UNIVERSIDAD DE MURCIA

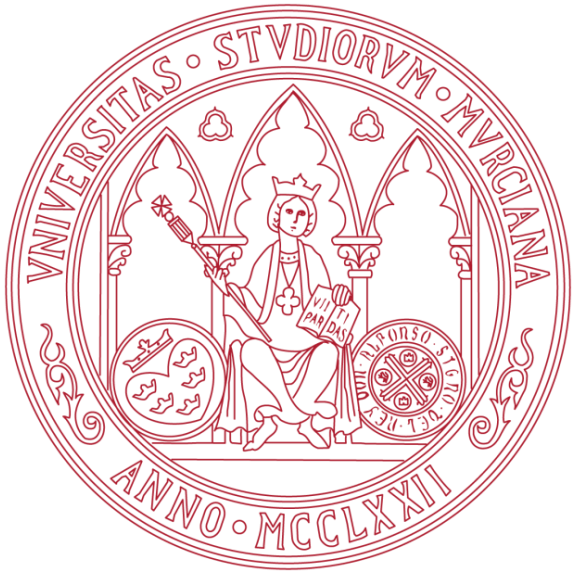
FACULTAD DE MEDICINA

Non-Canonical Function of the Telomerase in Metastasis. New Strategies for Tumor Aggressiveness Prediction

Función no Canónica de la Enzima Telomerasa en el Proceso de Metástasis. Nuevas Estrategias para Predicción de Agresividad en Tumores

D. Manuel Bernabé García

2017



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Non-canonical function of the telomerase in metastasis . New strategies for tumor aggressiveness prediction.

Memoria que presenta

D. Manuel Bernabé García

para optar al grado de Doctor Internacional

por la Universidad de Murcia

Junio 2017

Index

Abbreviations

ALT	Alternative Lengthening of Telomeres
ATM	Ataxia Telangiectasia Mutated
ATR	ATM and Rad3-related
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CFU	Colony Forming Units
CMV	Cytomegalovirus
CSF	Colony Stimulating Factor
CTC1	Conserved telomere maintenance component 1
CUL3	Cullin 3
DC	Dyskeratosis congenita
DDR	DNA Damage Response
DKC1	Dyskerin 1
D-loop	Displacement loop
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide Triphosphate
dpf	Days post-fertilization
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
*Ec*LPS	*Escherichia coli* Lipopolysaccharide
EDTA	Ethylenediaminetetraacetic acid

EF1α	Elongation factor 1□
EGFP	Enhanced GFP
EGTA	Ethylene glycol bis (2-aminoethyl ether)-N, N, N', N'-tetraacetic acid
F	Direct primer
FCS	Fetal Calf Serum
FISH	Fluorescence *In Situ* Hybridization
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GAR1	H/ACA ribonucleoprotein complex subunit 1
gDNA	Genomic DNA
GFP	Green fluorescent protein
GLI3	GLI Family zinc finger 3
GMCSF	Granulocyte/macrophage CSF
hpf	Hours post-fertilization
hpi	Hours post-infection
hpm	Hours post-microinjection
hpw	Hours post-wound
IL-6	Interleukin-6
IL-6R	Interleukin-6 Receptor
iPS	Induced Pluripotent Stem cells
LPS	Lipopolysaccharide
LTL	Leukocyte Telomere Length
lyz	Lysozyme
Luc	Luciferase
MCSF	Macrophage-CSF

mRNA	Messenger RNA
miR 500a	hsa-miR-500a-5p
miRNA	MicroRNA
MTDH	Metadherin
MYC	v-myc avian myelocytomatosis viral oncogene homolog
NAF1	Nuclear Assembly Factor 1 ribonucleoprotein
ND	Not Detected
NF-κB	Nuclear Factor-κB
NHEJ	Non-Homologous-End-Joining
NHP2	H/ACA ribonucleoprotein complex subunit 2
NOLA3	NOP10 encoding gene
NOP10	Nucleolar Protein 10
NR3C1	Nuclear receptor subfamily 3, group C, member 1
n.s.	Not significant
nt	Nucleotides
OCLN	Occludin
PAMP	Pathogen-Associated Molecular Pattern
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PLB	Passive Lysis Buffer
PMA	Phorbol 12-myristate acetate
POT1	Protection of Telomeres 1
PTCH1	Patched 1

PTU	pheniltiourea
Q-FISH	Quantitative-FISH
Q-TRAP	Quantitative-TRAP
R	Reverse primer
RdRP	RNA-dependent RNA Polymerase
RMRP	RNA component of Mitochondrial RNA Processing endoribonuclease
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNP	Ribonucleoprotein
ROS	Reactive Oxygen Species
rRNA	ribosomal RNA
RT	Room Temperature
RTA	Relative Telomerase Activity
RT-qPCR	Reverse transcription – quantitative polymerase chain reaction
SEM	Standard Error of the Mean
siRNA	Small interfering RNA
SKP1	S-phase kinase-associated protein 1
ssDNA	Single-stranded DNA
SPI1	Spleen focus forming virus (SFFV) proviral integration oncogene
STE	Stem-Terminus Element
Std	Standard
STN1	Suppressor of cdc thirteen homolog
SWI/SNF	SWItch/Sucrose NonFermentable
TLE4	Transducin-like enhancer of split 4

TBE	Template Boundary Element
TCAB1	Telomerase Cajal Body protein 1
TEN1	CST complex subunit TEN1
TERC	Telomerase RNA Component
TERRA	Telomeric-Repeat-containing RNA
TERT	Telomerase catalytic subunit
hTERT	Human TERT
TIF1γ	Transcription intermediate factor-1γ
TIN2	TRF1-Interacting Nuclear factor 2
TK	Thymidine Kinase
t-loop	Telomeric DNA loop
TLR	Toll-Like Receptors
TMG	Tri-Methyl Guanosine
TMM	Telomere Maintenance Mechanism
TPP1	TIN2 and POT1-interacting Protein 1
TR	Telomerase RNA component
TRF1	Telomeric Repeat binding Factor 1
TRF2	Telomeric Repeat binding Factor 2
UTP	Uridine triphosphate
*Va*DNA	*Vibrio anguillarum* genomic DNA
WISH	Whole-mount RNA *In Situ* Hybridization
wt	Wild type
ZIRC	Zebrafish International Resource Center
ZFNs	Zinc Finger Nucleases

zf	Zebrafish

Summary

The aim of this Doctoral Thesis is to contribute to the understanding the extracurricular role of the catalytic sub unit of the telomerase in tumor cancer cell and invasion, we have exploited the unquestionable advantages of the zebrafish vertebrate model in xenotransplantation to study tumor aggressiveness.

Firstly, we have demonstrated a non-canonical pathway of hTERT. We have investigated the regulation of miR 500 from telomerase. Own data confirm that a tumor are more metastatic when have more hTERT, this increase of hTERT produce more express of miR 500 and produce more invasion.

Data of luciferase assay and CHIP we can determinate the promoter region where hTERT regulate the expression of miR 500

Taken together, this Doctoral Thesis provides a hypothetical downregulation of tumor gene suppressor using different tumor cells line and the zebrafish model in tumor invasion.

In a second part of this work, we use xenograft assay with breast tumor cells in zebrafish embryos/larvae to see the invasion. The result is a rapid and sensitive technique that can be used as prognosis of metastasis in human patient. We confirm the predicted results of Pathologic Anathomy with zebrafish model even more in some cases zebrafish model can detect possible metastasis that Pathology Anathomy can´t do. We have showed several evidences supporting the zebrafish as a model to study the aggressiveness in breast tumor and design a follow-up of patients.

Introduction

1. Telomeres

Genomic stability is the prerequisite of species survival to ensure that all required information will be passed on to the next generations. In contrast to single-cell-species, higher order organisms, in order to preserve their genomic information, require more efficient DNA repair mechanisms due to later onset of reproduction. Therefore, a remarkable ability of cells to recognize and repair DNA damage and progress through the cell cycle, in a regulated and orderly manner, has been developed.

A vulnerable portion of the genome, especially in eukaryotic organisms whose genome is organized in linear chromosomes, is their edges called telomeres (after the greek words '*τέλος*' (*télos*) and '*μέρος*' (*méros*) meaning 'the ending part'). For this, telomeres form specialized structures at the ends of linear chromosomes that ensure their integrity by 'hiding' the free-ends of the chromosome from the mechanisms within the cell that monitor DNA damage. They are also needed to overcome the 'end-replication problem' (Greider, 1996).

Telomeres are long tracts of DNA at the linear chromosome's ends composed of tandem repeats of a guanine rich sequence motif that vary in length according to species. This motif is conserved in lower eukaryotes and in mammalian cells (Greider, 1998). Usually, but not always, the telomeric DNA is heterochromatic and contains direct tandemly repeated sequences of the form $(T/A)_xG_y$ where *x* is between 1 and 4 and *y* is greater than 1 (**Table 1**).

Table 1: The following table shows the diversity of telomeric DNA. *Adapted from Sfeir (2012).*

Organism	**Sequence**	**Length**
Homo sapiens	TTAGGG	5-15 kb
Mus musculus	TTAGGG	20-100 kb
Danio rerio	TTAGGG	12-20 kb
Schizosaccharomyces pombe	$GGTTACA_{0-1}C_{0-1}G_{0-1}$	5 kb
Saccharomyces cerevisiae	TG_{1-3}	300 bp
Organism	**Sequence**	**Length**
Trypanosoma brucei	GGGTTA	2-26 kb

Tetrahymena thermophila	TTGGGG	120-420 bp
Coenorhabditis elegans	TTAGGC	4-9 kb
Arabidopsis thaliana	TTTAGGG	2-5 kb
Oxytricha	TTTTGGGG	20 bp

Exceptionally, the chromosome ends of a few insect species (*Drosophila* and some dipterans), instead of telomeric motifs, possess tandem arrays of retrotransposons (Abad *et al.*, 2004).

1.1. Structure and function

Telomeric DNA is double stranded with a single-stranded terminus that is on average 50-300 nucleotides (nt) long in human cells. Under normal conditions, in most somatic cells of an adult organism, telomeres shorten in each cell division (i.e. in humans by about 50-150 nt). The basic telomere DNA repeat unit in vertebrates is the hexamer TTAGGG, in which the strand running 5'→ 3'outwards the centromere is usually guanine-rich and referred to as G-tail (**Fig. 1**).

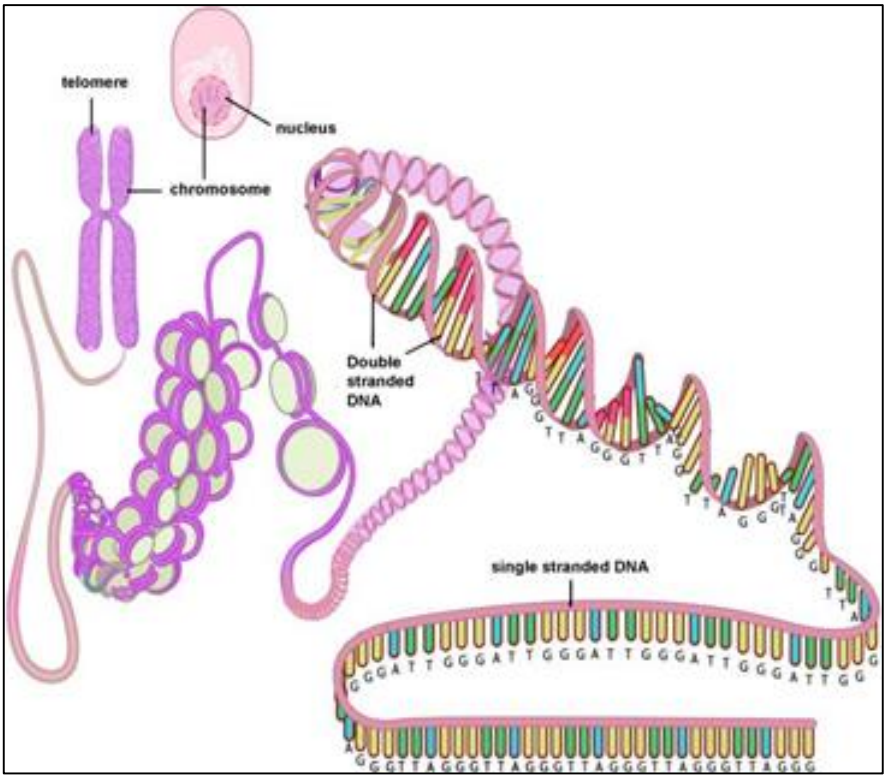


Figure 1: A Simplified diagram depicting the structure of the telomere and its location on the chromosome and in the cell. *This Figure was reproduced from (Zhu et al., 2011)*

In order not to leave exposed a single stranded overhang, this G-rich strand protrudes its complementary DNA-strand and, by bending on itself, it folds back to form a telomeric DNA loop (t-loop), while the G-tail 3' end invades into the double strand forming a triple-stranded structure called displacement loop (D-loop) inside the t-loop. As a result, the t-loop protects the G-tail from being recognized as a double-stranded DNA (dsDNA) break by sequestering the 3'-overhang into a higher order DNA structure (Griffith *et al.*, 1999), (**Fig. 2**).

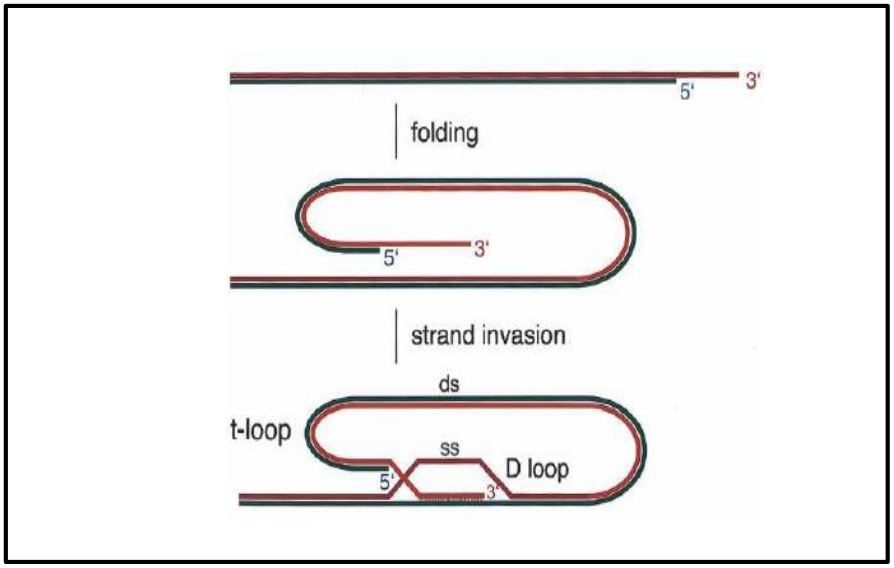


Figure 2: Telomere secondary structure scheme. The single stranded overhang folds back and forms a telomeric DNA loop, t-loop. Then, the 3' overhang is strand-invaded into the adjacent duplex telomeric repeat array, forming a D-loop. The size of the loop is variable. *Adapted from de Lange (2005).*

In vertebrates, the role of chromosome end protection in order to be distinguished from chromosome breaks is attributed to a specific complex of proteins collectively referred to as shelterin. Shelterin complex is basically composed by six proteins: TRF1 (telomeric repeat-binding factor 1), TRF2 (telomeric repeat-binding factor 2), POT1 (protection of telomeres 1), RAP1 (repressor activator protein 1), TIN2 (TRF1-interacting nuclear factor 2) and TPP1 (TIN2 and POT1-interacting protein 1). TRF1 and TRF2 bind directly to double stranded telomeric sequence, while POT1 binds single-stranded DNA (ssDNA). TRF2 interacts with and recruits RAP1, while TIN2 mediates TPP1-POT1 binding to the TRF1/TRF2 core complex. POT1 binds to and protects the 3′ ssDNA overhang of telomeres (G-tail), while TIN2 likely links the ssDNA and dsDNA binding

complexes, especially in the area of the telomeric D-loop formation [reviewed in de Lange, (De Lange, 2005) and (Stewart *et al.*, 2012), (**Fig. 3**).

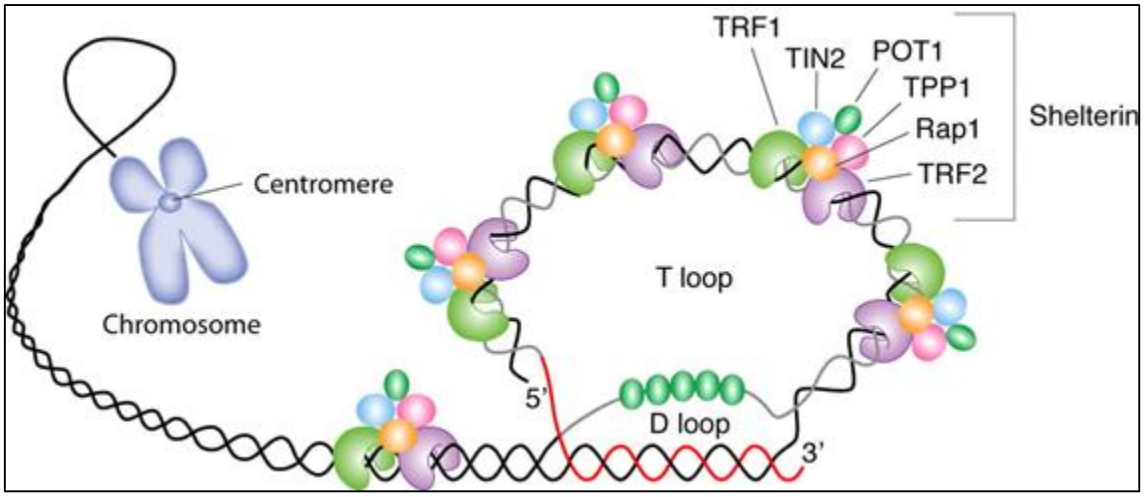


Figure 3: Speculative model for t-loop formation by shelterin. TRF1 has the ability to bend, loop, and pair telomeric DNA *in vitro* and could potentially fold the telomere. *Adapted from Rodrigo et al. (2008).*

It seems that this core shelterin complex is mainly located at the telomere end (also referred to as telosome) and serves both to stabilize t-loop structure and to protect it from being recognized by the DNA damage detection machinery as dsDNA breaks and repaired by Non-Homologous-End-Joining (NHEJ) repair activities. Additionally, shelterin regulates access to restoration processes of telomeric DNA after each genome replication. In general, shelterin complex seems to function as a platform regulating recruitment of a growing list of factors involved in chromatin remodelling, DNA replication, DNA damage repair, recombination and telomerase function, thus regulating telomere access/modification by diverse cellular processes, as reviewed by Diotti and Loayza (2011).

Apart from shelterin and interacting partners, another significant complex has recently emerged to be also involved in telomere biology, the CST complex. The CST complex is composed of three subunits: CTC1 (conserved telomere maintenance component 1), STN1 (suppressor of cdc thirteen homolog) and TEN1 (CST complex subunit TEN1), and has been attributed the rescue of stalled replication forks during

replication stress. The CST complex interconnects telomeres to genome replication and protection independently of the POT1 pathway (Miyake *et al.*, 2009).

An RNA molecules called telomeric-repeat-containing RNA (TERRA), has been identified as the third entity of the telomere nucleoprotein complex. TERRA transcription is mediated by DNA-dependent RNA polymerase II and is initiated from the sub-telomeric regions that are found near chromosome ends (Azzalin *et al.*, 2007); (Schoeftner e Blasco, 2008). TERRA levels are regulated during the cell cycle, and its localization at telomeres is modulated by the nonsense-mediated decay machinery (Porro *et al.*, 2010); (López De Silanes *et al.*, 2010). It has been recently demonstrated that this non-coding RNA acts as a bimolecular regulator to turn telomerase and the telomere on (replication-competent state) and off (protected state) during the cell cycle (Redon *et al.*, 2013).

The structure of telomeres is intrinsically dynamic, as chromosome ends should relax during genome replication and then re-establish their 'capped' state after replication. Consequently, telomeres may switch between off/protected and on/replication competent states during the cell cycle. Each state is governed by a number of interactions with specific factors and can lead the cell to either cell division or senescence/apoptosis under normal conditions, or to disorders/cancer in abnormal cases. Moreover, during development and in certain cell types in adults, telomere length should be preserved. Thus, multiple physiological processes guarantee functional and structural heterogeneity of telomeres concerning their length and nucleoprotein composition. A functional chromosome end structure is essential for genome stability, as it must prevent chromosome shortening and chromosome end fusion as well as degradation by the DNA repair machinery. Hence, structure and function of telomeres are highly conserved throughout evolution review by(Galati *et al.*, 2013).

