression level of genes involved in the DNA damage response, and this is thought to be associated with a decrease in spontaneous chromosome damage in G1 cells and improvement in the DNA repair kinetics [78]. Moreover, hTERT is also found to be associated physically with many DNA repair proteins and the telomere, thus enhancing the stability of the genome and DNA repair functions [79]. However, increasing evidence is emerging to indicate that the role of telomerase in the DNA damage response is not limited to DNA double-strand break repair but also associated with many other types of DNA repair, including via nucleotide excision [80]. These studies predict a role of telomerase in the DNA damage response independent of its classical activity of telomere length maintenance.

CONCLUSIONS

Accumulating evidence indicates that the telomerase complex performs several functions that do not depend on its classical function of telomere maintenance. All of these telomere-independent roles affect normal cell physiology and promote the proliferation of cancer cells. The exact pathways by which telomerase interferes with or enhances tumorigenesis point towards possible new targets for cancer treatment. Further research is needed to clarify the role of TERT as a mitochondrial RNA-dependent DNA polymerase. It is very necessary to develop the tools that would help to study functions of TERT in cellular physiological conditions to provide clear results. There is no direct molecular evidence to explain the role of telomerase in DNA repair, so a testable experimental model is needed to explain the telomere-independent role of telomerase in DNA repair.

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Global expression profile of telomerase-associated genes in HeLa cells

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ABSTRACT

Telomerase is a specialized nucleoprotein enzyme complex that maintains the telomere length. The telomerase reverse transcriptase (TERT) is the catalytically active component of the telomerase complex. In humans, the protein component (hTERT) and RNA component (hTR) are found to differentially express in cancer cells. In contrast to differentiated cells, most of the cancer cells overexpress hTERT, which is needed to maintain the proliferative potential of cells. The overexpression of telomerase is not proportionate to telomere length in cancer cells, suggesting that the immortalizing phenotype can be mediated through other factors in addition to telomere length. To investigate the role of hTERT in immortalizing process, loss of gene function studies were carried out. Short interfering RNA (siRNA) and short hairpin RNA (shRNA) against hTERT showed the reduction of hTERT transcript, reduction of telomerase activity and alteration of gene expression in HeLa cells. The molecular basis of proliferative capacity of hTERT was investigated by gene expression microarray. Analysis of microarray data for HeLa cells following siRNA and shRNA mediated knockdown of hTERT showed that 80 genes were upregulated and 73 genes downregulated. Out of these, 37 genes are known to be involved in cancer. Further analyses of previously known genes involved in cancer like KLF4, FGF2, IRF-9 and PLAU by Real Time PCR showed their upregulation. We are documenting for the first time the effect of knocking down hTERT on expression of KLF4 and FGF2. Interestingly, it has been earlier reported that KLF4 and FGF2 up-regulate the expression of hTERT in cancer cells. This suggests that hTERT may be subject to its own auto-regulatory effects.

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1. Introduction

Telomerase is an RNA–protein complex that synthesizes and maintains telomere length to counter the end replication problem by adding repeated oligomer sequence to the 3′ termini of chromosomal DNA (Greider and Blackburn, 1987). The human telomerase reverse transcriptase (hTERT) is the catalytically active component of the telomerase complex (Nakamura et al., 1997). hTERT catalyzes the telomere elongation that leads to increased genomic stability and enhanced DNA-repair (Sharma et al., 2003).

hTERT is found to be overexpressed in stem cells, germ cells and in most of the cancer cells, whereas in normal and differentiated cells there is minimal or undetectable level of hTERT (Poole et al., 2001). Activated telomerase helps in maintaining telomere length that provides replicative potential to the proliferating cells. As a proof of concept, expression of hTERT in normal cells also expands the life span and number of cell doublings. Therefore, finding out genes associated with inhibition

of expression/function of hTERT may provide potential therapeutic target for cancer treatment. Several small-molecule inhibitors (Damm et al., 2001; Hisatake et al., 1999; Naasani et al., 1998, 1999), compounds targeting putative telomeric DNA G-quadruplex structures (Neidle et al., 2000), dominant-negative hTERT genes (Hahn et al., 1999), antisense oligonucleotides targeting hTR or hTERT mRNA (Glukhov et al., 1998), ribozymes targeting hTR (Suresh Kumar et al., 2014; Wan et al., 1998) and hTERT mRNA (Kondo et al., 2001), result in limitation of proliferative potential, genomic instability and induction of apoptosis.