1.2. The 'end-replication problem'

The primary difficulty with telomeres is the replication of the lagging strand. Because DNA synthesis requires a RNA template (that provides the free 3'-OH group) to prime DNA replication, and this template is eventually degraded, a short single-stranded region would be left at the end of the chromosome (referred to as the 'end-replication

problem'). This region would be susceptible to enzymes that degrade ssDNA. In the absence of any compensatory mechanism, the result would be that the length of the chromosome would be shortened after every cell division, resulting in cumulative telomere attrition during aging (Watson, 1972); (Olovnikov, 1973). In addition, loss of telomere DNA also occurs due to post-replicative degradation of the 5' strand that generates long 3' G-rich overhangs (Wellinger *et al.*, 1996), (**Fig. 4**).

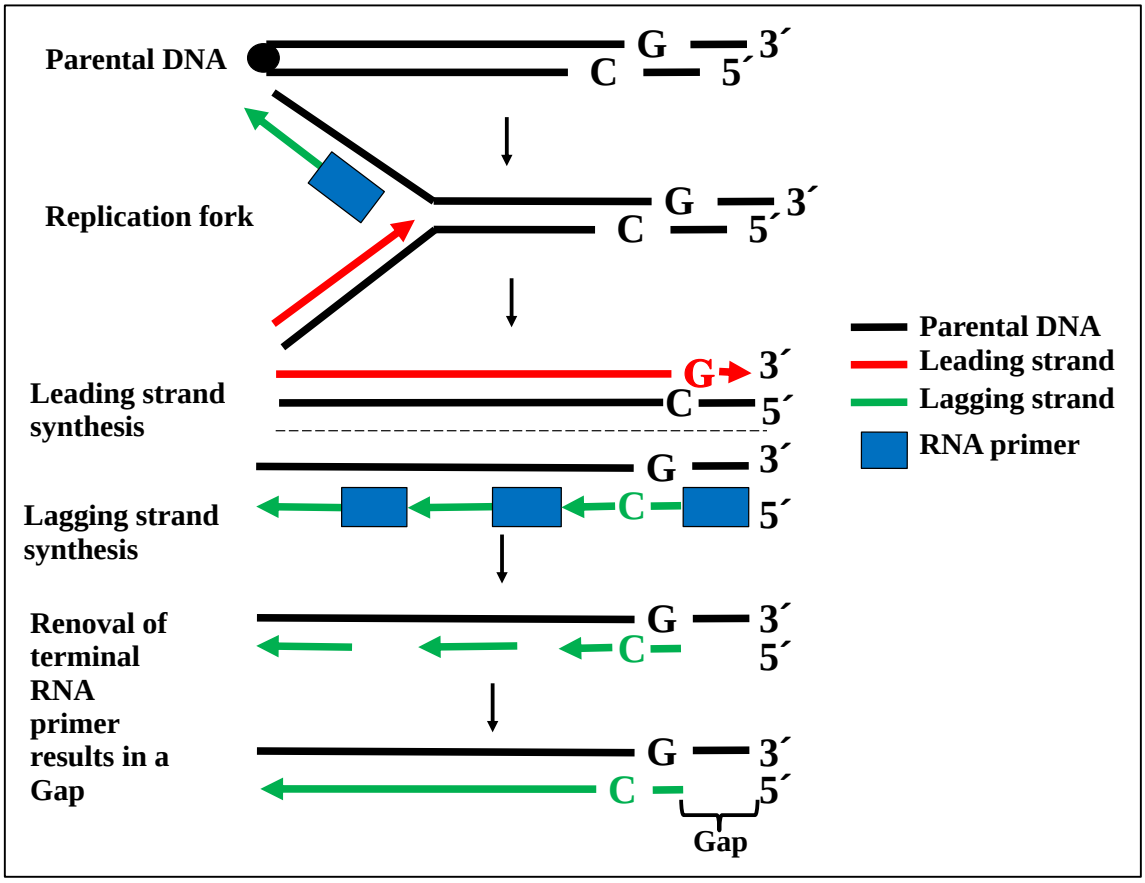


Figure 4: The 'end-replication problem'. DNA polymerase requires an RNA primer to initiate synthesis in the 5'→3' direction. At the end of a linear chromosome, DNA polymerase can synthesize the leading strand until the end of the chromosome. In the lagging strand, however, DNA polymerase's synthesis is based on a serie of fragments, called Okazaki, each requiring an RNA primer. Without DNA to serve as template for a new primer, the replication machinery is unable to synthesize the sequence complementary to the final primer event. The result is an end-replication problem in which sequence is lost at each round of DNA replication. *Adapted from the website http://jcs.biologists.org*

Upon each genome duplication, cells would otherwise keep losing genetic material, eventually resulting in premature cell death or replicative senescence, a critical problem for both the species and an individual's survival. This issue is even more prominent especially in multi-cellular organisms with late onset of reproduction.

2. Telomerase complex

During ontogenesis, eukaryotic organisms solved the 'end replication problem' by preventing telomere attrition in dividing cells, through recruitment of telomerase, a specialized and unique RNA-dependent DNA polymerase that synthesizes telomeric repeats at the end of eukaryotic chromosomes (Blackburn, 2005), thereby maintaining them at a 'constant' length, as a limited telomere length is a prerequisite for cell replication (Blackburn *et al.*, 2006), (**Fig. 5**).

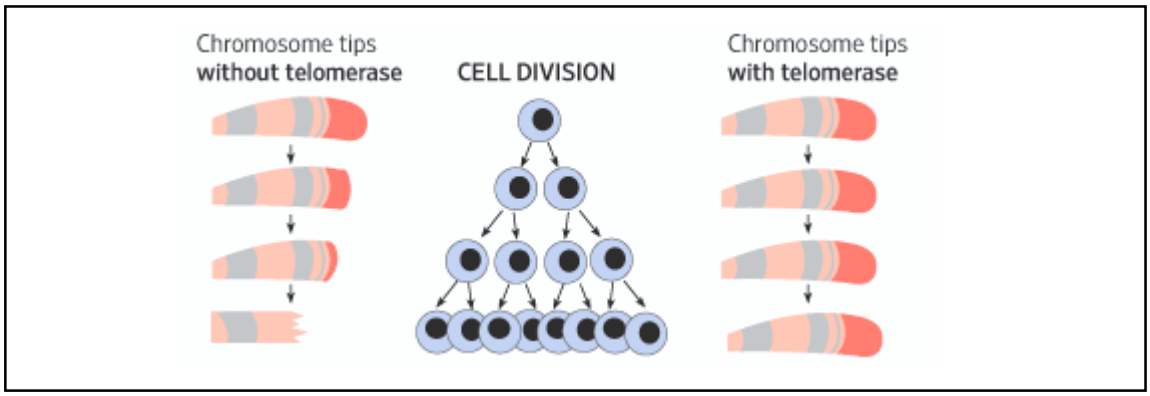


Figure 5: Telomerase synthesizes telomeres. Without telomerase, chromosomes get shorter over time and cells eventually stop dividing. *Adapted from The Nobel Committee for Physiology or Medicine.*

Telomerase is routinely active only during embryogenesis and development, while in adults is expressed only to rapidly dividing cells (i.e. proliferative skin and gastrointestinal cells, activated lymphocytes, specific bone marrow stem cells and dividing male germ cell lineages) (Ulaner e Giudice, 1997). In most adult cells telomerase is not expressed. Consequently, after a number of cell divisions, telomeres reach a critical length and chromosomes become uncapped (**Fig. 6**).

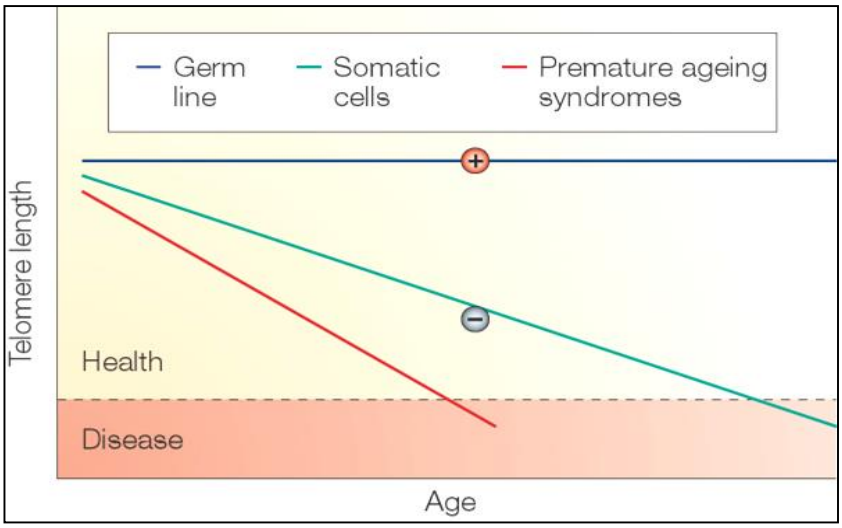


Figure 6: Telomeres undergo characteristic length changes over time in normal somatic and germ line cells, and in premature ageing syndromes. In contrast to germ cells, which have high telomerase activity (indicated by the plus symbol) and maintain telomere length with age, most somatic cells show progressive telomere shortening owing to low or absent telomerase activity (indicated by the minus symbol). In addition, several human premature ageing syndromes show an accelerated rate of telomere shortening, therefore resulting in an early onset of ageing-related pathologies. *Adapted from Blasco (2005).*

This progressive telomere loss eventually leads to critically short telomeres which leads, depending on the cellular context in which the uncapping occurs, a DNA damage response (DDR) that results in chromosomal end-to-end fusions or a permanent cell cycle arrest (termed cellular senescence) or apoptosis (programmed cell death) (Blasco, 2005); (Galati *et al.*, 2013). This loss of cell viability associated with telomere shortening is thought to contribute not only to the onset of degenerative diseases that occur during human ageing, but also to several age associated diseases such as cancer, coronary artery disease, and heart failure (Sherr e Mccormick, 2002); (Ogami *et al.*, 2004); (Starr *et al.*, 2007); (Donate e Blasco, 2011). Cells programmed to enter senescence may escape this procedure due to checkpoint dysfunction and instead continue infinite proliferation, leading to oncogenesis. In such cases genomic stability has to be re-established and telomere length has to be restored by a telomere maintenance mechanism (TMM). In most of tumor cells telomere maintenance is achieved by re-expression of telomerase. Interestingly, tumors have been described where telomerase could not be detected.

Further studies revealed that in addition to the role of telomerase in maintaining telomere length, homologous recombination constitute an alternative method (ALT 'alternative lengthening of telomeres') to maintain telomere DNA in telomerase-deficient

cells. ALT TMM, in contrast to telomerase dependent TMM, results in telomeres with high heterogeneity in length and at least in the well-studied model of *S. cerevisiae*, consists of two pathways. While the bulk of cancer and immortalized cells utilize telomerase re-expression to maintain telomere length, about 10-15% of tumors described operate using the ALT mechanism (Lundblad e Blackburn, 1993); (Teng e Zakian, 1999); (Teng *et al.*, 2000), (**Fig.7**).

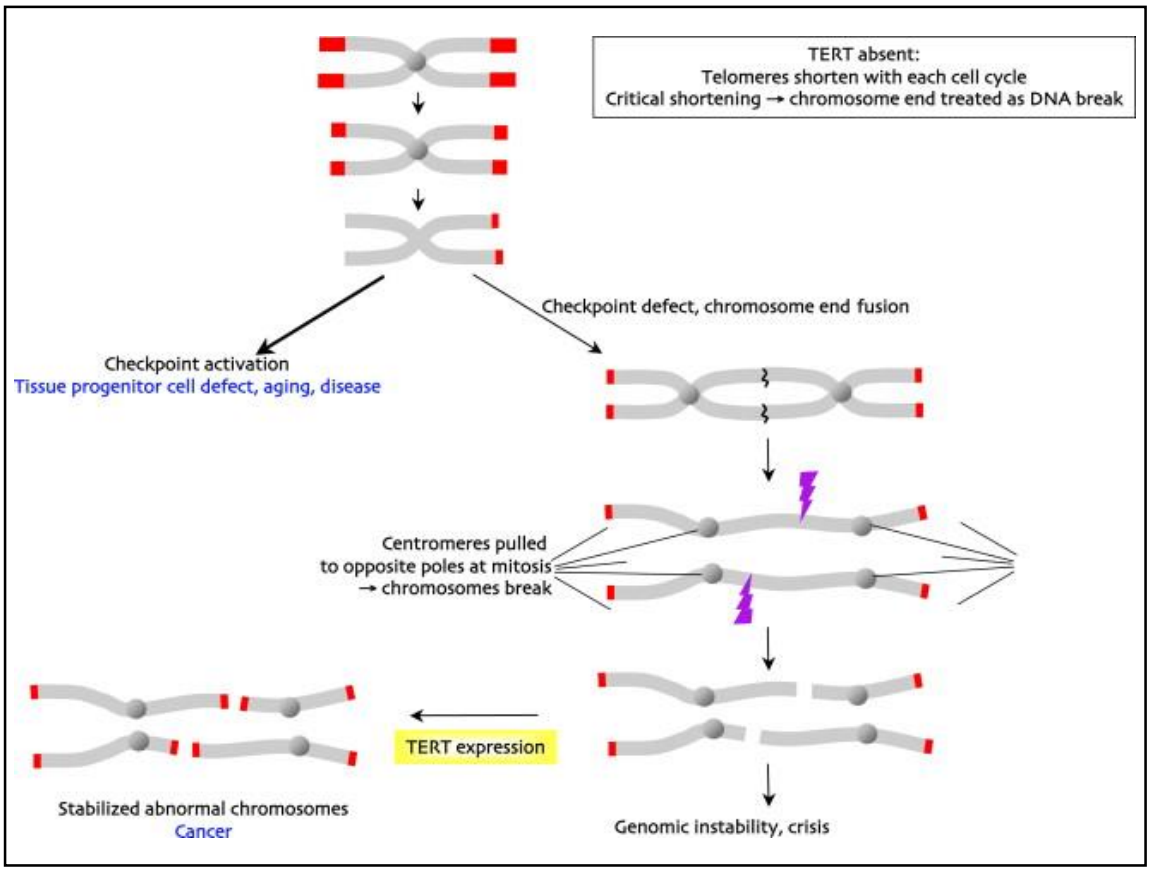


Figure 7: Telomere shortening causes senescence and chromosome instability. In cells with insufficient telomerase, DNA replication-dependent telomere attrition results in critically short telomeres, which activate the DDR, leading to senescence or apoptosis. However, in cells lacking a robust DNA-damage checkpoint response, inappropriate 'repair' reactions act on telomeres. One result of this is the end fusion depicted, which produces a dicentric chromosome that breaks randomly if the centromeres are pulled to opposite poles by the mitotic spindle. This scenario can lead to cell death or to widespread chromosomal rearrangement through cycles of chromosomal fusion and breakage, which can drive tumor development. Telomerase upregulation (or, rarely, an alternative telomere maintenance pathway) stabilizes the telomeres, allowing the propagation of aberrant genomes associated with many cancers. *Adapted from Artandi & Cooper (2009).*

2.1. Structure and function

The telomerase complex is a ribonucleoprotein (RNP) composed by a catalytic subunit (telomerase reverse transcriptase, TERT), a RNA component (*TR*) which acts as a template for the addition of the telomere sequence in the 3' end of the telomere, and species-specific accessory proteins that regulate telomerase biogenesis, subcellular localization and its function *in vivo* (Wyatt *et al.*, 2010). In human telomerase, seven associated proteins have been identified: Dyskerin, NHP2 (H/ACA ribonucleoprotein complex subunit 2), NOP10 (nucleolar protein 10), GAR1 (H/ACA ribonucleoprotein complex subunit 1), TCAB1 (telomerase Cajal body protein 1), Pontin and Reptin, (reviewed by (Gomez *et al.*, 2012), (**Fig. 8**).

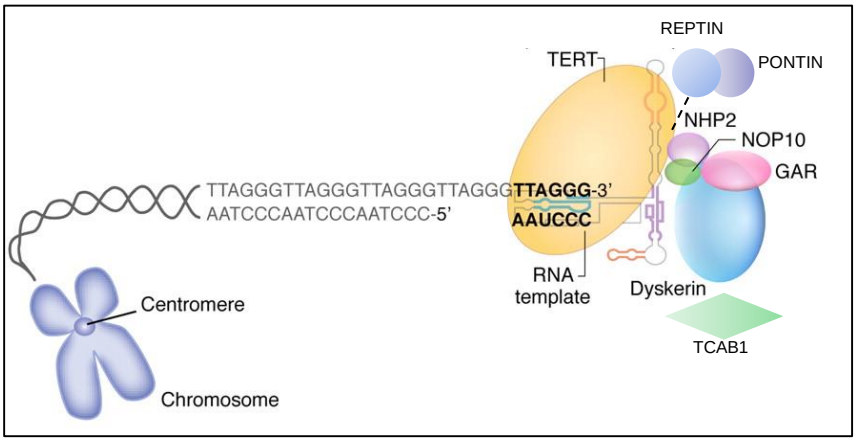


Figure 8: Schematic representation of human telomerase and its associated proteins. *Modified from Rodrigo et al. (2008).*

Dyskerin, NHP2 and NOP10 are required for the stability and accumulation of human *TR in vivo* [Fu & Collins, 2007]. Pontin and reptin are two closed ATPases necessary for the stability of dyskerin and *TR in vivo* (Veinteicher *et al.*, 2008). The current model is that dyskerin, pontin and reptin form a scaffold that recruits and stabilizes *TR*, and assembles the telomerase ribonucleoprotein particle. Once this complex is formed, pontin and reptin are believed to dissociate from the complex and release the catalytically active enzyme (Veinteicher *et al.*, 2008). The subcellular location of telomerase complex assembly is the cajal body, and it appears to be regulated by the TCAB1 protein (Zhong *et al.*, 2011). Also, while one study claims that the human telomerase holoenzyme contains only dyskerin, TERT, and *TR* (Cohen *et al.*, 2007), other studies establish that the human telomerase holoenzyme assembles all of the core proteins (Fu e Collins, 2007).

The action of the telomerase complex ensures that the ends of the lagging strands are replicated correctly. Elongation of the telomere by telomerase is a process that happens in different stages. First, the nucleotides of the 3' extreme of the telomeric DNA are hybridized to the end of the RNA template, inside the RNA domain of the telomerase complex. The template sequence of 11 nucleotides is complementary to almost two telomeric repeats. Next, the gap in the extreme of the template is completed by synthesis, using triphosphate nucleotides in the catalytic site of the enzyme (TERT). In this way, a complete hexanucleotidic repeat is assembled in the template. Then, telomerase relocates and the cycle is repeated, extending the telomere in the 3'-direction. Finally, the DNA polymerase can synthesize the lagging strand and thus, the end of the chromosome is faithfully replicated (Sfeir, 2012), (**Fig. 9**).

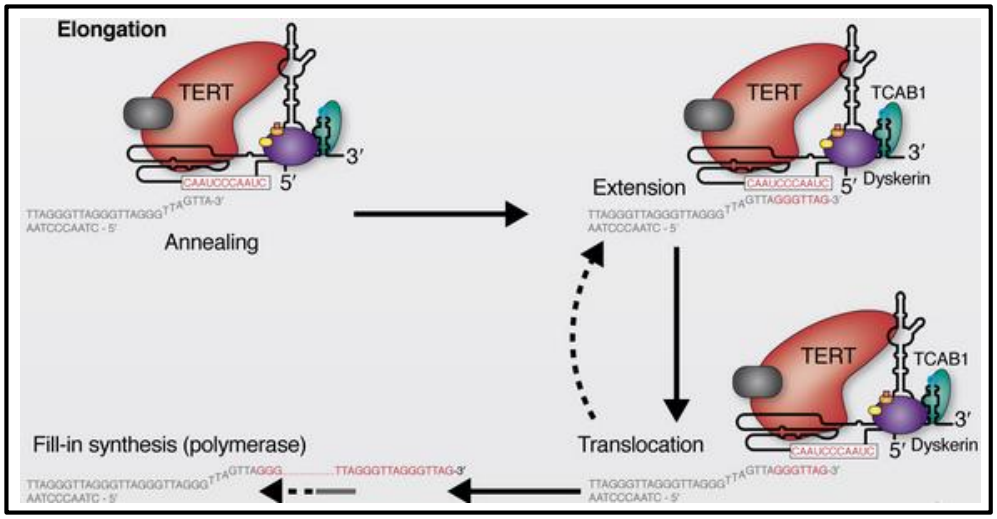


Figure 9: Telomere maintenance. Telomerase, consisting mainly of the protein (TERT) and RNA subunit (*TR*), binds to 3' flanking end of telomere that is complementary to *TR*, and bases are added using *TR* as template. Then, telomerase relocates and telomere is extended in the 3'-direction. Finally, the DNA polymerase can synthesize the lagging strand. This process can maintain telomere length or lead to telomere lengthening. *Adapted from Sfeir (2012).*

2.2. Catalytic subunit

Telomerase is a ribonucleoprotein polymerase that maintains telomere ends by addition of the telomere repeat TTAGGG. The enzyme consists of a protein component with reverse transcriptase activity, encoded by this gene, and an RNA component which serves as a template for the telomere repeat. Telomerase expression plays a role in cellular senescence, as it is normally repressed in postnatal somatic cells resulting in progressive

shortening of telomeres. Deregulation of telomerase expression in somatic cells may be involved in oncogenesis. Studies in mouse suggest that telomerase also participates in chromosomal repair, since de novo synthesis of telomere repeats may occur at double-stranded breaks. Alternatively spliced variants encoding different isoforms of telomerase reverse transcriptase have been identified; the full-length sequence of some variants has not been determined. Alternative splicing at this locus is thought to be one mechanism of regulation of telomerase activity.

Telomerase are located in 5p15.33 chromosome and are compound of 16 exons (**Fig. 10**).

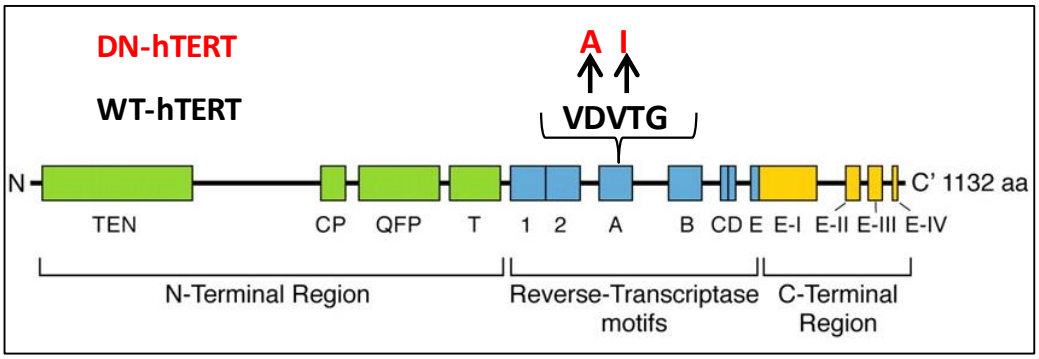


Figure 10: Linear structure of TERT, which is highly conserved among eukaryotes and consists of the central reverse transcriptase (RT) motifs (1, 2, A, B, C, D, and E), a large N-terminal region, and a short C-terminal region, all necessary for telomerase enzymatic function. The N-terminal region comprises a telomerase-essential N-terminal domain (TEN), the CP, and the QFP domains, required for RNA interaction, and a telomerase-specific T motif. The C-terminal region contains 4 conserved domains (E-I to E-IV). To generated the dominat negative hTERT they sustituyed the aspartic acid and valine residues at positions 710 and 711 in the third RT motif of hTERT with alanine and isoleucine. *Adapted from William et al. (1999).*

hTERT is a limiting subunit of telomerase in most human tissues. Whereas TERC RNA and other telomerase-associated proteins are expressed in most cell types, hTERT expression is highly regulated and its expression correlated with telomerase activity in many cell types. Studies have indicated that hTERT transcription is the primary step of telomerase regulation. hTERT transcriptional regulation is a complex process that involves both binding of transcription factors, such as Sp1 and c-Myc, to the promoter and epigenetic mechanisms that regulate chromatin environment of the hTERT locus.

hTERT expression is stringently regulated in most adult somatic tissues. It was previously shown that the hTERT gene was embedded in a condensed chromatin domain in many somatic cells

3. Telomere diseases

3.1. Telomere diseases, Tumor

This loss of cell viability associated with telomere shortening is thought to contribute not only to the onset of degenerative diseases that occur during human aging, but also to several age associated diseases such as cancer, coronary artery disease, and heart failure (Donate e Blasco, 2011); (Ogami *et al.*, 2004); (Starr *et al.*, 2007), (**Fig. 11**).

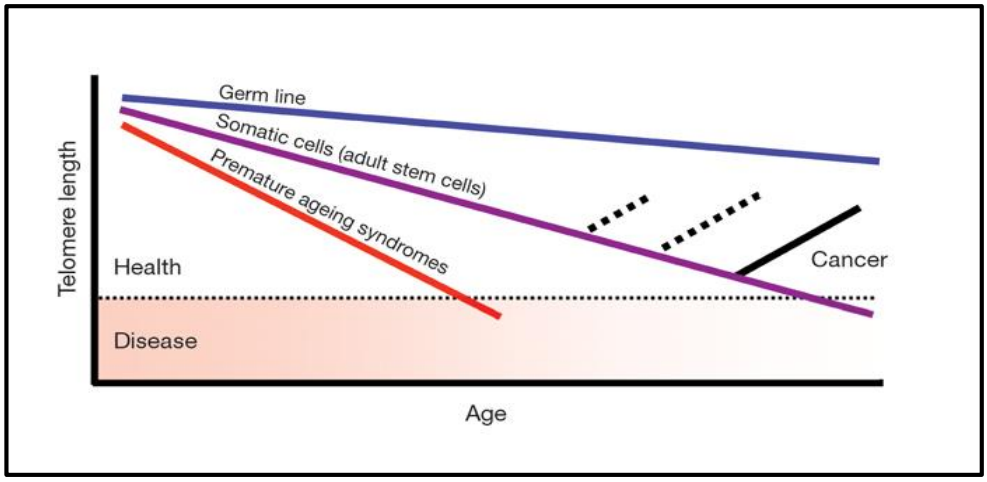


Figure 11: Telomeres undergo characteristic length changes over time in normal somatic and germ line cells, and in premature aging syndromes. Normal somatic cells, including adult stem cells, suffer progressive telomere attrition coupled to cell division or to increasing age of the organism. This attrition has been proposed to contribute to multiple age-related pathologies. In germline cells, telomere shortening is attenuated owing to high levels of telomerase activity. By contrast, telomere shortening is accelerated in several human premature aging syndromes, and patients with dyskeratosis congenita and aplastic anemia show decreased telomerase activity and shortened telomeres owing to mutations in the *TERC* and *TERT* telomerase genes. Psychosocial and environmental factors such as perceived stress, social status, smoking and obesity have also been shown to accelerate telomere attrition. In contrast to normal somatic cells, most immortalized cultures cell lines and more than 95% of human tumors aberrantly activate telomerase to achieve immortal growth. Although telomerase activity has been shown to be rate-limiting for mouse aging and lifespan, it is unknown whether increased telomerase activity will be able to extend the lifespan of organisms. *Adapted from Finkel et al. (2007).*

Telomere function is directly implicated in cellular senescence and therefore is expected to play a fundamental role in aging processes. Large epidemiological studies have reported an association between shorter telomere length in peripheral leukocytes and several inflammatory diseases of the elderly including diabetes, atherosclerosis and, recently, periodontitis (Steffens *et al.*, 2013). To date, leukocyte telomere length (LTL) serves in many cases as a predictor of age-related diseases and mortality. The potential role of telomere attrition in the onset or evolution of chronic inflammatory diseases, although requiring further investigation, could serve as a monitor of disease progression and effectiveness of treatment schemes. Furthermore, a recent work provides preliminary evidence in humans, supporting a correlation of maternal psychological stress during pregnancy with the setting of newborn leukocyte telomere length (Shalev et al., 2013).

Telomerase mutations have been detected in the context of several premature aging syndromes: dyskeratosis congenita (DC), a multisystem disorder characterized by defects in skin, blood, and lung, among other tissues (Walne e Dokal, 2008); aplastic anemia, a hematological disorder characterized by reduced red blood cell counts, bone marrow failure and liver and lung disease (Vulliamy *et al.*, 2002); Hoyeraal-Hreidarsson syndrome, a multisystem disorder characterized by bone marrow failure, immunodeficiency and severe growth retardation (Nishio e Kojima, 2010); and idiopathic pulmonary fibrosis, a chronic, progressive, and fatal disease that is defined by irreversible lung fibrosis (Armanios, 2012). The unifying molecular characteristic of these diseases is that patients harbor telomeres that are significantly shorter than age-matched control subjects (Armanios *et al.*, 2009). Telomerase mutations have also been identified in familial cases of pulmonary fibrosis and in sporadic cases of aplastic anemia. The full spectrum of telomerase mutations in human disease remains to be identified, and additional work in this area will continue to provide new insights into the pathophysiology of degenerative diseases and human aging (Garcia *et al.*, 2007) (**Fig.12**).

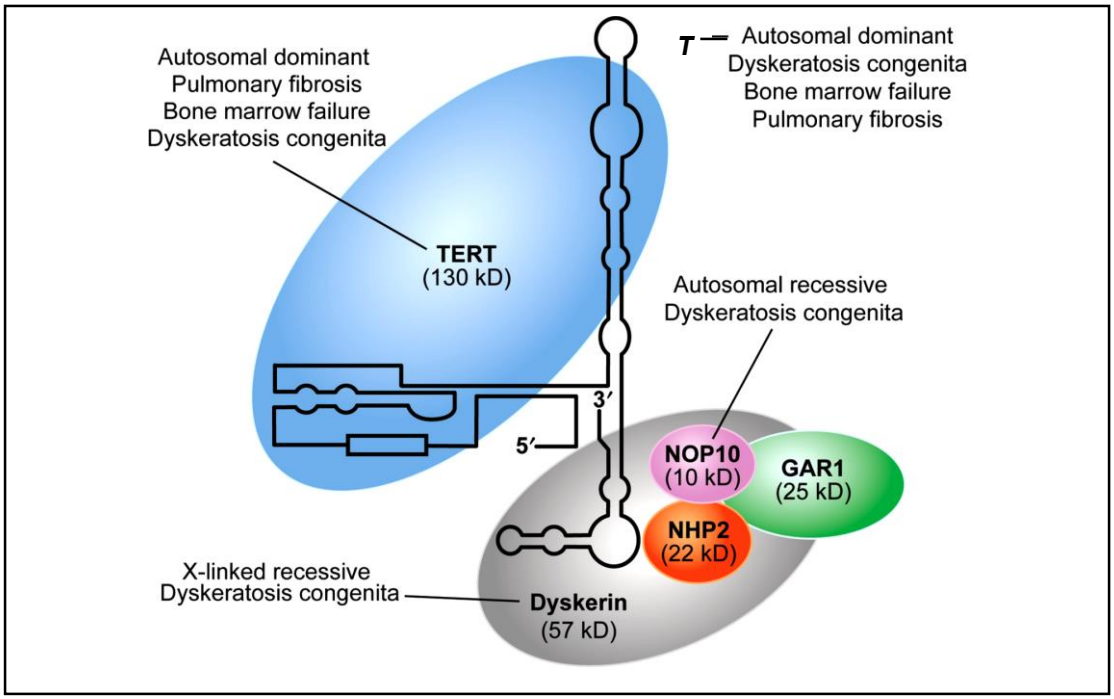


Figure 12: Schematic structure of the telomerase complex and diseases associated with mutations in the genes encoding each protein within the complex. *Adapted from García et al. (2007).*

Apart from aging and specific syndromes directly related to telomere dysfunction, abnormal telomere biology critically interferes with cancer (Prescott *et al.*, 2012). One of the hallmarks of cancer is unlimited cell proliferation, therefore tumor cells require a telomere maintenance mechanism in order to retain the ability of infinite propagation. Telomere maintenance in cancer is achieved by two major mechanisms. In most of the cases telomere attrition in cancer cells is counteracted by telomerase upregulation (Shay e Bacchetti, 1997) but in about 10-15% of tumor telomeres are preserved by telomerase independent mechanisms referred to as the ALT pathways which are based on homologous recombination (Royle *et al.*, 2009); (Cesare e Reddel, 2010), (**Fig. 13**).

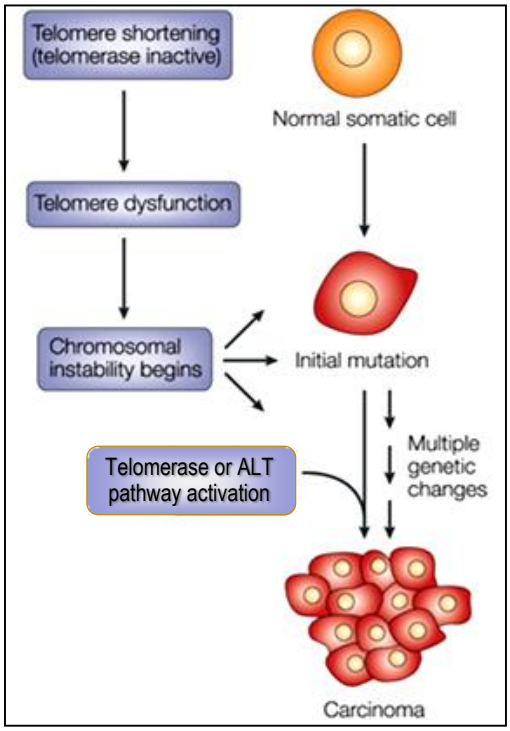


Figure 13: Telomere shortening in a simplified cancer-progression model. Normal somatic cells lose telomeric repeats as they divide in the absence of telomerase. Eventually, telomeres become dysfunctional and can cause chromosomal instability. Chromosomal instability occurs very early in tumorigenesis. It begins either before or soon after the initial mutation in a tumor suppressor or oncogene. Chromosomal instability then drives the multiple genetic changes that are required for the formation of a carcinoma. Telomerase or ALT pathway activation occurs late in tumorigenesis, increasing the replicative potential of a tumor by facilitating tumor growth. *Adapted from (Feldser et al., 2003).*

The relevance of telomere shortening induced by dysfunctional telomerase to physiological aging is suggested by the phenotypes of a telomerase deficient mouse model (Blasco *et al.*, 1997);(Lee *et al.*, 1998). Early generations of mouse *TR* deficient ($mTR^{-/-}$) mice do not show abnormalities presumably because laboratory mice have a much longer telomere length (20–150 kb) than humans (5–15 kb). However, late generations of $mTR^{-/-}$ mice have defects in cell viability of highly proliferative tissues. They have a shorter life span compared with wild-type (wt) mice and show hair loss or early graying of hair, decreased capacity for wound healing and a slight increased incidence of cancer. However, the telomerase deficient mouse models may not completely mirror human diseases of telomerase dysfunction as they have longer telomeres (Blasco *et al.*, 1997); (Zijlmans *et al.*, 1997), express telomerase activity in most tissues (Prowse e Greider, 1995); (Martín-Rivera *et al.*, 1998) and do not use telomere shortening as a counting mechanism (Shay e

Wright, 2000). For these reasons, an alternative animal model able to reproduce the human symptoms of telomere diseases is needed.

3.2. Telomeres and telomerase in aging

Organismal aging is characterized by the declining ability to respond to stress, increasing homeostatic imbalance and increased risk of disease that eventually results in mortality. Such functional decline can result from the loss or diminished function of post mitotic cells or from failure to replace such cells by a functional decline in the ability of cells to sustain replication and cell divisions (Aubert e Lansdorp, 2008).

One of the central mechanisms responsible for the aging of cells is the shortening of telomeres, as telomere attrition has been shown to contribute to a persistent DDR, which contributes to p53 activation and cellular responses to stress during replicative senescence, leading to the irreversible loss of division potential of somatic cells where telomerase is not expressed. Telomerase expression, robust in pluripotent stem cells and early stages of embryonic development, is however restricted to stem cell compartments in the context of the adult organism (Blasco, 2005).

Adult stem cells reside at specific compartments within tissues, which are enriched in cells with the longest telomeres (Flores *et al.*, 2008), then with sufficient telomere reserve in young or adult organisms to efficiently repopulate tissues and repair lesions. In old organisms, telomeres of adult stem cells may be too short (Flores *et al.*, 2008), and critically short telomeres are recognized as DNA damage. This triggers a complex signaling cascade with several steps: (i) activation of DDR proteins (53BP1, NBS1 and MDC1); (ii) activation of ATM and ATR kinases in senescent cells; (iii) activation of CHK1 and CHK2 kinases; (iv) phosphorylation and activation of several cell cycle proteins; and (v) the p53-mediated DNA damage signalling response impairs stem cell mobilization. These changes can induce a transient proliferation arrest allowing cells to repair their damage or lead to apoptosis or senescence if the DNA damage persists and exceeds a certain threshold (Kuilman *et al.*, 2010).

DNA damage can be caused by either exogenous or endogenous sources from the organism's own metabolism, which generates reactive oxygen species (ROS). Damage to

telomeric DNA by ROS produced by either dysfunctional mitocondria (Harman, 1988); (Wallace *et al.*, 2005), or by signalling pathways predispose cells to apoptosis or senescence. DNA damage signals originating from telomeres could be replication-independent and the sensitivity of cells to DNA damage could increase as the overall telomere length declines. Then, telomeres are important targets for stress and this has important consequences for the aging process (Aubert e Lansdorp, 2008).

In addition to a decline in stem cell function and accumulation of DNA, there are several others mechanisms that influence the aging process, as accumulation of protein damage, alterations in gene expression, checkpoint responses, then damage to multiple cellular constituents accounts for aging process (Kirkwood, 2005), and several biomarkers as senescence associated β-galactosidase (SA-β-gal), lipofuscin (Porta, 2002); (Terman e Brunk, 1998) and others stress-associated markers are used for assessing signs of aging and/ or oxidative stress.

The relevance of telomere shortening induced by dysfunctional telomerase to physiological aging is suggested by the phenotype of the telomerase deficient mouse models (Blasco *et al.*, 1997); (Lee *et al.*, 1998). Early generations of *TR* deficient mice ($mTR^{-/-}$) do not show abnormalities presumably because laboratory mice have a much longer telomere length (8 to 10 times) than humans (5–15 kb). However, late generations of $mTR^{-/-}$ mice have defects in cell viability of highly proliferative tissues. They have a shorter lifespan compared with wild-type mice and show hair loss or early graying of hair, decreased capacity for wound healing and a slight increased incidence of cancer as a consequence of chromosomal instability but, however, resistance to induction of skin cancer (González-Suárez *et al.*, 2000). Similarly, after successive generations of TERT-deficient ($mTERT^{-/-}$) mice, telomere shortening causes a decrease in life expectancy and the regenerative capacity (Strong *et al.*, 2011). However, the telomerase deficient mouse models do not reproduce the symptom that is the leading cause of death in DC patients, the bone marrow failure, as they have longer telomeres (Zijlmans *et al.*, 1997) and show telomerase activity in most tissues (Prowse e Greider, 1995); (Martín-Rivera *et al.*, 1998). Although there are two mouse models showing bone marrow failure, like the one combining mutations in *TR* and in one of the telomere binding proteins (POT1) (Hockemeyer *et al.*, 2008), and the one lacking TRF1 (Beier *et al.*, 2012), that help to clarify the mechanism by which bone marrow failure occurs, to date, has not been described any mutation neither in POT1 neither in TRF1 in the DC patients. For these reasons, an

animal model complementary to the mouse which is capable to reproduce the symptoms of human telomere diseases would be extremely useful to study the telomere biology.

In summary, shortening of telomeres associated with organismal aging, is sufficient to impair stem cells mobilization and tissue regeneration and it is proposed to be a key determinant of organismal longevity, then telomere length measurement may be useful to monitorize the healthspan of the cells in aging.

3.3. 'Extracurricular' roles

Telomerase is essential for the long-term proliferation potential of stem cells and cancer cells, and for normal tissue renewal. However, other functions have been described beyond its action at the telomeric level, so-called 'extracurricular' or non-canonical roles. Indeed, human TERT can function as a transcriptional modulator of the Wnt-β-catenin signaling pathway, as a cofactor in a β-catenin transcriptional complex through interactions with BRG1, which is an SWI/SNF (SWItch/Sucrose NonFermentable) related chromatin remodeling protein [Park *et al.*, 2009]. In addition, human TERT forms a complex with RMRP (RNA component of mitochondrial RNA processing endoribonuclease) and acts as an RNA-dependent RNA polymerase. The TERT-RMRP complex acts as an RNA-dependent RNA Polymerase (RdRP) and processes RMRP into double-stranded RNA (dsRNA), which is then processed by the endoribonuclease Dicer into small interfering RNA (siRNA), which controls RMRP endogenous levels (Maida *et al.*, 2009). Some evidence has been found for a role for telomerase in the regulation of apoptosis in a telomere maintenance-independent manner (Cong e Shay, 2008). Human TERT contains a mitochondrial localization signal peptide at its N-terminal that targets TERT to mitochondria where it is active. Furthermore, it was shown that telomerase sensitizes mitochondrial DNA to hydrogen peroxide-induced oxidative damage, probably through the modulation of metal homeostasis (Santos *et al.*, 2004). The mitochondrial localization of telomerase also has an important role in apoptosis (García-Santos *et al.*, 2006). Moreover, human TERT contribution to epithelial proliferation, tumorigenesis and aging is also mediated by a telomere length-independent mechanism (Choi *et al.*, 2008); (Sarin *et al.*, 2005); (Stewart, 2002); (Geserick e Blasco, 2006).