The role of telomerase in cellular biology is becoming increasingly complex and includes functions other than telomere maintenance (Choi et al., 2008; Kraemer et al., 2003; Xiao et al., 2005). Telomerase influences normal cellular physiology in cells having long telomeres e.g., mice lacking functional telomerase are unable to maintain proper tissue homeostasis, particularly in tissues of high cell turnover, such as the bone marrow, skin, liver, and gastrointestinal tract (Herrera et al., 1999; Lee et al., 1998; Rudolph et al., 1999). Other studies have demonstrated that ectopic expression of telomerase even in cells that are endogenously telomerase positive results in increased resistance to apoptosis (Holt et al., 1999). Non-correlation of telomerase expression status and telomere length in these studies has given enough evidence that telomerase has extra-curricular activities in cellular physiology.

To delineate the extra telomeric function of hTERT, loss of gene expression studies was carried out. Knockdown of hTERT using siRNA and shRNA lowered the hTERT transcript as well as telomerase activity and altered the global gene expression in cancer cells. Microarray studies revealed that expression of certain annotated genes gets altered upon knockdown; 80 genes were upregulated and 73 genes downregulated by both agents. Among genes with known role in cancer, 25 were upregulated and 12 genes downregulated. Real Time PCR showed that four genes viz., KLF4, FGF2, PLAU and IRF9, seemingly important for cancer progression, were upregulated upon knockdown of hTERT. Among them KLF4 and FGF2 are known to be activator of hTERT expression, reflecting a possible feed-back loop mediated autoregulation of expression of hTERT. The finding provides further insight in to the potential role of hTERT in cellular physiology in functions other than telomere lengthening.

2. Materials and methods

2.1. Construction of shRNA hTERT in to pPRIME vector

pPRIME vector system was used for cloning and expression purposes (kind gift from Dr. Stephen Elledge of Howard Hughes Medical Institute) (Stegmeier et al., 2005). shRNAs against hTERT were generated using the miR30 based design and cloned in to pPRIME vector to target hTERT transcripts. The following target (sense) sequences were used in the design of shRNAs for hTERT: shRNA–hTERT (5'TTTCATCAGCAAGTTTGGA-3') cloned in to pPrime vector. The designing of miR-30 embedded shRNA–hTERT was essentially followed as described (Stegmeier et al., 2005). The flanking regions are used as universal flanks for primer annealing, whereby the entire miR-30-styled shRNA is amplified to produce a PCR product that is cloned in to XhoI and EcoRI site of pPRIME vector. The clones were verified by sequencing.

2.2. Cell culture

HeLaTet-ON cervical cancer (kindly provided by Dr Manoj Bhat, NCCS, Pune) cell line stably expressing the reversed tetracycline-controlled trans-activator (rtTA) was used for this purpose. These cells, upon transient transfection and doxycycline addition, trigger the shRNA expression from a Tet responsive promoter. Doxycycline (Sigma-Aldrich, Oakville, ON) at 1 μg/ml concentration was used for transcriptional activation of the pPRIME vector via control over the Tetracycline Transcriptional Activator.

2.3. Transfection of siRNA and shRNA against h-TERT in cancer cells

All siRNA molecules were ordered purified and pre-annealed from Sigma. Lyophilized siRNA (10.5 nmol) was dissolved in DEPC-treated water at a concentration of 50 pmol/μl. The mixture was vortexed for 30–40 s. Cells were seeded in 6-well plates 1 day prior to transfection at 2×10^4 to 10×10^4 cells per well and grown in media supplemented with 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). siRNA was complexed with lipofectamine (Invitrogen, Carlsbad, CA) and transfected according to the manufacturer's protocol. After 7–8 h of transfection fresh complete medium was replaced and cultures were maintained for 24–72 h.

2.4. Real Time RT-PCR

Q RT PCR was performed for transcripts of hTERT, KLF4, FGF2, PLAU, IRF9 and GAPDH using Sybr green, with gene specific primers (primer sequence can be provided upon request). Real Time PCR was performed on Applied Biosystems 7500 with cycling condition of 95 °C for 15 s, 60 °C for 1 min (for primer annealing and elongation) for 40 cycles, followed by a melting point determination or dissociation curve. Real Time data were normalized with GAPDH.

2.5. Determination of telomerase activity by conventional TRAP assay

Telomerase activity was assayed following telomere repeat amplification protocol (TRAP) (Piatyszek et al., 1995) using a TRAPeze kit (Chemicon) according to the manufacturer's protocol. The autoradiographs of the PCR products were viewed following electrophoresis in 10% non-denaturing polyacrylamide at 800–1000 V (Sambrook and Russell, 2001).