In contrast to TERT, repressed at the transcriptional level in most somatic cells, *TR* is constitutively expressed in human cells, and it ubiquitously assembles as a stable RNP complex (Cong *et al.*, 2002) ; (Cairney e Keith, 2008), suggesting a non-canonical role also for *TR*. For example, the cancer promoting activity of *TR* may also occur independently of telomerase activity (Blasco *et al.*, 1996); (Cayuela *et al.*, 2005); (Fragnet *et al.*, 2003); (Li *et al.*, 2005). Although the mechanism involved in this activity of *TR* is largely unknown, a recent study identified 2198 *TR* binding sites in the genome using chromatin isolation by RNA purification (ChIRP), which represents a large resource to study potential non-canonical functions of human *TR* and TERT (Chu *et al.*, 2011)(Chu *et al.*, 2011)(Chu *et al.*, 2011. *TR* occupied multiple *Wnt* genes directly and several binding sites near the *MYC* (v-myc avian myelocytomatosis viral oncogene homolog) gene, concordant with previously documented binding sites of TERT (Chu *et al.*, 2011).

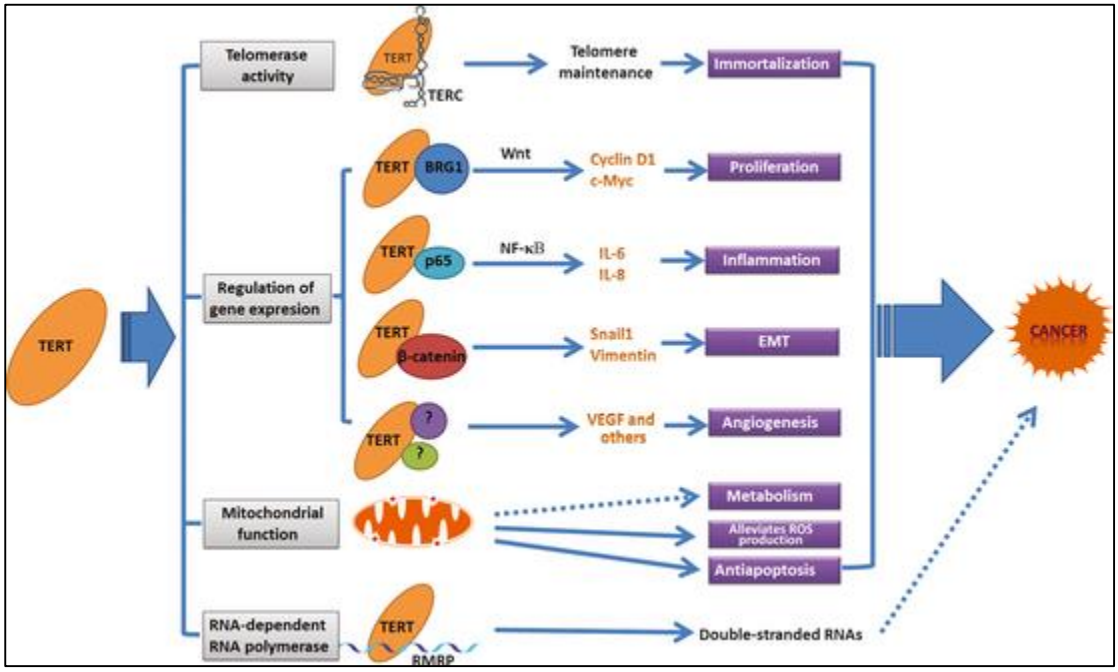


Figure 14: The functions of hTERT in cancer progression. hTERT functions as the catalytic component of telomerase in maintaining telomere homeostasis, and also forms complexes with different cellular factors involved in several fundamental cellular functions in a telomereindependent fashion, which may provide transformed cells with cancer-specific capacities during multiple stages of tumor development. IL, interleukin**.** *Adapted from (Ding et al., 2013).*

TERT plays multiple roles in both physiological and pathological processes, not only by affecting telomere homeostasis, but also by regulating small RNA homeostasis, including both endogenous siRNA and miRNA.

3.4. Cancer and metastasis

The cancer stem cell is defined by its capacity to self-renew, the potential to differentiate into all cells of the tumor and the ability to proliferate and drive the expansion of the tumor.

Approximately 90% of all cancer deaths arise from the metastatic spread of primary tumors. Current models view metastasis as a highly dynamic process that occurs in multiple steps. The initial steps involve disruption of cell–cell adhesions, the migration of cells away from the primary tumor, and intravasation into the vasculature. Later translocate through the vasculature, arrest in distant capillaries, extravasate into the perivascular tissue, and finally proliferate from micrometastases into macroscopic secondary tumors. The individual steps in the metastatic cascade remain incompletely understood. This is especially true of the initial steps leading to intravasation, when small developing tumors and micrometastases are not easily detected. Most of our understanding of these early steps during the metastatic cascade has been derived from static images of large, advanced-stage tumors and their associated metastases. Consequently, fundamental questions remain as to how invasive cancer cells navigate through complex tissues, locate vessels, and intravasate. Thus, there is a crucial need to understand invasive mechanisms and angiogenic programs that facilitate metastasis so that therapeutic strategies can be developed to block disease progression.

Invasiveness and early formation of metastases are the main reasons why for example pancreatic cancer continues to have a dismal prognosis, with a 5 year survival rate of <5% and a mean life expectancy of <6 month.

3.5. Xenotrasplant in zebrafish embryos

Zebrafish embryos have been employed in several useful models for therapeutic drug research and preclinical studies, as well as high throughput screening for several reasons: fish are inexpensive to maintain, breed in large numbers (100–300 embryos per week/couple), develop rapidly ex vivo, embryos are transparent, have short generational cycles (2–3 mo), are immunodeficient until day 11 post-fertilization (dpf) and require small amount of drugs per experiment.

Analysis of tumor metastasis in an in vivo model depends on intrinsic tumor cell properties, host factors and the experimental techniques used.

This model has become recently interesting for oncology research as a xenograft system for transplantation of malignant melanoma cells that showed that cells were not rejected, survived and showed motility. In another study, the group of (Haldi *et al.*, 2006) established the conditions where human melanoma cells proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in transplanted zebrafish embryos.

Using 2 days post-fertilization (dpf) zebrafish embryos as transplant recipients, we can see cells grown from breast carcinoma cell lines migrate to the tail of the embryo and form masses with a significantly higher frequency than parental monolayer populations.

Currently, the gold standard functional assay to demonstrate tumor initiating ability is the cell xenograft in immunodeficient mice, although cancer stem cell frequency measurement depends on the system used. This animal model presents several caveats at the practical level, such as dedicated expensive animal facilities, including maintaining a pathogen free area and trained pathologists and veterinarians; the number of animals that can be used in one experiment, usually limited to a few dozens, and the length of tumor formation extends over a period of months.

The zebrafish as a robust in vivo model for investigating invasiveness and metastatic behaviour of human primary tumors. It is known that early zebrafish embryos do not reject xenotransplanted human cells, whereas 1 month old zebrafish already need to be immune suppressed. The early embryos and larvae used here did not reject the primary tumor xenografts, most likely due to the fact that their immune system is not fully developed. It has been observed that while lymphopoiesis and lymphoid organogenesis are initiated at the middle to late embryo period, they remained in their rudimentary and immature form throughout the early larval stages. The major maturation events leading to immune competence occur between 2 and 4 weeks post fertilisation (wpf), coinciding with the larval to juvenile transitory phase. The observed metastasis in an animal model primarily should reflect the intrinsic metastatic ability of the tumor cells, but may depend to some extent also on the experimental system. Other experimental animal systems have demonstrated that only a small subset of metastatic cells (approximately 2%) survive and grow at secondary sites. The significantly higher percentage of micrometastases observed using

fish embryos may in part reflect the absence of the humoral immune response and/or other selective pressures on tumors cells which would lead to tumor cell death following extravasation into secondary organs. The transparency of the fish embryo enables an investigation of fluorescently labelled tumor cells in real time and at high resolution. The unique availability of transgenic zebrafish without a functional vasculature further allowed us to show that the metastatic spread of tumor cells in zebrafish embryos involves the vascular system. Even the very early steps of invasion, circulation of tumor cells in blood vessels, colonization at secondary organ sites and metastasis formation can be observed this way-something which to date cannot be investigated in established mouse tumor models. Advantages of the model system such as good accessibility, easy handling, low costs and short incubation times make it a promising system for future functional studies in primary tumors. The experiments described here provide the basis for the future development of a screening methodology of drugs, which inhibit invasion and metastasis of human tumors. Recently, adult zebrafish with an almost entirely transparent body have been described, as a novel tool for in vivo transplantation analysis. These will be of interest for additional comparative analysis of metastasis formation of primary tumors in the immune competent animal.

Thus, full recapitulation of the genetic complexity of human tumors may not be possible using zebrafish animal models and certain genetic diseases may be restricted to humans making verification of results in primary human cells mandatory. Due to obvious ethical and practical limitations, in vivo studies on human cells are limited to xenografts. While these assays are so far best established in immunopermissive mice, several reports document the feasibility of xenografting human cells to zebrafish. This technique has the potential of marrying the clinically relevant context of human-derived cells with the unique opportunities for imaging and genetic manipulation provided by the zebrafish. Here, we summarize available data on zebrafish as hosts for human xenotransplant assays and discuss the potential of this model to complement and, in some cases, surpass murine systems.

The zebrafish model provides unique tools for visualization of tumor cell behavior and interaction with host cells. Zebrafish embryos develop ex utero and up to one month of age, their larvae are transparent, allowing direct imaging of development, organogenesis, and cancer progression.42,43 In adults, transparent zebrafish lines enable tracking of

transplanted cells down to the single cell level.44 Several other transgenic lines are available and can facilitate studies on interactions between human cells and specific host factors, as well as exposure to genetically modified host tissue niches. For example, Tg(fli1-eGFP) embryos in which the fli1 promoter is able to drive the expression of EGFP in all blood vessels throughout embryogenesis can be used for analyzing tumor-induced angiogenesis. Other available transgenic lines labeling blood vessels are Tg(flk1:mCherry) or Tg(vegfr2:g-rGFP). More recently, the Tg(mpx:GFP)i114 line was used to examine neutrophil-mediated experimental metastasis. Other transgenic lines that specifically label macrophages Tg(mpeg1:eGFP) or platelets Tg(cd41:eGFP) could similarly be employed to study the impact of these components of the host's inflammatory response on tumor cell behavior.

If tumor engraftment rates in zebrafish prove to be similar surrogate markers for clinical disease aggressiveness they can provide readout within a few days. In leukemia, differential engraftment of putative CD34+ stem cells versus CD34– primary myeloid blasts was observed only four days after xenotransplantation into zebrafish embryo. Similarly, human chronic myeloid leukemia (CML) K562 and acute promyelocytic leukemia (APL) NB-4 cell lines that were injected into 48-hpf embryos could be tracked for up to seven days. During this time, engraftment and circulation of these leukemia cells were monitored by live-cell microscopy and proliferation was quantified by enumerating fluorescently labeled humancancercellsat24-and72-hpostinjection (hpi). Using this proliferation assay, we observed a reproducible increase in leukemia cell numbers within the embryo of approximately threefold after 48 hours (see also Fig. 1B). These developments hearken at the potential for the zebrafish system as experimental readout for leukemia proliferation and progression in real time.

Thus, higher zebrafish maintenance temperatures around 34–35 ◦C should be considered when evaluating biological characteristics of xenotransplated human cells.

Another concern relates to cellular size. Zebrafish cells, as well as zebrafish vessels and other anatomic structures are smaller than the corresponding human structures. However, circulation of leukemic cells and migration of even larger human solid tumor cells, such as sarcoma cells, has been repeatedly demonstrated in zebrafish indicating that passage is possible.

4. MicroRNA

Are small, highly conserved non-coding RNA (NcRNAs) molecules which are not translated into proteins. NcRNAs play critical roles in regulating gene expression at transcription, RNA processing, and translation levels (Macfarlane e Murphy, 2010) in various biological processes. Depending on the length and functions, ncRNAs can be classified into three groups: very small RNAs (18 - 25 nucleotides) – microRNAs (miRNAs) and small interfering RNAs (siRNAs); small RNAs (smRNAs, 20-200 nucleotides); and medium and large RNAs (piRNAs, 200-10000 nucleotides) (Esteller, 2011). MicroRNAs are single-stranded noncoding RNA molecules and approximately 21–25 nucleotides in length, that play crucial roles in posttranscriptional regulation of gene expression (Bartel, 2004). MicroRNAs are partially or fully sequence-complementary to mRNA targets, and their main function is reduce stability, expression and/or translation of mRNAs in a variety of manners, including mRNA cleavage, translational repression, and deadenylation (Bartel, 2009).

4.1. Biogenesis

Most of miRNAs has known to be located in the intron region of their host genes and share their mRNAs and regulatory elements resulting in a similar expression pattern. (**Fig. 15**) shows canonical biogenesis of miRNA (Esquela-Kerscher e Slack, 2006). The process has several steps to produce a mature form of a miRNA from its host gene. The miRNA host gene is transcribed to a large RNA precursor (pri-miRNA) with of a 5’ cap and poly-A tail 3’ in nucleus, and then processed to a precursor miRNA (pre-miRNA) by the complex of Drosha (RNase III enzyme) and Pasha/DGCR85 (double-stranded-RNA-binding protein). The pre-miRNAs becomes the imperfect stem-loop structure of about 70-nucleotides in length, and is exported into the cytoplasm by the karyopherin exportin 5 (Exp5) and Ras-related nuclear protein (Ran)-GTP complex. Next pre-miRNA is further processed to a miRNA-miRNA duplex with 22 nucleotides by the RNAse III enzyme Dicer. After one strand of this duplex is degraded, a mature miRNA with approximately 22 nucleotides is generated and binds to RNA-induced silencing complex (RISC) leading to gene silencing and RNA interference (Paroo *et al.*, 2007).

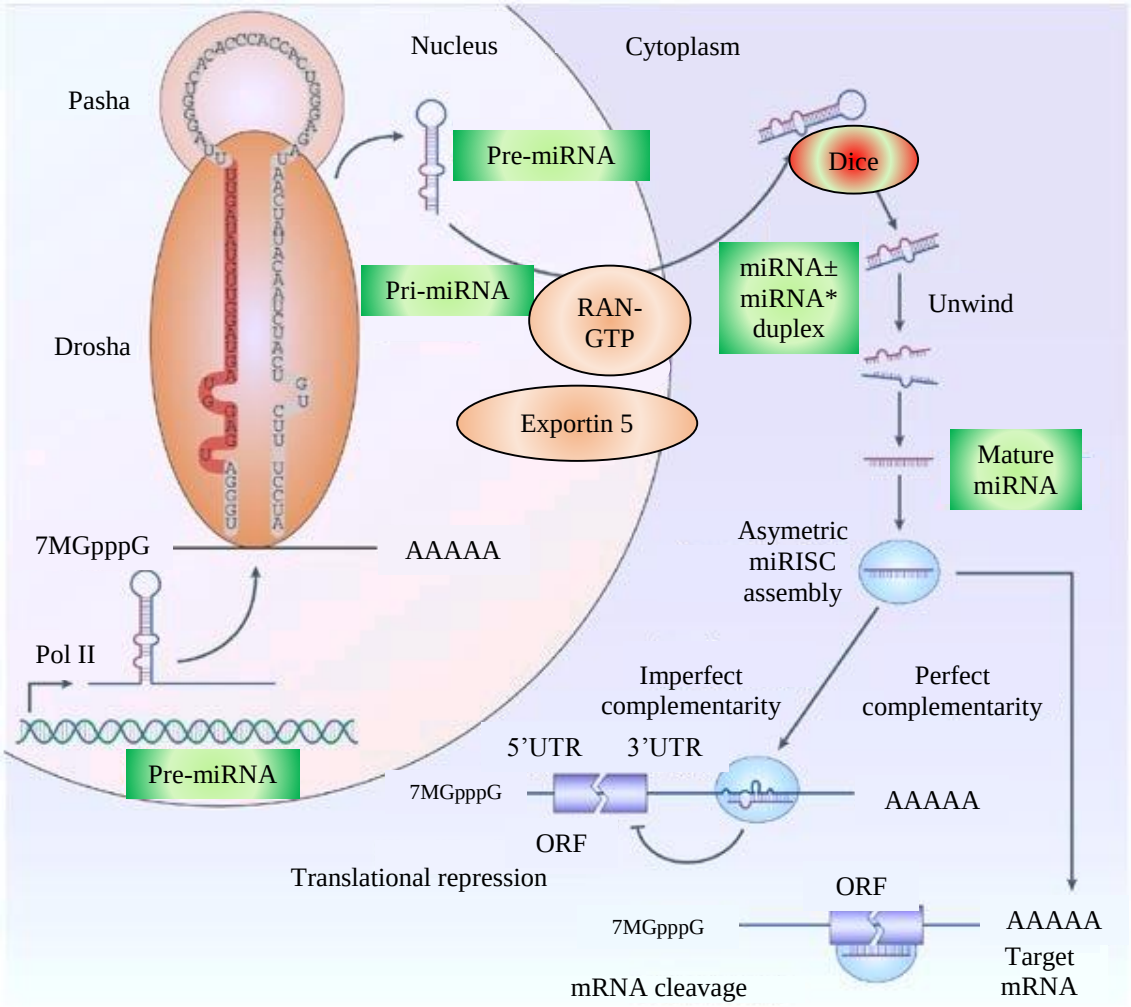


Figure 15: Canonical biogenesis of miRNA. MicroRNA (miRNA) genes are generally transcribed by RNA Polymerase II (Pol II) in the nucleus to form large pri-miRNA transcripts, which are capped (^{7}MGpppG) and polyadenylated (AAAAA). These pri-miRNA transcripts are processed by the RNase III enzyme Drosha and its co-factor, Pasha, to release the ~70-nucleotide pre-miRNA precursor product. (Note that the human *let-7a-1* miRNA is shown here as an example of a pre-miRNA hairpin sequence. The mature miRNA sequence is shown in red.) RAN–GTP and exportin 5 transport the pre-miRNA into the cytoplasm. Subsequently, another RNase III enzyme, Dicer, processes the pre-miRNA to generate a transient ~22-nucleotide miRNA:miRNA* duplex. This duplex is then loaded into the miRNA-associated multiprotein RNA-induced silencing complex (miRISC) (light blue), which includes the Argonaute proteins, and the mature single-stranded miRNA (red) is preferentially retained in this complex. The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation (lower left). However, recent evidence indicates that miRNAs might also affect mRNA stability (not shown). Complementary sites for miRNAs using this mechanism are generally

found in the 3' untranslated regions (3' UTRs) of the target mRNA genes. miRNAs that bind to their mRNA targets with perfect (or nearly perfect) complementarity induce target-mRNA cleavage (lower right). miRNAs using this mechanism bind to miRNA complementary sites that are generally found in the coding sequence or open reading frame (ORF) of the mRNA target.Modified (Esquela-Kerscher e Slack, 2006).

4.2. MicroRNA and mRNA target interaction

In miRNA target prediction using sequence based computational approaches, the critical step is to identify the miRNA-mRNA target interaction. There are four main characteristics to predict this interaction as followings: seed match, conservation, free energy and site accessibility (Kandoth *et al.*, 2013). The seed sequence of a miRNA is the first 2-8 nucleotides from the 5' end (Asgari, 2011). Most of miRNA target prediction tools use this seed sequence to identify the targets of a miRNA by Watson-Crick (WC) match (Asgari, 2011) (**Fig. 16**). Base paring pattern is important to predict miRNA targets (Mazière e Enright, 2007). The potential binding sites can be defined into three groups such as 5'-dominant seed canonical, 5'-dominant seed only, and 3'-compensatory (Enright *et al.*, 2003); (Lewis *et al.*, 2005) (Figure 16 D). Depending on the prediction algorithms, several types of seed matches are used: 6 mer, 7-mer-m8, 7mer-A1, and 8mer (Brennecke *et al.*, 2005).