2.6. Gene expression arrays

HeLa cells were seeded in 6 well plates in DMEM containing 10% FBS and transfection was performed in three independent sets with 100 pmol siRNA hTERT, scrambled siRNA and shRNA–hTERT using lipofectamine 2000. Total RNA was harvested 72 h after transfection using a Qiagen kit. Affymetrix microarray (PrimeView™ Human Gene Expression Array chip) hybridization was performed with the help of iLife Discoveries.

3. Results

3.1. siRNA and shRNA-mediated knockdown of hTERT expression in cancer cells

Transient knockdown methods can reveal the silencing of target gene and it also helps to understand the associated changes in immediate early gene expression profile. To investigate the immediate early effects of siRNA against hTERT, HeLa cells were transfected with 80 pmol siRNA and harvested 72 h posttransfection. The sense and anti-sense sequences of siRNA are shown in Fig. 1A. In parallel, equimolar concentrations of siRNA against GAPDH and scrambled siRNA were used as controls. Real Time PCR using hTERT specific primers showed 60% reduction of hTERT transcript level in cells transfected with siRNA against hTERT (Fig. 1B). HeLa cells transfected with hTERT siRNA showed no morphological changes (Fig. 1C).

To further assess the potential of shRNA targeting different target sites of hTERT, the duplex representing the shRNA along with miRNA loop was constructed in pPRIME vector under a Tet regulated promoter (Fig. 2A). Schematic representation of predicted RNA folds for simple stem-loop and miR-30–based hTERT shRNA is shown in Fig. 2B. As reported earlier, the miR30 loop can increase the processivity and knockdown efficiency by presenting silencing RNAs in its natural format. In addition, the transfected shRNA can be monitored with GFP expression (Fig. 2C). HeLaTet-On cell lines were transfected with shRNA mirhTERT construct with Dox and 80% reduction in the hTERT levels was observed (Fig. 2D). The silencing efficiency of siRNA and shRNA was comparable and the latter was used in subsequent experiments.

3.2. Attenuation of telomerase activity by shRNA against hTERT

The observed reduction of hTERT transcript level should reflect in its functional activity. To assess shRNA efficiency in telomerase inhibition, extracts from transfected constructs were analyzed with Telomeric Repeat Amplification Protocol (TRAP) assay with serially diluted (100 ng to 1000 ng) cellular protein. The TRAP assay with 500 ng protein showed visible reduction in signal intensity of telomerase extended ladders in HeLa Tet-On cells transfected with shRNA hTERT when compared to control (Fig. 2E) showing that shRNA against hTERT indeed reduces the telomerase activity in HeLa cell line.

3.3. Global profiling of gene expression pattern following knockdown of hTERT

In addition to telomere maintenance, telomerase also modulates gene expression by hitherto unrevealed mechanisms. In order to develop an impression of various players influenced by telomerase vis-à-vis
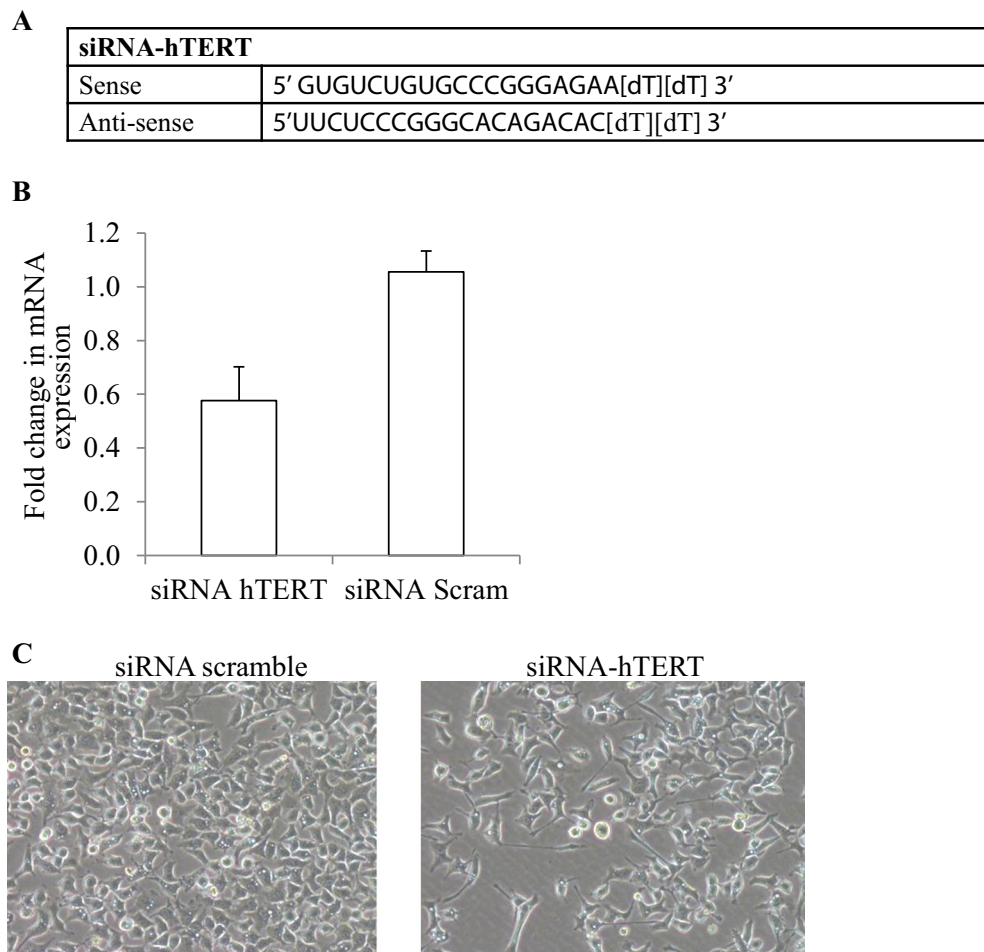
A

siRNA-hTERT	
Sense	5′ GUGUCUGUGCCCGGGAGAA[dT][dT] 3′
Anti-sense	5′UUCUCCCGGGCACAGACAC[dT][dT] 3′

B

C

Fig. 1. siRNA mediated knockdown of hTERT expression in HeLa cells.(A) Sequence of siRNA used for knockdown of hTERT expression in HeLa cells. (B) hTERT expression level in siRNA–hTERT and scrambled siRNA treated cells was determined by quantitative Real Time PCR and normalized with GAPDH. HeLa cells were transiently transfected with 80 pmol of siRNA against hTERT for 72 h showing 55% knockdown. (C) Phase contrast images of HeLa cells transfected with hTERT siRNA did not show any conspicuous morphological changes (magnification 100× and scale bar = 50 μm).

those influencing telomerase expression, it becomes pertinent to make a genome-wide expression profile of various genes associated with change in telomerase expression.

The cDNA expression microarray analysis was performed on HeLa cells 72 h after transfection with siRNA hTERT, Tet regulated shRNA–hTERT or scrambled siRNA. To characterize the transcriptomic profiles under the above treatment conditions, the log2 values of normalized transcript intensities of all the three treatments in triplicate were plotted against the basal expression range and the distribution of the total number of probes/genes analyzed.

Differential gene expression was analyzed by pair wise comparison for each experimental set. Out of 34,398 only 952 genes were found differentially expressed that were statistically significant (after ANOVA, p = 0.05). Among these, the total number of genes expressed at fold change ≥2.0 was 388 (Fig. 3A and B). Out of 388 genes the number of upregulated genes was 215 after siRNA hTERT treatment and 183 after shRNA hTERT treatment; among these, 80 genes were commonly upregulated (the degree of concordance was 21%, Fig. 3C). On the other hand, the number of downregulated genes was 173 after siRNA–hTERT treatment and 205 after shRNA–hTERT treatment; among these, 73 genes were commonly downregulated (the degree of concordance was 19%) as shown in Venn diagram (Fig. 3D).

3.4. In-silico functional analysis of differentially expressed genes

Enrichment analysis was done with 80 genes commonly upregulated and 73 genes commonly downregulated in between siRNA and shRNA mediated knockdown of hTERT with MetaCore software. From the gene expression data, pathway maps were constructed and genes were found to have role in different pathways (Supplementary Table S1). The pathways are related to development, angiotensin, HGF signaling, cell adhesion, plasminogen signaling and AP1 transcription in metabolic pathway signaling.