Sequence conservation across species is one of factors to predict miRNA targets. Generally miRNA seed regions are highly conserved compared with non-seed region in a miRNA (Network, 2012). Gibbs free energy in binding between a miRNA and its target mRNA can be used for a prediction measure. If it has the lower energy the stability is increased meaning more likely to be a true target (Nair *et al.*, 2014). Site accessibility of a miRNA to a mRNA target is one of measurements for target prediction. Depending on the secondary structure of a target mRNA, miRNA:mRNA hybridization can be predicted (Network, 2008). Although many miRNA target prediction algorithms have been developed, it is still challengeable due to the thousands of binding possibility per a miRNA depending on various conditions.

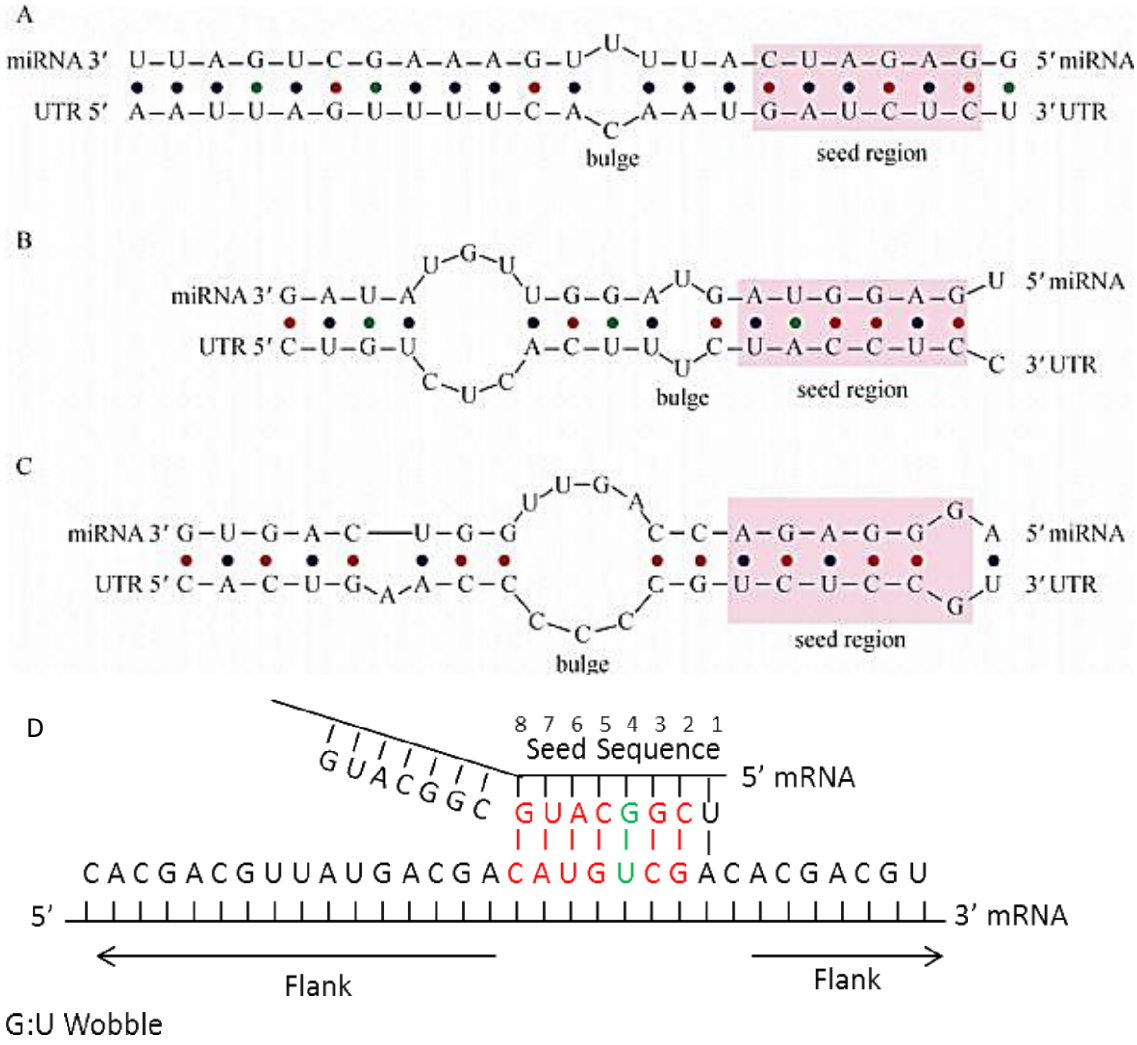


Figure 16: Schematic overview of a miRNA-mRNA target interaction and secondary structures. Canonical sites have good or perfect complementarity at both the 5′ and 3′ ends of the miRNA, with a characteristic bulge in the middle. (A). Dominant seed sites have perfect seed 5′complementarity to the miRNA but poor 3′ complementarity (B). Compensatory sites have a mismatch or wobble in the 5′ seed region but compensate through excellent complementarity at the 3′end (C). The nucleotides 2-8 of the miRNA seed region play an important role in binding to a target mRNA. Flank represents the outside sequence of the seed region. A G-U wobble pair in green is shown in the middle of the interaction (D). Modified from (Peterson *et al.*, 2014); and (Huang *et al.*, 2010).

4.3. MicroRNA action mechanism

Currently, 28645 miRNAs has been registered in miR Base (Kozomara *et al.*, 2014). miRNAs silence gene expression by making partial base-paring with 3' untranslated region of target mRNAs (Bartel, 2009). Because imperfect base pairing with target mRNAs is

sufficient for inhibition, single miRNA can target a number of genes and also multiple miRNAs can target single mRNA (Vlachos *et al.*, 2012).

The regulatory mechanism of MicroRNAs can repress mRNA translation and destabilize mRNA transcripts in the processing body (P-body) in which miRNA-target mRNAs are isolated from translational process and degraded (Fazi e Nervi, 2008); (Romero-Cordoba *et al.*, 2014). The complexes repress mRNA translation, degrade mRNAs and destabilize by deadenylation. Scissors indicate the cleavage on pri-miRNA or mRNA. RISC: RNAinduced silencing complex. Furthermore miRNA regulation mechanisms can be classified to cis- and transregulation (Nikitina et al., 2012). (**Fig. 17**). In cis-regulation, miRNAs directly bind to target mRNA sequences and regulate the gene expression and translation. In trans-regulation, miRNAs can indirectly regulate from gene to protein levels by targeting the mRNAs of transcription factors, RNA regulating proteins and interacting proteins.

Recent studies estimated that each miRNA can regulate more than 200 genes (Bussey *et al.*, 2006); (Gennarino *et al.*, 2009), implying that miRNAs regulates a large number of biological processes that are frequently altered in many human diseases. Therefore, to understand the functional roles of miRNAs in disease, it will be axiomatic to accurately identify target mRNAs.

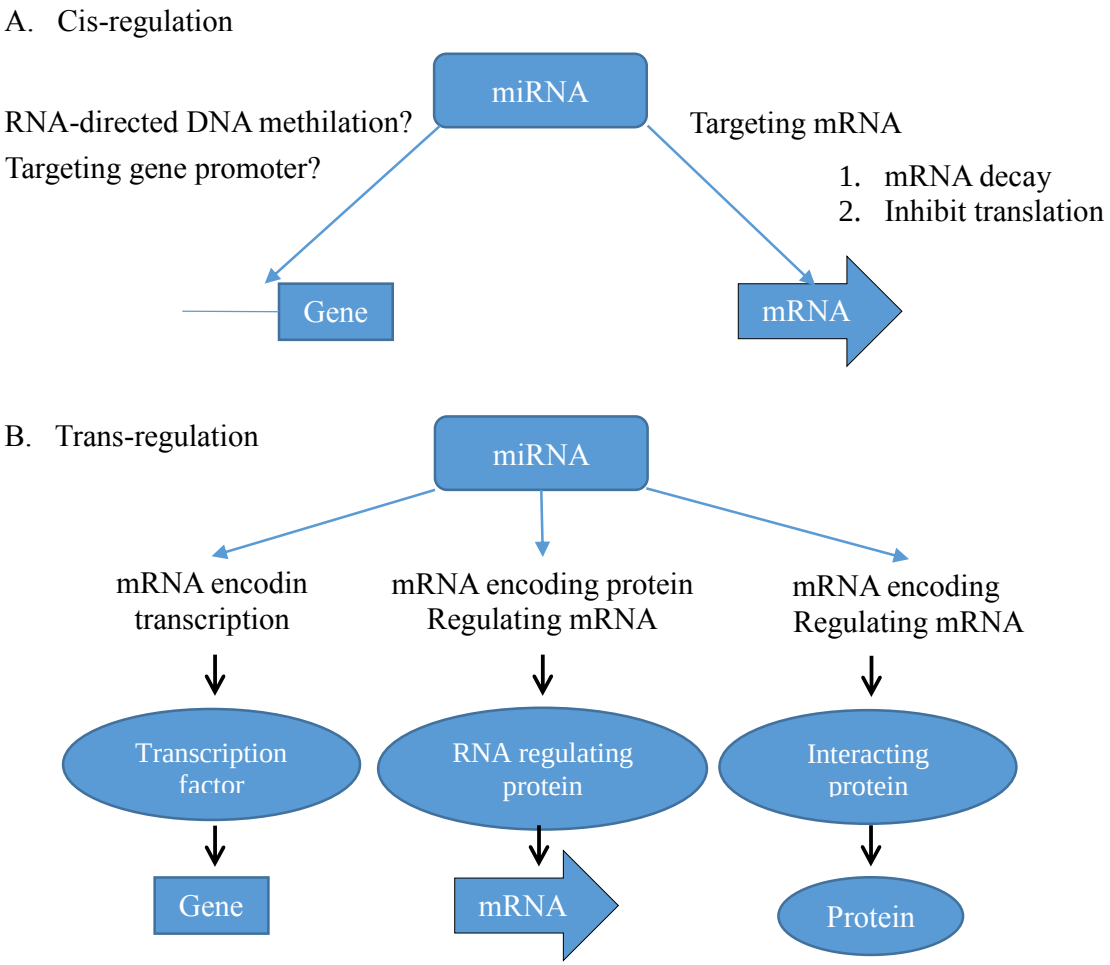


Figure 17. **Potential microRNA regulation mechanisms by multifactorial and encompassing interactions.** Cis-regulation. MicroRNAs directly target the mRNA, and control the expression of the target gene at post-transcriptional levels by mRNA degradation and inhibiting translation. (A). Transregulation. MicroRNAs regulate the expression changes of the targeted specific genes such as transcription factors, RNA regulating protein coding genes, and interacting protein coding genes (B). Modified from (Nikitina *et al.*, 2012).

4.4. miRNA cluster

Around 50 % of all miRNA genes are located within 50 kb in length on the genome and transcribed together as a cluster (Mcgraw *et al.*, 2007); (Becker *et al.*, 2012); (Chan *et al.*, 2013). These clusters range from 2 to several dozens of miRNAs. miRNAs in a cluster frequently shows similar sequence homology in the seed region. This results in identical targets of a miRNA cluster. The length of miRNA clusters depend on species (Chan *et al.*, 2013) (**Fig. 18**). This cluster family functions in cell proliferation, apoptosis, development and cancer oncogenesis (Mogilyansky e Rigoutsos, 2013). Moreover, miRNA clusters can coordinately regulate the different genes or the downstream effectors such as transcription factors in a specific signaling pathway or protein complex (Inui *et al.*, 2010).

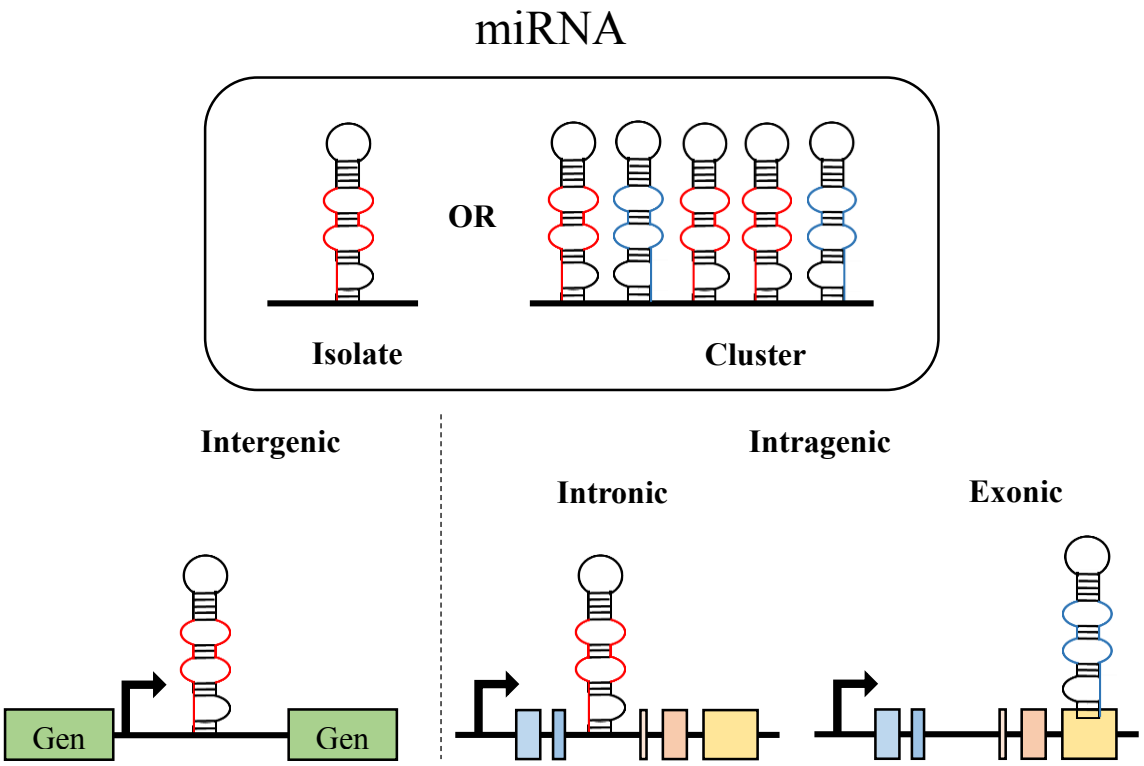


Figure 18. Genomic locations of miRNAs. miRNA genes, isolated or in clusters, are located in intergenic *(ex: miR-494)* or intragenic genome regions, including exons of non-coding *(e.g. miR-155)* or coding (e.g. miR-985) genes and introns of non-coding (e.g. the miR-15a ~16-1 cluster) or coding *(e.g. miR-126)* genes

Objectives

The specific objectives of the present work are:

1. Characterization of the mechanism by which TERT overexpression influences the increased invasion capacity and metastasis of tumor cells.

2. Validation of the zebrafish model to study the invasion capacity of human tumor cells by xenograft experiments.

CHAPTER I:

Telomerase non-canonical function in metastasis process

Abstract

Human telomerase reverse transcriptase (hTERT) plays a key role in tumor invasion and metastasis, but the molecular mechanism is not well understood. In this study we were able to increase the invasion capacity of an osteosarcome cell line, which maintains telomere length independently of telomerase activity, through h*TERT* overexpression. We found that the up-regulation of h*TERT* in these cancer cells changes the microRNA expression profile. In particular, hTERT regulates the expression of miR-500a. Moreover, the overexpression or inhibition of miR-500a enhanced or decreased the invasiveness in a xenograph experiment, respectively. Finally, we demonstrated that hTERT regulates the miR-500a expression through direct binding to its promoter region. Taken together, our results suggest that hTERT may promote cancer cell metastasis through the transcriptional regulation of miR-500a, a key component in this process.

1. Introduction

Telomerase activation is a common feature of most types of human cancers. Although several studies have shown that activation of telomerase might participate in the progression of tumors, the molecular mechanism remains unclear.

Telomerase is a reverse transcriptase that carries its own template and synthesizes DNA telomere repeats to maintain telomere length. These repeats are composed of 1000–2000 non-coding tandem repeats of the TTAGGG sequence and serve as protective 'caps' at the ends of chromosomes, protecting them from degradation and thereby maintaining chromosome stability, enhancing cell proliferation and promoting cell immortality (Liu *et al.*, 2004); (Blackburn *et al.*, 2006). In most cell types, after each round of DNA replication, telomeres are shortened. However, telomere length is stabilized by the telomerase enzyme in stem cells, germ cells and cells from tissues with a high proliferative capacity. Telomerase activation is a very common occurrence in tumor cells (Kim *et al.*, 1994); (Nugent e Lundblad, 1998). In humans, the active telomerase is a ribonucleoprotein complex with two main components: i) human telomerase RNA (hTR), which is expressed in most cells and contains the template sequence for reverse transcription; and ii) human telomerase reverse transcriptase (hTERT), a reverse transcriptase that catalytically synthesizes telomere DNA, whose expression seems to be restricted to telomerase-positive tissues, which indicates that hTERT is the limiting factor for telomerase activity (Feng *et al.*, 1995); (Chang *et al.*, 2002).

For many years it was widely believed that telomerase activation was the only telomere lengthening mechanism involved in tumorigenesis, until the unveiling of the alternative lengthening of telomere (ALT) mechanism (Feng *et al.*, 1995); (Nakamura *et al.*, 1997), which is independent of telomerase. The ALT mechanism is characterized by instability at a specific minisatellite locus and high rates of telomeric recombination exchange (Smogorzewska e De Lange, 2004). Nowadays is known that, in approximately 10% of tumors, telomere maintenance relies on the ALT mechanism. Recent studies have showed that hTERT alone is sufficient to restore telomerase activity and this restoration results in tumorigenesis in telomerase negative cells, such as epithelial cells and human fibroblasts (Qi *et al.*, 2011); (Hahn *et al.*, 1999). Most tumors express high levels of hTERT (80–90%) (Harley, 2008), suggesting that the reverse transcriptase activity of hTERT plays an important role in tumor occurrence and development.

Although telomerase and ALT are involved in the maintenance of telomeres, malignant phenotypes are significantly different in these two types of cells. *Chang* and colleagues (Yu *et al.*, 2010) confirmed that, although telomere dysfunction provokes chromosomal aberrations that initiate carcinogenesis, telomerase-mediated telomere maintenance enables such initiated cells to efficiently achieve a fully malignant endpoint, including metastasis.

Most research on hTERT has been focused on its crucial function on telomere maintenance. However, there are many phenomena that cannot be explained by its reverse transcriptase activity. Recent research has discovered that hTERT has other functions unrelated to its reverse transcriptase activity, such as increasing the anti-apoptotic capacity of cells, enhancing DNA repair, maintaining stem cells and regulating gene expression (Cong e Shay, 2008). Non-canonical roles of hTERT have also been revealed. These non-canonical roles of hTERT are referred to as its non-reverse transcriptase activity (Hanahan e Weinberg, 2011).

If superior/higher proliferation ability is the main feature of early primary tumors, then metastasis is the main feature of end-stage cancer. Metastasis directly threatens the lifes of cancer patients and is the cause of 90% of cancer deaths (Gupta e Massagué, 2006). The multi-step process of tumor invasion and metastasis, referred to as the invasion-metastasis cascade, includes: i) loss of cellular adhesion, ii) increased motility, iii) entry into and survival in the circulation, iv) exit into new tissue and v) eventual colonization at a distant site (Talmadge e Fidler, 2010); (Fidler, 2003). Tumor invasion and metastasis are associated with a variety of factors and processes, including: i) the epithelial-mesenchymal transition (EMT), ii) heterotypic contributions of stromal cells and iii) plasticity in the invasive growth program. EMT plays a critical role in cancer metastatic progression and it has been postulated to be an absolute requirement for tumor invasion and metastasis (Kalluri e Weinberg, 2009). EMT refers to the physiological and pathological situations occurring during cell epithelial-mesenchymal transition, accompanied by cell morphology and gene expression changes. It is characterized by the loss of epithelial proteins, including E-cadherin, γ-catenin and β-catenin, and is often accompanied by the increase of mesenchymal proteins such as vimentin, fibronectin and smooth muscle actin (Hanahan e Weinberg, 2011); (Huber *et al.*, 2005).

E-cadherin expression is a marker of epithelial cells and it is an initiating factor for EMT since the down-regulation, inhibition, or loss of function of E-cadherin can activate this process. E-cadherin also helps maintain cancer cell adhesion to prevent tumor invasion

and metastasis. A variety of factors have been shown to regulate E-cadherin expression, including somatic mutations, promoter hypermethylation, the Snail protein and the ZEB family (Berx e Van Roy, 2009).