To further narrow down enrichment analysis to genes having role in cancer progression, we found 25 (31%) genes out of 80 commonly upregulated genes and 12 (16%) genes out of 73 commonly downregulated genes to be known for their role in cancer (Fig. 4A and B). Detailed functional roles of these 25 upregulated and 12 downregulated genes are listed in Supplementary Table S2. Next, we validated four genes viz., KLF4, FGF2, IRF9 and PLAU, known for their role in cancer progression, by Real Time PCR (Fig. 5). We found that all four genes get upregulated after hTERT knockdown by Real Time PCR that is in concordance with microarray data. Most of the genes including PLAU, PLAT, KLF4 and FGF2 altered upon knockdown of hTERT were observed and documented for their association for the first time.
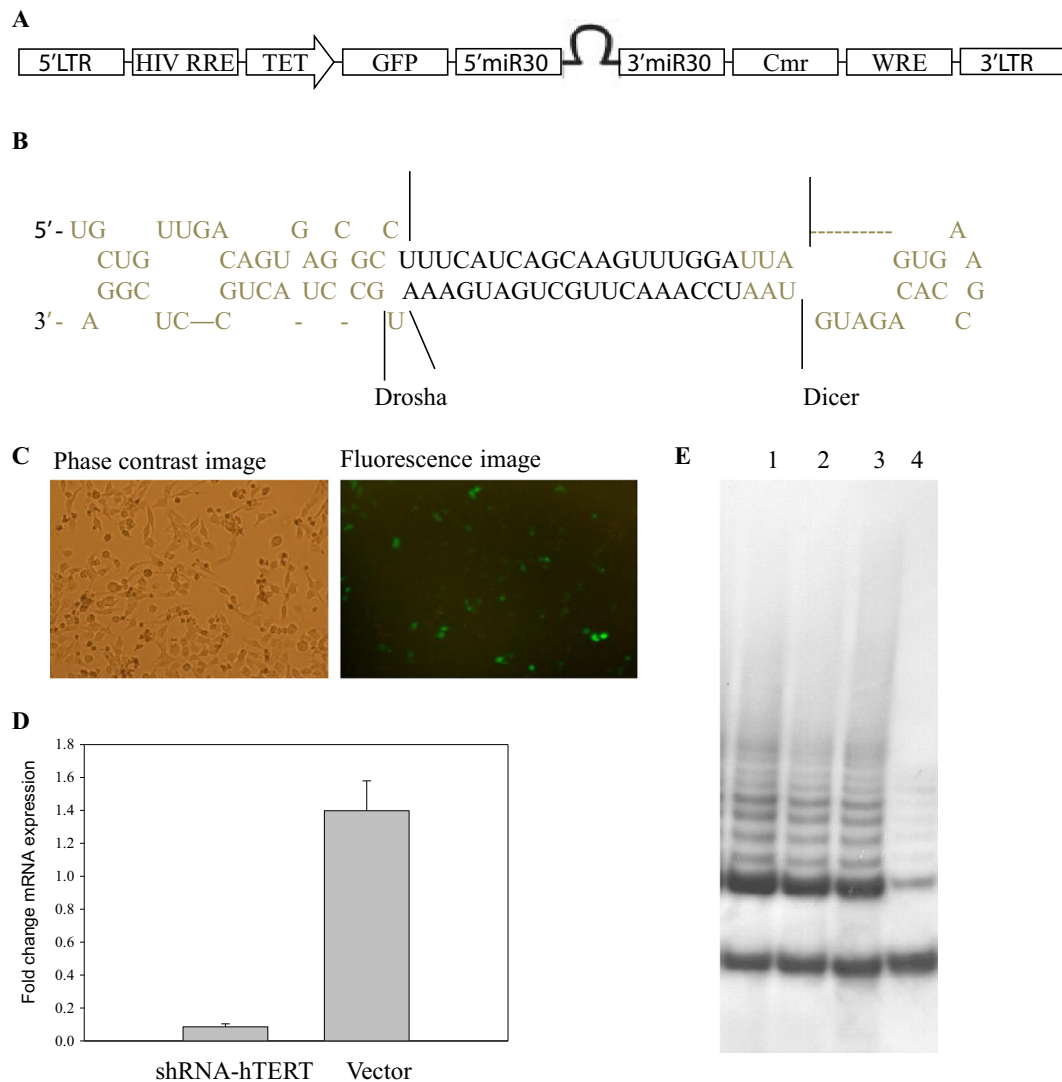
Fig. 2. Knockdown of hTERT expression in HeLa cell line by miR30 based shRNA–hTERT. (A) Schematic representation of pPRIME vector used for construction of miR30 based shRNA–hTERT. In this vector shRNA was cis-conjugated downstream of GFP under a tetracyclin regulated promoter. (B) Secondary structure prediction of the miR30-based shRNA–hTERT, the antisense strand, which encodes the targeting siRNA. (C) HeLa cells that were transiently transfected with shRNA–hTERT for 72 h showed green fluorescence indicating expression of shRNA–hTERT. (D) hTERT expression level in shRNA–hTERT and vector treated cells was determined by quantitative Real Time PCR and normalized with GAPDH showed 80% knockdown after 72 h. (E) TRAP assay was performed to measure the telomerase activity in HeLa cells expressing shRNA–hTERT showed discernible reduction in telomerase activity when compared with vector at 500 ng cellular protein extract. Lane 1 vector − Dox, lane 2 vector + Dox, lane 3 shRNA hTERT − Dox, lane 4 shRNA hTERT + Dox.

4. Discussion

Previous studies from our laboratory and those of others showed that telomerase has functions other than telomere length maintenance (Bilsland et al., 2009; Choi et al., 2001; Kraemer et al., 2006; Suresh Kumar et al., 2014). We have earlier shown that knockdown of hTR by targeted ribozyme leads to decrease in telomerase activity while no change in telomere length is observed. Differential display of hTR knockdown HeLa cells showed decrease in expression of RPL23a and RPL35, indicating the role of telomerase in ribosome biogenesis (Suresh Kumar et al., 2014). In a study conducted by Choi et al. (2008), which addressed the enhanced telomerase activity despite possessing long telomeres, overexpression of mTERT in mice developed invasive mammary carcinoma. Expression of oncogenic H-Ras in ALT cells was insufficient to confer tumorigenicity, where as subsequent ectopic overexpression of hTERT or a catalytically active mutant form of hTERT that has lost the ability to elongate telomeres results in

transformation (Stewart et al., 2002). These data reinforce the view that telomerase performs extra telomeric functions to promote carcinogenesis.

Various approaches have been used to study these extra functions of telomerase e.g., inhibiting the kinase regulating the hTERT expression (Bilsland et al., 2009), inhibitor of hTERT (Damm et al., 2001; Kraemer et al., 2006; Xiao et al., 2005), Tet-regulated expression of TERT in mice (Choi et al., 2008), retrovirus-mediated overexpression of hTERT (Choi et al., 2001), siRNA against hTR (Li et al., 2005) and ribozyme against hTR (Bagheri et al., 2006). Studies on genome-wide expression patterns associated with telomerase add to the value of available information.

In this study we have used siRNA and shRNA to knock down hTERT and assess its association with global gene expression pattern by micro-array analysis. We found that 388 genes were differentially expressed (p = 0.05 and fold change >2) upon hTERT knockdown by both methods. Out of these, 73 genes (19%) were commonly downregulated
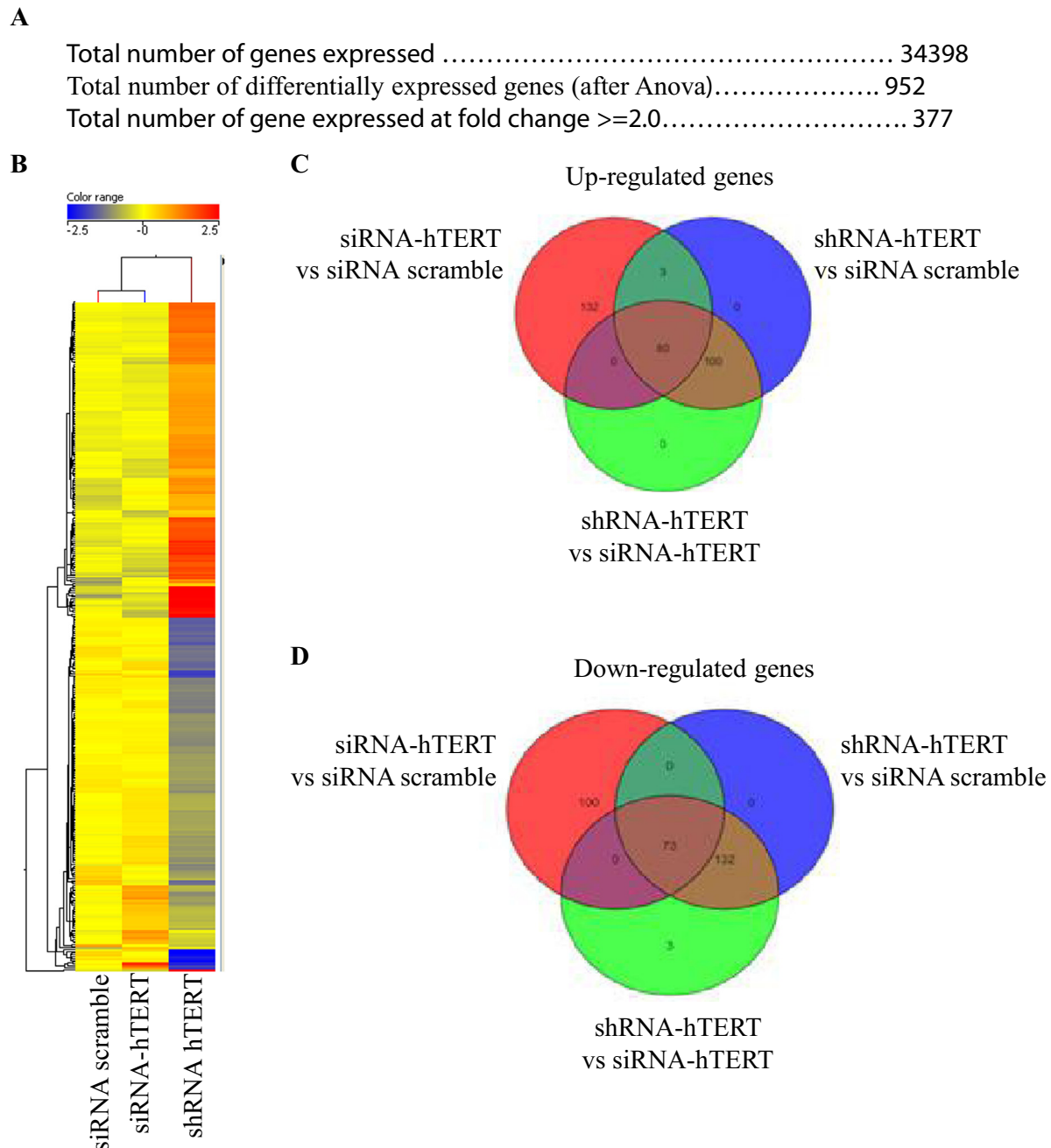
A