Several evidences show that hTERT can promote the metastasis of cancer cells and this capability may be independent of its reverse transcriptase activity. Upon h*TERT* transfection into U2OS osteosarcoma cells, which is a telomerase-negative cell line, the invasion and metastasis capacity of tumor cells were increased (Yu *et al.*, 2009). In human esophageal squamous cell cancer, h*TERT* activation increased migration and invasion when compared with control cells. It has been shown that hTERT regulates the glycolytic pathway in melanoma cells, improving the energy supply state of the tumor cells thus contributing to tumor invasion and metastasis (Okawa *et al.*, 2007); (Bagheri *et al.*, 2006). Recent studies have indicated that exogenous expression of h*TERT* also leads to upregulation of *MMP9* and *RhoC* and promotes the invasiveness and metastasis of HepG2 cells *in vitro* (Chen *et al.*, 2013).

hTERT promotes not only tumor formation, but also tumor metastasis. Therefore, it is possible that hTERT promotes tumor metastasis through the EMT pathway. Transfection of h*TERT* into *Xenopus* caused faster embryonic limb and neuron development compared to controls, and promoting embryonic development is one of the three main functions of EMT, which also plays a central role in embryogenesis (Park *et al.*, 2009); (Thiery e Sleeman, 2006; Kalluri e Weinberg, 2009). It has also been demonstrated that hTERT can affect TGF-β1-mediated β-catenin induction and nuclear accumulation, which enhances Wnt signaling pathway activation and promotes EMT (Liu *et al.*, 2013). hTERT can form a complex with the brahma-related gene 1 (BRG1) and nucleostemin (NS) through upregulation of Twist to increase EMT and this complex does not directly contribute to telomere maintenance (Okamoto *et al.*, 2011). In summary, hTERT plays a role in tumor invasion and metastasis by promoting EMT and this function is independent of its reverse transcriptase activity.

On the other hand, an unexpected role for TERT as a regulatory molecule modulating transcription complements the more widely appreciated function of telomerase in maintaining telomere repeats at chromosome ends (Chu *et al* 2009). Through chromatin immunoprecipitation of the endogenous TERT protein from mouse gastrointestinal tract, *Chu* and colleagues showed that TERT physically occupies gene promoters of Wnt-dependent genes. These data revealed an unanticipated role for telomerase as a transcriptional modulator of the tumorogenesis signaling pathway.

All these studies show that hTERT is involved in the entire process of tumorigenesis, and the introduction of telomerase into an ALT cell line promotes or enhances tumor invasion *in vitro*. However, more studies are necessary for clarify the cellular mechanism of this outcome.

In this Doctoral Thesis, in order to explore the non-canonical role of hTERT in the process of invasion and metastasis of tumors at the cellular level, we transfected a vector containing the full-length cDNA of h*TERT* into an ALT osteosarcoma cell line (SAOS 2) and compared the effect of hTERT on aggressiveness through a xenograph assay. We found that h*TERT* transfection increases the invasive ability of SAOS 2 cells. We also demonstrate that hTERT acts as a regulatory molecule, modulating the transcription of several micro RNAs, in particular miR-500a.

2. Materials and methods

2.1. Animals

Zebrafish (*Danio rerio* H., Cypriniformes, Cyprinidae) were obtained from the Zebrafish International Resource Center and mated, staged, raised and processed as described (Westerfield, 2000).

The experiments performed comply with the Guidelines of the European Union Council (86/609/EU). Experiments and procedures were performed as approved by the Bioethical Committee of the University Hospital "Virgen de la Arrixaca" (Spain) and by the Children's Hospital Boston institutional Animal Care and Use Committee (USA).

2.2. Human breast samples

Female patients in the region of Murcia, with breast cancer, who underwent surgery, have an anatomical report of the HUVA (Hospital Universitario Virgen de la Arrixaca), examination of tumor biopsy and axillary node status and have Signed informed consent, between the years 2011-2014.

2.3. Culture cells

All cell lines were cultured in growth medium containing DMEM supplemented with 10% FBS, 2 mM glutamax (Life Technologies), 100 units/mL penicillin, and 100 µg/mL streptomycin at 37 °C with 5% CO_2. Cells were grown in 10-cm dishes and passaged every 3–4 days. At every passage, cells were counted, and 0.3 million cells were seeded/plate in fresh growth medium.

HEK-293 cell line was purchased from the ATCC (#CRL-1573.3). pBABE-SAOS 2 and hTERT-SAOS 2 cell lines were obtained by transfection of the SAOS 2 cell line (ATCC #HTB-85) with pBABE-puro and pBABE-puro-h*TERT* plasmids, respectively, and followed by selection with puromycin.

The plasmid pBABE-puro was a gift from Hartmut Land & Jay Morgenstern & Bob Weinberg (Addgene plasmid #1764) and the okasmids pBABE-puro-h*TERT* and pBABE-puro-DN-h*TERT* were a gift from Bob Weinberg (Addgene plasmids #1771 and #1775, respectively).

2.4. Transfection assay for PNA-antimiR 500

One day before transfection, seed 5.0 x 10^4 cells per well in 0.5 ml of the appropriate complete growth medium without antibiotics. Incubate cells at 37 ºC with 5% CO_2 overnight. Prepare the stock solution of PNAsTM miRNA inhibitors of 100 µM in RNase-free water. Store at - 20 ºC until used. Before use, incubate PNA for 10 min at 70 ºC in a water bath or heating block. Cells were transfected with LipofectamineTM 2000 following manufacturer's instructions (Invitrogen, Cat# 11668-027). Briefly, dilute 200 ng target DNA, 20 ng control DNA and PNAsTM miRNA inhibitor in 50 µl Opti-MEM® I Medium. PNAsTM miRNA inhibitor is added into culture medium at a final concentration of 500 ~ 2,000 nM. Mix gently [suspension 1]. Dilute 1 µl of LipofectamineTM 2000 in 50 µl Opti-MEM® I Medium and mix gently [suspension 2]. Incubate each tube for 15 minutes at room temperature. Mix [suspension 1] and [suspension 2] gently by pipetting and incubate for 15 minutes at room temperature [suspension 3]. Add [suspension 3] onto each well containing cells and medium and mix gently by rocking the plate back and forth. 7. Incubate the cells at 37oC in 5% CO2 incubator for 48 hours.

2.5. Telomerase activity assay (TRAP)

The TRAPezeH XL Telomerase Detection Kit (Millipore, Cat.#S7707) was used to qualitatively measure the telomerase activity of human cells extracts. The protein extracts were obtained according to the manufacturer's instructions. Human carcinoma cells (included in the telomerase detection kit) were used as a positive control. As a specific negative control, the higher protein concentration assayed of every sample extract was incubated with 1 mg of RNAse A (QIAGEN) at 37 ºC for 20 min.

2.6. Analysis of gene expression

Total RNA was extracted from 10^6 cells with Qiazol reagent (Qiagen, ID: 79306) Joint (ankle) tissue was used to extract RNA by the thioisocyanate method. cDNA generated by the miScript II RT Kit (Qiagen) following the manufacturer's instructions and treated with DNase I, amplification grade (1 U/µg RNA; Invitrogen) was used to synthesize first-strand cDNA (1 µg of total RNA at 50°C for 50 min). Real-time qPCR was performed with a MyiQ™ instrument (BIO-RAD) using miScriptSYBR® Premix Ex Taq™ (Perfect Real Time) (Takara). Reaction mixtures were incubated for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C, and finally 15 s at 95°C, 1

min at 60°C, and 15 s at 95°C. For each mRNA sample, gene expression was normalized to U6 snRNA content in each sample using the comparative *Ct* method ($2^{-\Delta\Delta}$*Ct*). The primers used are shown in **Table I.** In all cases, each PCR was performed with triplicate samples and repeated at least with two independent samples.

Table I: Gene accession numbers and primer sequences used for gene expression analysis.

Gene	Gene accession number	Primer name	Sequence (5'-3')
GADPH	NM_002046	QT0119 2646	QuantiTect Primer Assay (QIAGEN)
TERT	NM_003219	F1 R1	TGACACCTCACCTCACCCAC CACTGTCTTCCGCAAGTTCAC
TERT-R-pre-mir 5	MI0000067		UCAGAGUGAGGUAGUAGAUUGUAUA GUUGUGGGGUAGUGAUUUUACCCUG UUCAGGAGAUAACUAUACAAUCUAU UGCCUUCCCUGA
TERT-R-miR 5	MIMAT0004486		CUAUACAAUCUAUUGCCUUCCC
TERT-R-pre-mir 20	MI0006443		GUGAGCAGUCUCCACCACCUCCCCU GCAAACGUCCAGUGGUGCAGAGGUA AUGGACGUUGGCUCUGGUGGUGAUG GACAGUCCGA
TERT-R-miR 20	MIMAT0005950		ACGUUGGCUCUGGUGGUG
TERT-R-pre-mir 25	MI0006428		AGGGGGCACCGGGAGGAGGUGAGUG UCUCUUGUCGCCUCCUCCUCUCCCCC CUU
TERT-R-miR 25	MIMAT0005939		UCGCCUCCUCCUCUCCC
TERT-R-pre-mir 26	MI0006328		GUGAGUGGGAGCCCCAGUGUGUGGU UGGGGCCAUGGCGGGUGGGCAGCCC AGCCUCUGAGCCUUCCUCGUCUGUC UGCCCCAG
TERT-R-miR 26	MIMAT0005593		CUUCCUCGUCUGUCUGCCCC
pre-mir-500a	MI0003184		1GCUCCCCCUCUCUAAUCCUUGCUAC CUGGGUGAGAGUGCU 2GUCUGAAUGCAAUGCACCUGGGCA AGGAUUCUGAGAGCGAGAGC
miR-500a	MIMAT0004773		UAAUCCUUGCUACCUGGGUGAGA
pre-mir-500b	MI0003184		1CCCCCUCUCUAAUCCUUGCUACCUG GGUGAGAGUGCUUUC 2UGAAUGCAGUGCACCCAGGCAAGG AUUCUGCAAGGGGGA
miR-500b	MIMAT0016925		AAUCCUUGCUACCUGGGU

miR-502	MIMAT0002873		AUCCUUGCUAUCUGGGUGCUA
miR-362	MIMAT0000705		AAUCCUUGGAACCUAGGUGUGAGU
miR-532	MIMAT0002888		CAUGCCUUGAGUGUAGGACCGU
U6		hU6f	GAGGGCCTATTTCCCATGATT
		hU6r	TAATTAGAATTAATTTGACT

2.7. Analysis of *miR-500* gene promoter activity

A 2 Kb genomic DNA sequence upstream of *miR-500a* +1 position was amplified using the following primers: forward 5´ CAGTGTTGTGGTTTTGGTCCAGGCG 3´ and reverse 5´ CCGGACACCGAGCACCGGCGAGCCGCC 3´. The DNA fragment was cloned in the *SmaI* site of the pGL3basic vector (Promega, E1761) driving the expression of firefly luciferase gene (*miR-500a promoterPGLe Luc+)*

For the luciferase assay (Promega, Cat# E1910), remove growth medium from the cultured cells, and gently apply a PBS to rinse the bottom of the culture vessel. Prepare 1 X Passive Lysis Buffer (PLB) just before use. Apply 100 µl of 1 X PLB into each well and lyse the cell. Transfer the lysate to a new tube or vial. For firefly luciferase assay, carefully transfer 10 µl of cell lysate and 10 µl of Lucifearase Assay Reagent II (LAR II) into luminometer tube. Mix by pipetting two times. Place the tube in the luminometer and initiate reading. For Renilla luciferase assay, remove the sample tube from the luminometer, add 10 µl of Stop & Glo® Reagent and mix briefly. Replace the sample in the luminometer and initiate reading. Normalize the firefly luciferase activity to the Renilla luciferease activity.

2.8. Chromatin immunoprecipitation (ChIP) assay

Both pBabe-SAOS 2 and hTERT-SAOS 2 ($1*10^7$ cells) were cross-linked with 1% paraformaldehyde (Sigma Aldrich) in culture medium for 10 min at room temperature. After, aldehydes were quenched with PBS containing 200 mM glycine (Sigma Aldrich) for 5 min followed by a PBS wash. The cells were centrifugate at 200 × g for 10 minutes at 4°C to pellet. Then, they were resuspended in Lysis Buffer containing Protease Inhibitors (Sigma-Aldrich) and the lysate was sonicated using a sonication system Bioruptor® Plus (Diagenode) for 30 cycles of 30 seconds ON, 30 seconds OFF (conditions obtained in a previous set-up). The sonicated lysate was centrifuged at 20,000 x *g* for 10 min at 4°C, and the supernatant was transferred to new tubes. For confirming

DNA fragment sizes we used a 1,7% agarose gel. ChIP dilution buffer (100 uL) was added to the supernatant, and 10 uL of the supernatant was set aside for input. Binding the chromatin to the Antibody-Dynabeads® complexes, reverse the formaldehyde crosslinking of the chromatin and purifying the DNA were performed as described in the protocol MAGnify™ Chromatin Immunoprecipitation System" (Invitrogen).

The results of the ChIP were analyzed by quantitative PCR, since the chromatin present in the sample was amplified, which will be the zones that have been bound to the antibody. The following primers of the positive (c-Myc) and negative (c-Myc-3'UTR) controls were designed for the Sigma-Aldrich commercial house, the sequences of the primers that used are show in Table II.

Table II: Sequence of the different primers used for the analytical qPCR of ChIP.

c-Myc Positive control TBE2-F 5'-CGTTT TCCTC CTTAT GCCTC TATC-3' TBE2-R: 5'-GTACC AGGCT GCAGG GCGCC TCGCT-3' c-Myc-3'UTR Negative control F: 5'-CTAAT GTATC ACAAA GTCCT TTA-3' R: 5'-GTGAT CTGCT CTGTT AGCTT TTGA-3' hTRF3-R: Negative control R: 5'-TTGCTTCCTGTACGGCTTG-3' F: 5'-ACAGCCAAACCTGTGAGACC-3'
TCF/LEF: TCF_F: ATCCAGCTTTTCCCTGGGC TCF_R: CTCAGCCACCAACCACTTTG
hsa-mir-500a promoter in kidney cells: TSSkydney_F: TTGACCAATTCATCTAGAGCCAA TSSkydney_R: CAGCAATGAAAACCCTGTACACC
hsa-mir-500a promoter in lungs cells : TSSlung_F2: GGGGGACAACTGTTCTCTGA TSSlung_R2: AACTGTGGGGCTATGTGGAT TSSlung_F1: ACTGGATGTTTTTCCCCCAGC TSSlung _R1: CATGCAGTCTCTGCACCAAAT
hsa-mir-500a promoter in gastric cáncer cells: TSSintestine_F: GGCCTAACCTGTTGTCCTCT

TSSintestine_R: TTTACCACAGCAGCAACCAC
hsa-mir-500a promoter in célls with leukemia: TSSleukemia_F: CAGGGGTGATTCCGGTTCAG TSSleukemia_R: GCAGCAGTTTCTTGCGTTTG
hsa-mir-500a Upstream: Up-mir-500a_F1: TGCACACATGCTGGGGATAC Up-mir-500a_R1: GAGACAGCTCACTGCCCTTT Up-mir-500a_F2: CAGGAGTTTCACCATCCCCC Up-mir-500a_R2: ACCTTGGTTCAAATGCACGC Up-mir-500a_F3: TACCTGGGTGAGAGTGCTGT Up-mir-500a_R3: AAGACGCTCTGTCCTACACG

To perform the quantitative PCR, the commercial kit "*Power* SYBR® Green PCR Master Mix" (Applied Biosystems), 2x "QuantiTect SYBR Green PCR Master Mix" 10 µl, 10x "miScript Universal Primer" 2 µl, 10x primer *hsa-mir-500a 2 µl,* H_2O RNAsa free 4 µl and 1:10 cDNA diluied. The reaction was perform in qPCR ABI PRISM 7500 instrument (Applied Biosystems). Reaction mixtures were incubated for 15 min at 95°C, followed by 40 cycles of 15 s at 94°C, 30 s at 55°C, and finally 30 s at 70°C, 1 min at 95°C, 1 min at 60°C and 10 s 60ºC 72 cycle.

2.9. Targets identification

The miRBase algorithm (Griffiths-Jones *et al*., 2006), available at http://microrna.sanger.ac.uk (accessed March 10, 2015) was used to search for possible miR-500 targets. This algorithm tries to predict the 3'-UTR target regions of the different miRNAs. To do this, through statistical models assigns *p* values to individual target sites of the miRNAs, to multiple sites in a single 3'-UTR target region and to sites in the 3'-UTR region which are conserved in multiple species. These *p* values allow the user to evaluate the confidence in the prediction.

Target genet were analyzed by using MetaCore software, available at https://portal.genego.com/ (accessed June 5, 2015).

2.10. Zebrafish model of micrometastasis / Xenograft assay

Cells were seeded in 6-well plates, grown to confluency trypsinized (without EDTA for EpRas cells or with EDTA for all other cells used). Subsequently, cells were washed with 67% DPBS (GIBCO, Invitrogen), transferred to 1.5 ml Eppendorf tubes and

centrifuged 5 min, at 1500 rpm. Cells were re-suspended in DPBS containing either CMDil (4 ng/ul final concentration). Cells stained with CM-Dil (red fluorescence, Invitrogen) were incubated 4 min at 37°C and then 15 min at 4°C. After this period cells were centrifuged 5 min at 1500 rpm, the supernatant discarded and cells re-suspended in 100% FCS, centrifuged again and washed 2 times with 67% DPBS. Cells were suspended in 67% DPBS for injection into 2 dpf zebrafish embryos previously dechorionated and anesthesized with tricaine (Sigma). Using a manual injector (Eppendorf; Injectman NI2), the cell suspension was loaded into an injection needle (15 µm internal- and 18 µm external-diameter). Cells were now injected in 2 dpf Wild type zebrafish embryos that previously are treated with PTU. After injection, embryos were incubated for 1 h at 31°C and checked for cell presence at 2 hpt. Fish with fluorescent cells outside the implantation area at 2 hpt were excluded from further analysis. All other fish were incubated at 35°C for the following days.

2.11. Statistical analysis

Data were analyzed by analysis of variance (ANOVA). The differences between two samples were analyzed by the Student *t*-Test. The percentage of cell invasion in zebrafish larvae was analyze by chi square (Fisher's exact test).

3. Results

3.1. Overexpression of h*TERT* correlates with tumor cell line invasion.

Transfected a telomerase-negative osteosarcoma cell line U2OS (hTERT/U2OS) (Song-Tao Yu et al Int J Oncol. 2009) increased the cellular adhesion capacity to the extracellular matrix. Transwell matrigel assay confirmed an increased invasion ability in hTERT/U2OS cells. These results strongly suggest that hTERT transfection promotes the invasion of telomerase-negative cells. Telomerase-mediated telomere maintenance enables these cells to achieve a fully malignant endpoint, including invasion and metastasis.

The plasmid pBABE-h*TERT* was transfected into SAOS 2 cell line. Simultaneously, the plasmid pBABE-puro was transfected as a blank control. Several stable cell clones with high expression of EGFP formed after 2 weeks of selection by puromycin the new cell lines obtained were named h*TERT* SAOS 2 and pBABE SAOS 2.

To characterize these cell lines, we first used a TRAP assay to determine the telomerase activity. Telomerase activity was detected in h*TERT* SAOS 2 cells and was similar to the positive control. As expected, pBABE SAOS 2 cells did not show telomerase activity (Fig. 1?). This result verify that the transfection of h*TERT* into an ALT cell line results in the activation of telomerase expression. Quantitative PCR for telomere length.

Next, we used a RT-qPCR assay to study the expression of h*TERT* and we found that h*TERT* SAOS 2 cells have a statistically significant incrase of h*TERT* expression, while pBABE SAOS 2 cells have no positive signal (**Fig. 2**).

Finally, we also analyzed the consequences of the expression of h*TERT* on SAOS 2 invasion capacity *in vivo* by using the zebrafish model of micrometastasis[34,35]. 72 hours after the xenograft, we found that sixty-one per cent (61%) of zebrafish embryos injected with pBABE SAOS 2 cells have micrometastasis. This number was increased to approximately 87% of the embryos presenting micrometastases when injected with h*TERT* SAOS 2 cells, resulting in an increase in the zebrafish colonization index by X±0.08 fold (*n*=3) (**Fig. 3**).

Therefore, h*TERT* SAOS 2 and pBABE SAOS 2 cell lines were selected as the model for the subsequent functional studies.

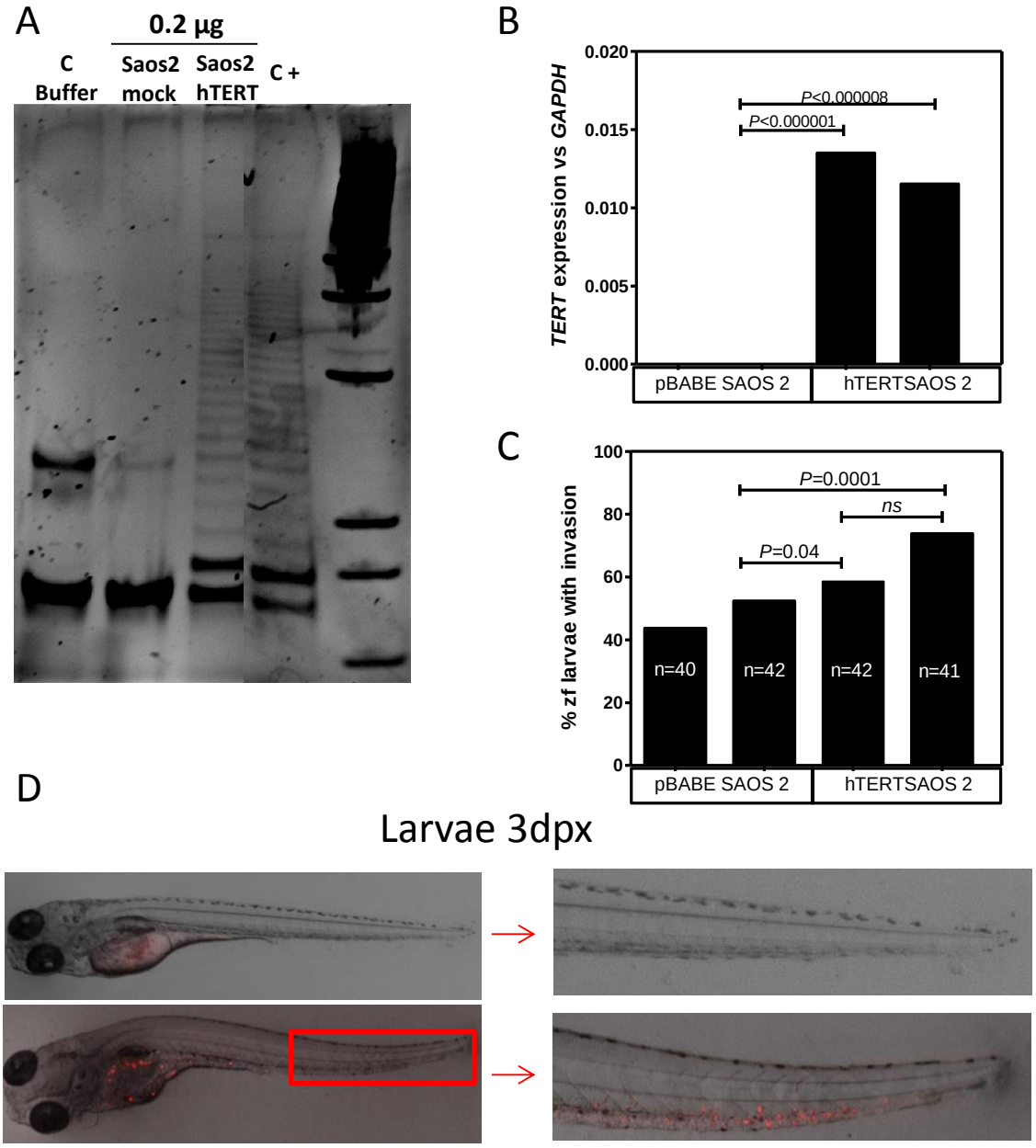


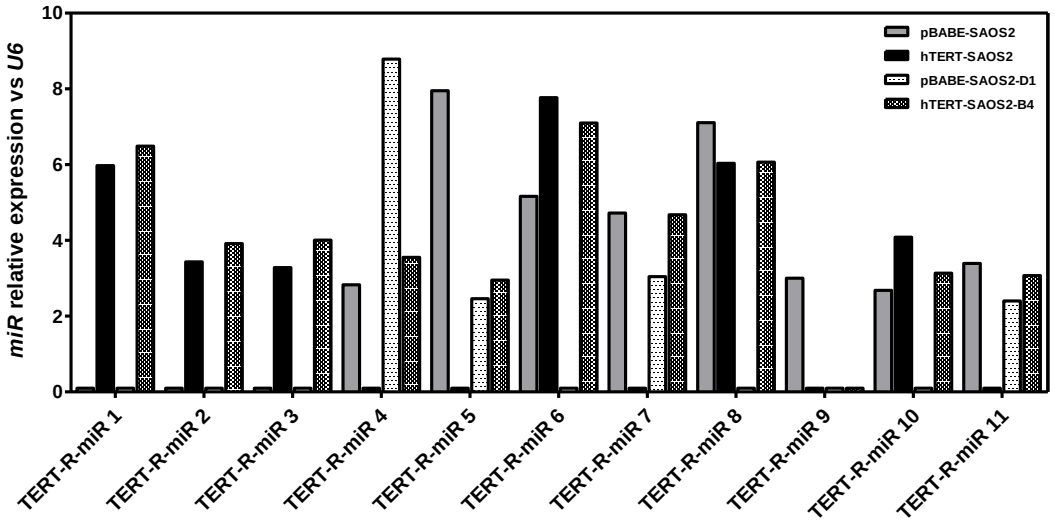
Figure 2: hTERT overexpression in SAOS 2 increases their invasion capacity. (A) Telomerase activity was measured quantitatively in hTERT –SAOS 2 and PBABE –SAOS 2 cells by TRAP using 0.1 mg of protein extract. Results are expressed as the mean value ± s.e.m. from triplicate samples relative to telomerase-positive cells. Statistical significance was assessed using Student's *t*-test ($P<0.05$). To confirm the specificity of the assay, a negative control is included for each sample, treated with 1 µg of RNase at 37°C for 20 minutes. (B) The mRNA levels of *hTERT* gene were determined by real-time RT-PCR in cell lines of the indicated genotypes. Gene expression is normalized against *GAPDH*. Each bar represents the mean ± s.e.m. from triplicate samples. (C) Xenograft assay with the different clones in Yolk sac implantation of CM-DiI labeled tumor cells into zebrafish embryos two days post-fertilization(dpf) and the percent of the larvae positive with invasion 3 days post injection from the yolk sac to the tail of the larvae. (D) Zebrafih xenotransplantation assay in red square we can see a zebrafish with positive invasion at 6pdf. hTERT-dependent *in vivo* invasion of SAOS2 cells from the yolk sac of zebrafish embryos.,. Histograms showing the percentage of positive zebrafish embryos for SAOS2 cells

expressing hTERT(shRD) or not . The total of embryos is indicated inside the figure for each condition (values are mean ± SEM of three to five independent experiments; *, $P < 0.05$; ***, $P < 0.001$). D) representative images of zebrafish embryos negative or positive for SAOS2 cell invasion.

3.2. Effect of h*TERT* gene overexpression on the miRNA expression profile

MicroRNAs play key roles in transcriptional regulation and tumor metastasis. Several studies have shown that microRNAs can regulate a number of tumor-related transcription factors that are involved in tumor invasion and proliferation (Cong e Shay, 2008). Therefore, we hypothesized whether invasiveness of cancer cells is regulated by h*TERT* through the control of miRNA(s), which would trigger tumor metastasis. Based on this hypothesis, miRNA microarray tests (with a capacity to measure thr expression of 939 miRNAs) were used to scan the h*TERT*-regulated miRNA. A total of 47 miRNAs were found as potential targets regulated by h*TERT* (Fig. 2a and). These miRNAs were called "hTERT-regulating miRNAmir" (hTERT-R-miRNA) from 1 to 47.

Next, we used RT-qPCR to validate the microarray results, and we found that mir500 expression was significantly increased in h*TERT* SAOS 2 cell line.



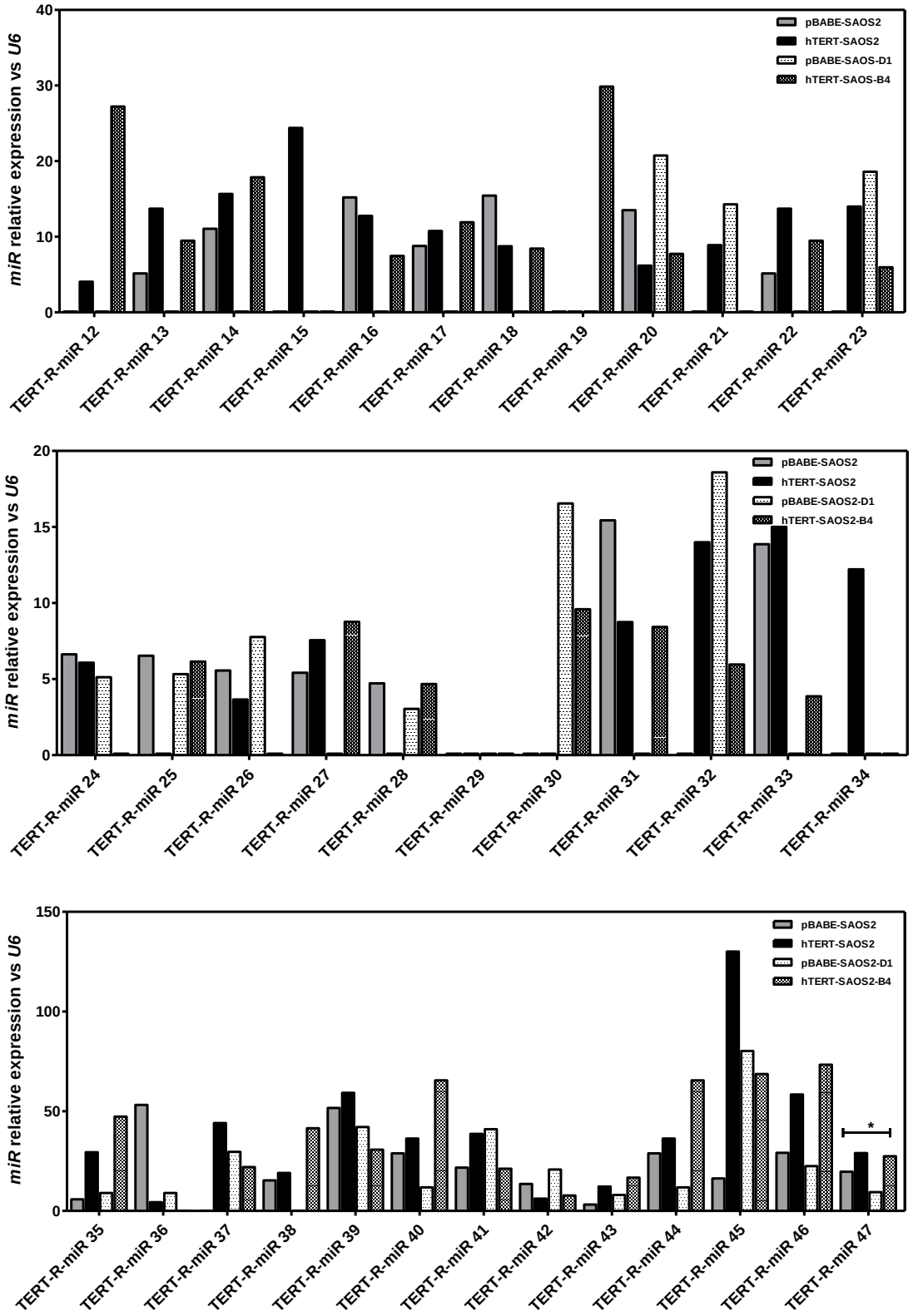


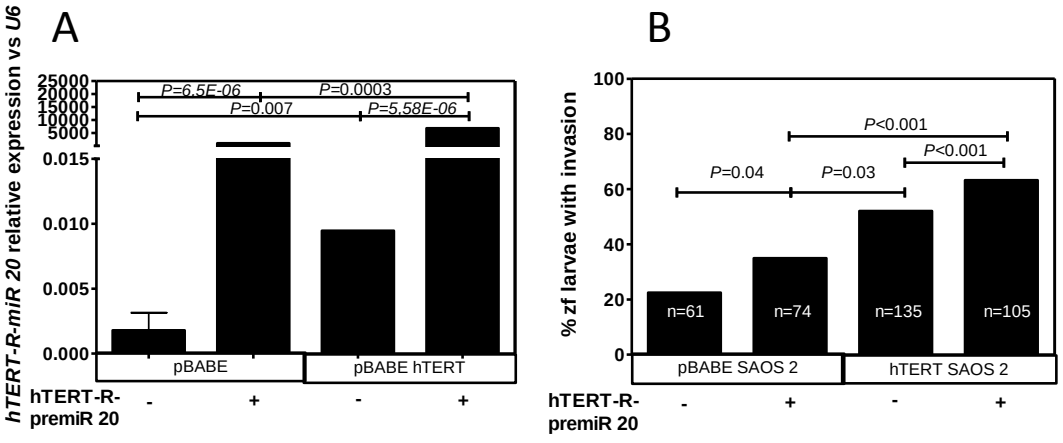
Figure 3: Effect of h*TERT* overexpression on miRNA expression profile. We used 2 different clone cell line from hTERT-SAOS and pBABE-SAOS. A total of 18 miRNAs were found that may be regulated by hTERT. (table V).

Table V. **The list of 18 possible miRNAs may be regulated by hTERT in the pBABE-SAOS 2 and hTERT-SAOS 2 miRNA verified by RT-qPCR**.

Up-regulated	Down-regulated
TERT-R-miR 1	TERT-R-mir 5
TERT-R-miR 2	TERT-R-mir 25
TERT-R-miR 3	TERT-R-mir 26
TERT-R-miR 12	TERT-R-mir 43
TERT-R-miR 13	TERT-R-mir 46
TERT-R-miR 32	
TERT-R-miR 35	
TERT-R-miR 38	
TERT-R-miR 44	
TERT-R-miR 46	
TERT-R-miR 47	

3.3. Validation of h*TERT*-down-regulated miRNAs

Four miRNAs which were down-regulated in h*TERT*-overexpression conditions, where validated by RT-qPCR. Then, we performed a xenograft assay to study the invasion capacity of the cells in hTERT-SAOS2 and pBABE –SAOS2. Surprisingly, the overexpression of miRNAs that are down-regulated in hTERT-SAOS2 cells produces a decrease in the cell invasion capacity (figure 3A,B and C). We were not able to validate the microarray data with TERT-R-mir 20. Reciprocally, the inhibition of TERT-R-mir 5 increases the cell invasion capacity *in vivo* (Figure 4D).



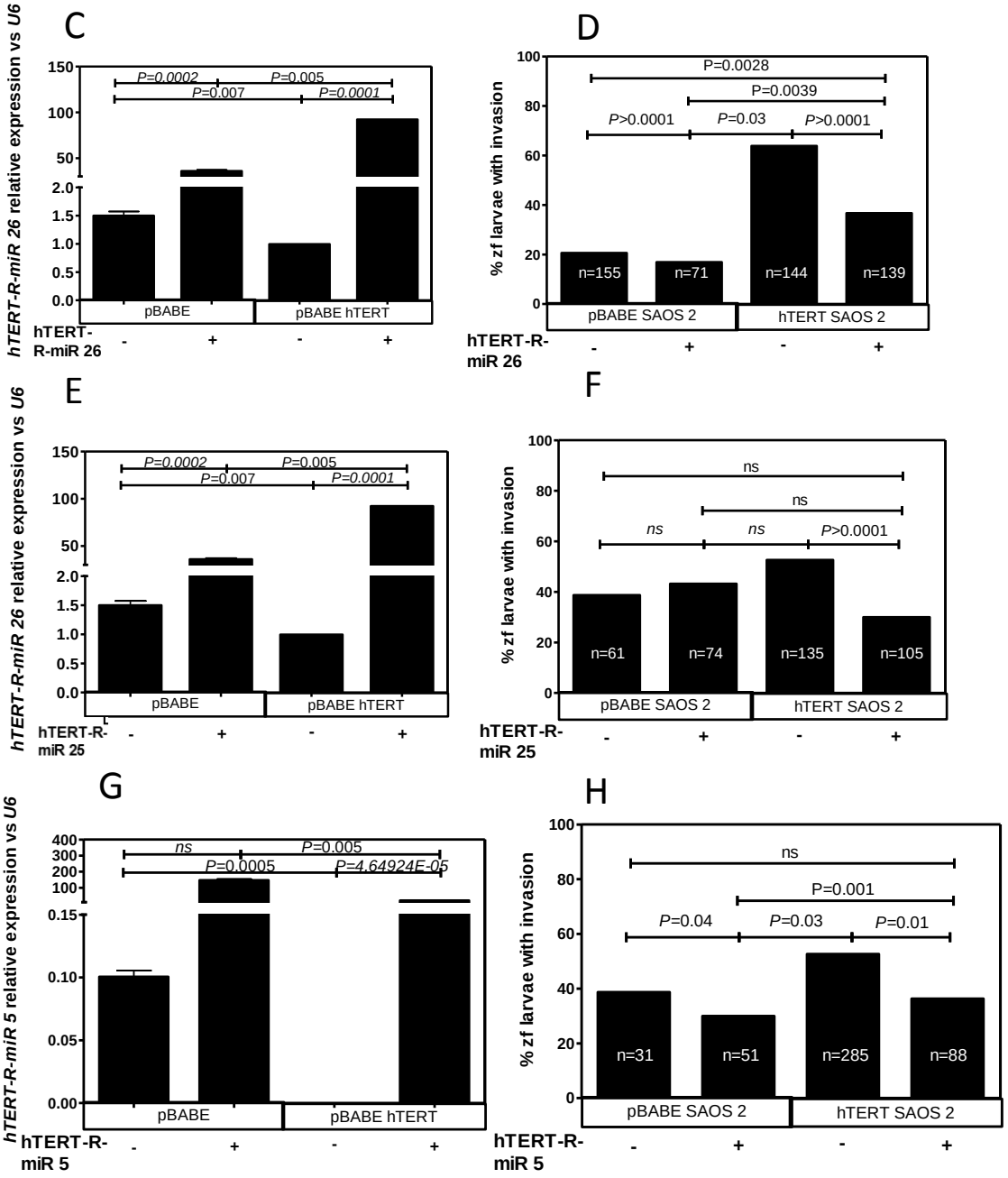


Figure 4: microRNA validation in invasion behavior in vivo The overexpression of telomerase produce a decrease expresion of , TERT-R-mir 26, TERT-R-mir 25 and TERT-R-mir 5. The over expression of all these mircoRNAS hTERT-SAOS an pBABE-SAOS decrease its invasiveness capacity in a xenograth in zebrafish larvae. (4D, F and H).

3.4. Validation of h*TERT*-up-regulated miRNAs

Analysis of miRNA array data showed that miR-500a levels were significantly up-regulated in the h*TERT* SAOS 2 cell line compared with pBABE SAOS 2. We verified this result by RT-qPCR, finding that miR-500a levels were increased in h*TERT*-overexpression conditions compared to control. Then, we performed a xenograft assay to study the invasion capacity of the cells in hTERT-SAOS2 and pBABE –SAOS2. As expected, the overexpression of miR-500a in hTERT-SAOS2 cells produces an increase in the cell invasion capacity (figure 3A,B and C). Collectively, these results indicate that overexpression of telomerase produces an increase of miR-500a expression, which leads to an increase in the invasion capacity of this cell line.