Total number of genes expressed ……………………………………… 34398
Total number of differentially expressed genes (after Anova)……………….. 952
Total number of gene expressed at fold change >=2.0……………………. 377

B

C

Up-regulated genes

D

Down-regulated genes

Fig. 3. Genome wide expression profiling of HeLa cells following knockdown of hTERT. (A) Summary result of microarray experiment. (B) Heat map of hierarchical clustering of genes regulated in the hTERT knockdown cell line compared with the control cell line showed little difference with siRNA–hTERT while greater difference with shRNA in comparison to control (scrambled siRNA). Red color shows up-regulated genes (fold change > 2.0) while blue color shows down-regulated genes (fold change genes < 2.0). (C & D) Venn diagram between siRNA–hTERT vs scrambled siRNA and shRNA–hTERT vs scramble siRNA shows that out of 377 differentially expressed genes, (C) 73 genes were commonly downregulated and (D) 80 genes commonly upregulated.

and 80 genes (21%) were commonly upregulated. The uncommon gene expression between these two methods may be attributed to the differences in transfection efficiency and stability of naïve siRNA compared with shRNA bearing miRNA loops.

Since our model system is cancer cells (HeLa), we have visualized telomerase association with other cellular genes and their expression levels in cancer cells. The number of genes reported to have a role in cancer came down to 37 out of which 12 genes were downregulated and 25 genes were upregulated (Supplementary Table S2).

Cancer progression is mediated by various classes of proteins including growth factors, transcription factors and extra cellular matrix proteins. Therefore we selected KLF4, FGF2, IRF9 and PLAU genes from

our enriched gene list. These genes were found upregulated following knockdown of hTERT. Further analysis of these four genes by Real Time PCR showed their up-regulation. Interestingly, FGF2 was reported to induce the expression of hTERT through SP1 in human endothelial and neural precursor cells (Haik et al., 2000; Kurz et al., 2003) and KLF4 induced the expression of hTERT through beta-catenin (Hoffmeyer et al., 2012). This indicates that there may be a feedback loop mechanism by which hTERT regulates its own expression by bringing down the expression of FGF2 accompanying its own buildup. In contrast to our observations, FGF2 was also reported to be overexpressed, when hTERT was overexpressed in HMEC (Smith et al., 2003). This behavior of FGF2 might be due to multiple regulatory factors in addition to hTERT. PLAU is a serine
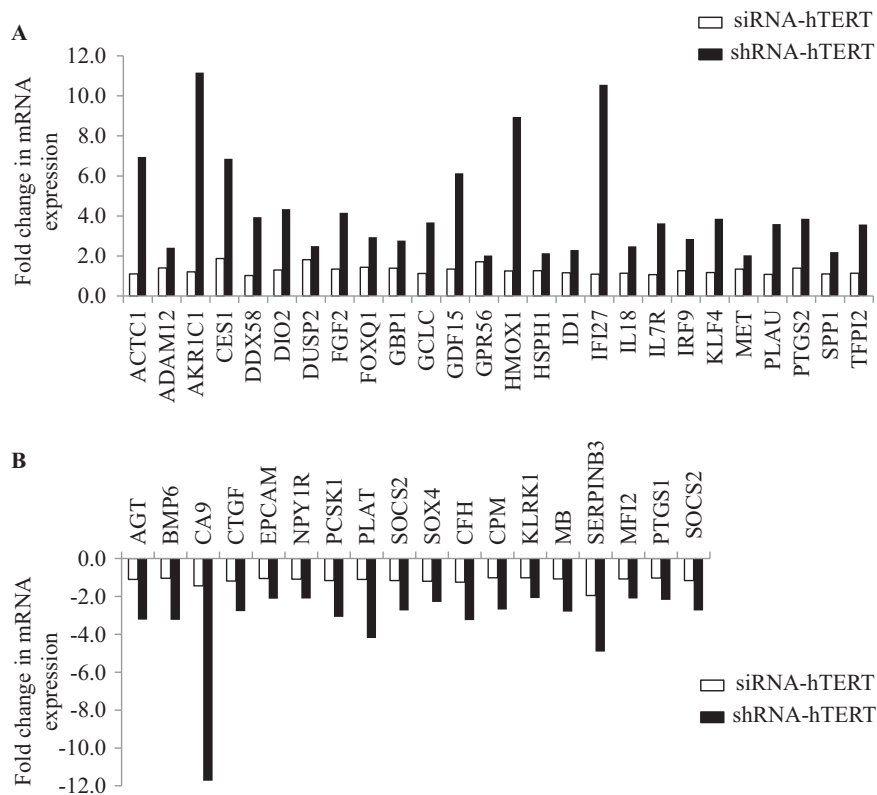
Fig. 4. An overview of differentially expressed genes after hTERT knockdown. Histograms are showing fold change of (A) 26 genes from 80 commonly up-regulated genes, and (B) 18 genes from 73 commonly downregulated genes found to be involved in cancer after siRNA (white bar) and shRNA (black bar) mediated knockdown of hTERT expression in HeLa cells.

protease enzyme involved in degradation of the extracellular matrix and activates plasmin (a known activator of FGF2) and Akt pathway. This indicates that PLAU is also involved in FGF2-mediated autoregulation of hTERT. To the best of our information, this is the first evidence that hTERT maintains its expression in the presence of its inhibitors through an auto-regulatory feed-back loop mechanism in the proliferating cells.
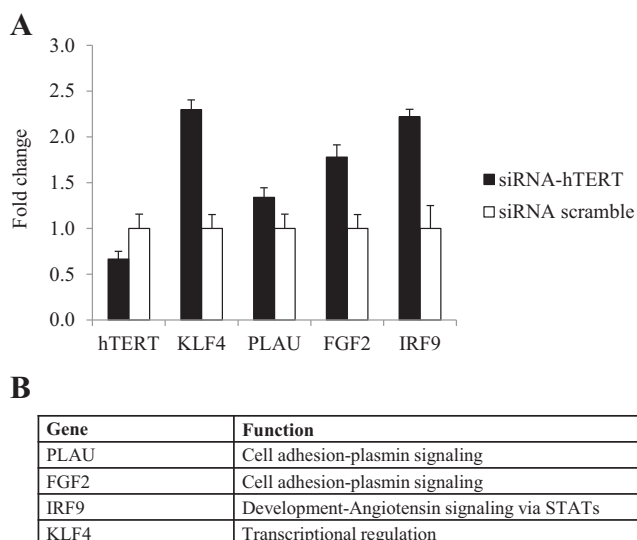

Fig. 5. Real Time PCR validation of differentially expressed genes. (A) Four differentially expressed genes belonging to various functional categories were chosen for Real Time PCR validation. All the genes selected for validation had similar fold changes by Real Time PCR to that seen on the microarray. (B) Functional characterization of four up-regulated genes using MetaCore™ annotation.

5. Conclusion

In conclusion knocking down the hTERT mRNA by siRNA and shRNA causes associated genome wide alteration in gene expression as shown by microarray analysis in HeLa cells. We observed reduction in hTERT transcript level, lower telomerase activity and alteration in gene expression pattern after treatment with siRNA/shRNA. Differentially expressed genes after knockdown of hTERT show possible association of telomerase in cellular functions other than telomere synthesis. By the enrichment of microarray results, we observed upregulation of PLAU, KLF4 and FGF2, which are known activators of hTERT. On this basis we suggest an autoregulatory feedback loop mechanism by which hTERT increases its expression in the presence of its inhibitor while its accumulation may suppress its own expression. Targeting TERT and its regulatory genes may provide potential therapeutic targets for arrest of metastasis and sensitization of cancer cells for radiation and/or chemotherapy.

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Conflict of interest

The authors did not have conflict of interest.

Acknowledgment

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