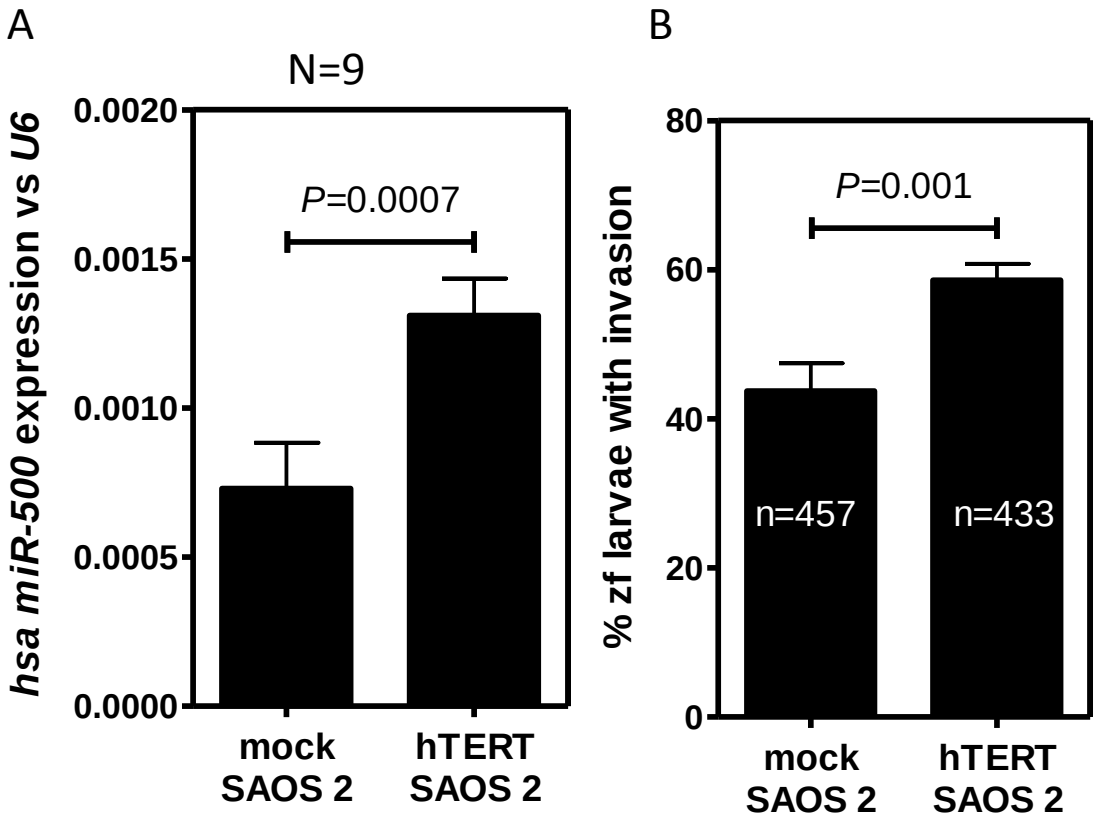


Figure 5. miR 500 a is regulated by hTERT in SASO 2 cells. We confirm by RT-qPCR the up-regulation of miR500a by hTERT and the increase in the percentage of invasion in vivo

3.5. Hsa-mir 500 role in invasiveness

Based on the previous results, we decided to focus on Hsa-mir500 characterization. Therefore, we tested whether has-miR 500 inhibition in hTERT-SAOS cells were able to affect the cells invasiveness in a zebrafish xenograft assay interestingly the mir-500 inhibition in pBABAE saos 2 showed no effect on invasiveness.

These results showed that miR 500 could increase the malignant activity of SAOS 2 cells *in vivo*, indicating that inhibition of Mir500a has a specific effect in cell which are over-expression hTERT.

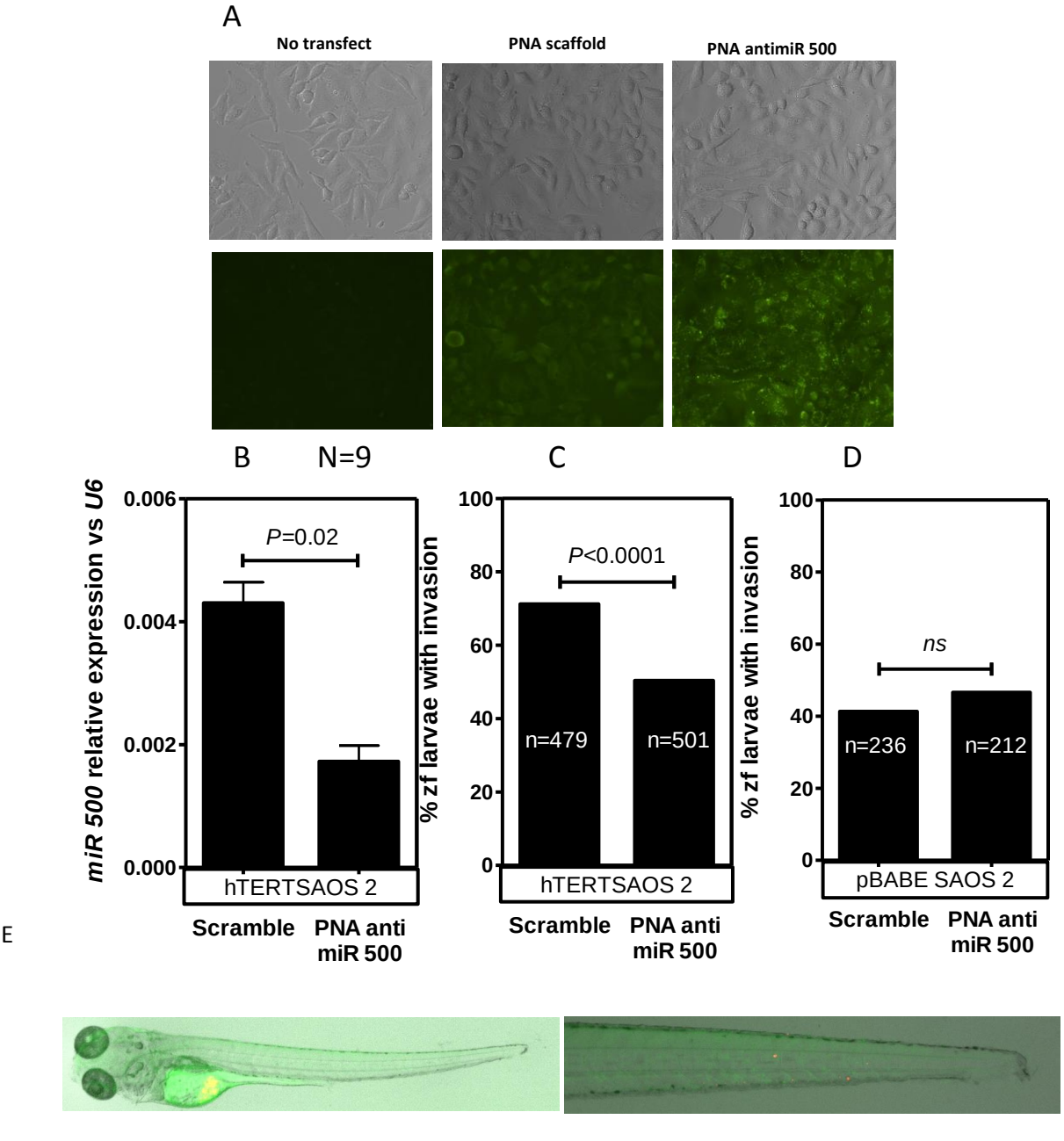


Figure 6: SAOS 2 cell line transfected with PNA-probe anti miR500 decrease the percent of invasion in SAOS2 cell line. We transfected the cell with a PNA-antimiR 500a label with FITC (green fluorescence) showing a correct PNA transfection (2A). We confirm the inhibition of miR 500a by RT-qPCR relative express versus U6 gene expression (2C).Quantification of the number of embryos with evident mass formation 3 days post injection of the hTERT -SAOS cells, incubated at 35ºC (2D) or pBABE-SAOS (2E). Representative image of an 5 dpf embryo with the injected cells in the yolk sac and showing dissemination of the injected cell to the tail, the cells are stained in green for the PNA (antimir500 or control) and in red SAOS cell staining with CM-DIL(2E).

To further study the role of miR 500 in the malignant activity in cancer cells, hTERT- SAOS 2 and pBABE-haTERT were transfected with pre-mir 500. Xenograft experiment showed that overexpress miR 500 can significantly increase the migration of SAOS 2 cells in vivo. We microinject zebrafish embryos with pre-miR 500 transfected pBABE-SAOS 2. The result showed that the percent of zebrafish larvae with invasion increased when the cells contain high level of 500.

We found that over-expression of hsa-miR-500 markedly increase hTERT-SAOS and pBABE –SAOS migration (Figure 7). Inhibition of hsa-miR-500 suppressed the invasiveness only in hTERT-SAOS cell line

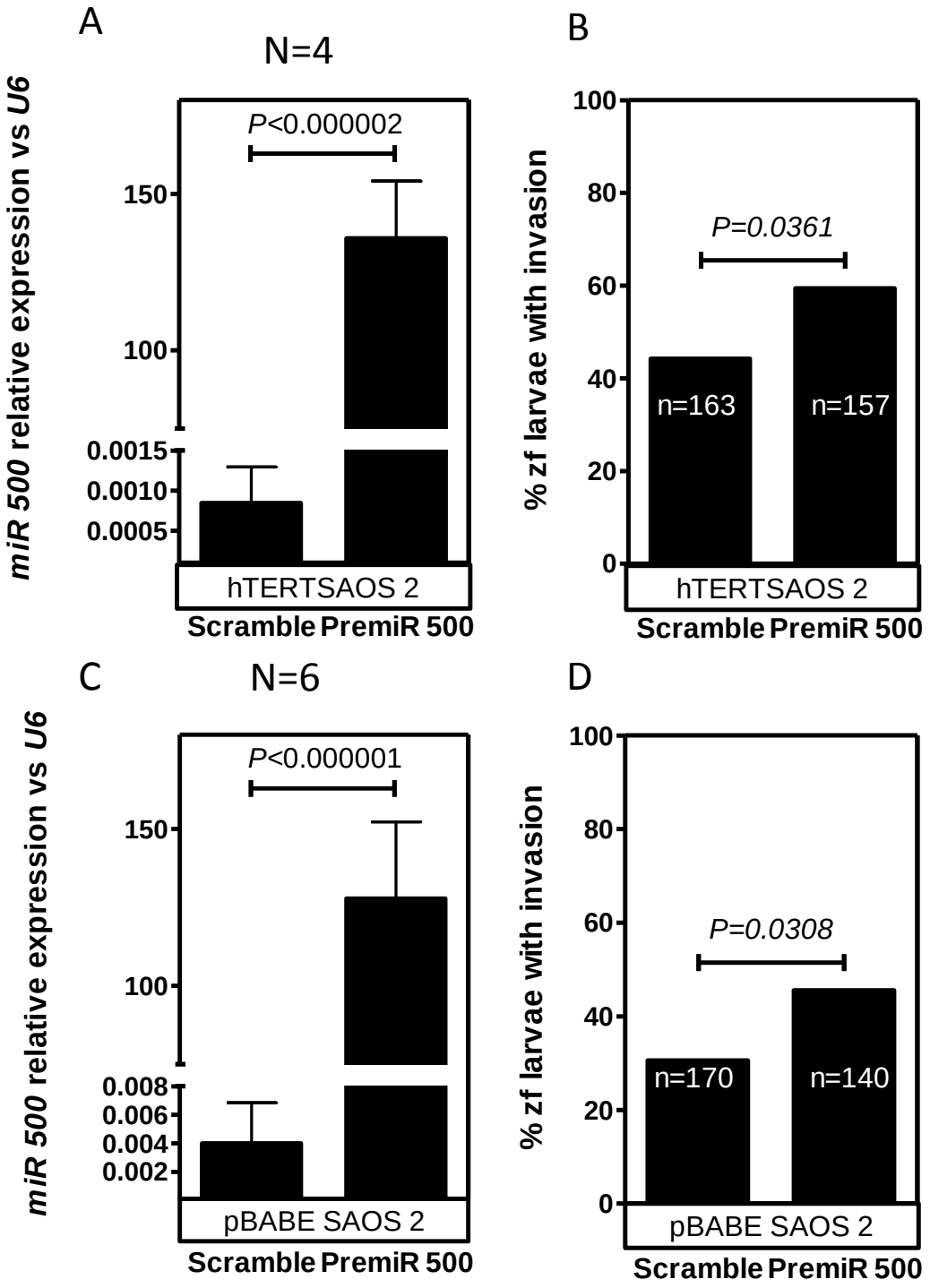


Figure 7: miR 500a increase tumor cells invasion . The expression levels of miR 500a in hTERT -SAOS 2 and pBABE –SAOS transfected with premiR 500 and scrambled by qRT-PCR.(A,C) Comparison of invasion for SAOS 2 hTERT and pBABE-SAOS cells in zebrafish over 3 dpt Statistic was doing with square chi for xenograft assay and p values are indicated on graph.

3.6. Telomerase activity is not involved in upregualtion of miR 500a and its invasiveness.

As described above, the TERT protein has a conserved structural organization that is divided into four functional domains; the N-terminal extension domain, the TR-binding domain, the catalytic RT domain, and the C-terminal extension domain **(**introduction figure). Each of these domains is required for full activity (Kelleher *et al.*, 2002). To investigate if telomerase activity is necessary during invasion and mir500 up regulation, we used a mutant hTERT , a dominant negative mutant based upon previous studies of hTERT (Bachand e Autexier, 2001) and examined whether these mutant proteins could still increase invasion in a invasion zebrafish assay.

Human TERT (hTERT) and DN-hTERT were transfected in SAOS 2 cell lines. Significant recovery of invasiveness was obtained with two cell lines indicating not telomerase activity is necessary for increase invasion and over-expression of mir 500

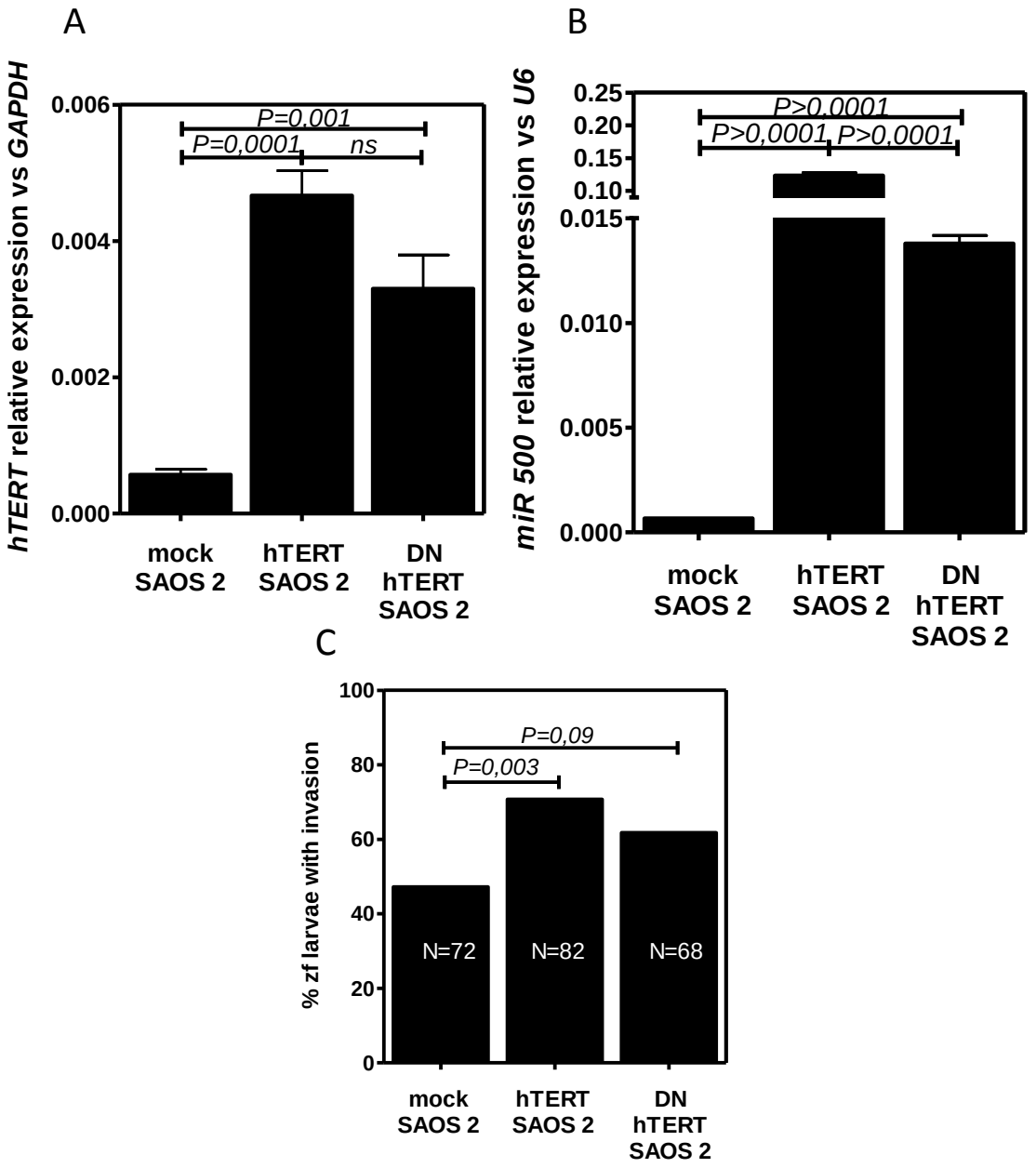


Figure 8: SAOS 2 cell line transfected with hTERT and Dominant negative hTERT. Validation of overexpresión of TERT in SAOS(A) miR 500 relative expression versus endogenous gene U6 (B). Micrometastasis of hTERT-SAOS and DN-hTERT indicate significant differences from the control condition at P> 0.05

3.8. miR 500 is regulated by hTERT by direct bind to promoter region

Regulation of microRNAs is unknown. It has been reported that the promoter region may be found in an intergenic or intragenic zone. When there is an intragenic zone, the microRNA can be inserted into an exon or an intron, which depends on its regulation, where can be regulated by the promoter of the gene where it is inserted or by its own promoter.

It is known that hsa-miR-500 are in a cluster into the short arm of the X chromosome (Xp11.23) and within the gene *CLCN5* in the I3-4 region. So next, we studied wether the expression of h*CLCN5* gene was affected by hTERT. We found that h*CLCN5* expression is not modified in h*TERT* SAOS 2 compared to control (Figure6A), indicating that hsa-miR-500a is not regulated by telomerase through the regulation of h*CLCN5* promoter.

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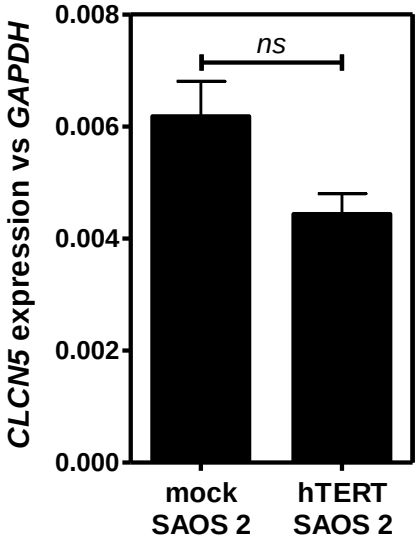

Figure 11: h*CLCN5* gene is not regulated by hTERT. The expression level of h*CLCN5* is similar in pBABE SAOS 2 and h*TERT* SAOS 2.

It has been reported that telomerase regulates Wnt program of self-renewal, proliferation or survival. This regulation is carried out by DNA interaction in TCF/LEF binding sites. Chromatin immunoprecipitation (ChIP) experiments revealed that stably expressed Flag–TERT was also associated with the TBE (TCF binding elements)-containing promoter fragments of the cyclin D1 and *Myc* promoters. This unexpected role for TERT as a regulatory molecule modulating transcription, complements the more widely appreciated function of telomerase in maintaining telomere repeats at chromosome ends. The non-reverse transcriptase activity of hTERT plays a very important role in tumor formation and metastasis

To address the mechanism by which hTERT regulates transcription of hsa-*miR-500a*, we investigated hTERT occupancy of *miR-500* promoters by ChIP experiments. Upon lithium treatment of h*TERT* SAOS 2 and control cells, a hTERT antibody (by Kenkichi Masutomi from Tokio's National Center for Cancer Investigation was used for ChIP (Maida *et al.* 2014) using $10x10^6$ of SAOS 2 cells.

As positive control for ChIP experiments we use the region from promoter fragments containing known TCF binding elements (TBEs) within Myc since has been decribed that hTERT bind to this regions. At the same time, we use as a negative control the 3' UTR region of c-MYC, which is known that it is not recognized by hTERT. This point was previously described

ChIP experiments revealed that stably expressed Flag–TERT was also associated with the TBE-containing promoter fragments of the *cyclin D1* and *Myc* promoters in a lithium-dependent manner (Fig. 12 a, b). Furthermore, sequential ChIP revealed that Flag–TERT was bound to the same promoter elements as BRG1 and β-catenin. TERT binding to TCF elements was analyzed further through quantitative ChIP experiments using primer pairs spanning 20 kb upstream of the *Axin2* and *Myc* genes at average intervals of 1 Kb. The profile of Flag–TERT binding closely resembled that of TCF3 at both the *Axin2* and *Myc* genes in HeLa cells stimulated with lithium. Notably, the Flag antibody ChIP performed on parental HeLa cells lacking expression of Flag–TERT did not detect these promoter fragments, indicating that the Flag–TERT ChIP signals depended on the presence of stably overexpressed TERT protein. These data reveal that

Flag–TERT physically associates with TBE-containing promoter fragments, along with BRG1 and β-catenin.

The data obtained by Marsico *et al.* (2013) designed a new approach for miRNA promoter annotation based on a semi-supervised statistical model trained on deepCAGE data and sequence features. This research predicts possible promoters of different miRNAs, such as hsa-*miR-500a*. Different primers were designed attending the predicted promoters (Fig 9).

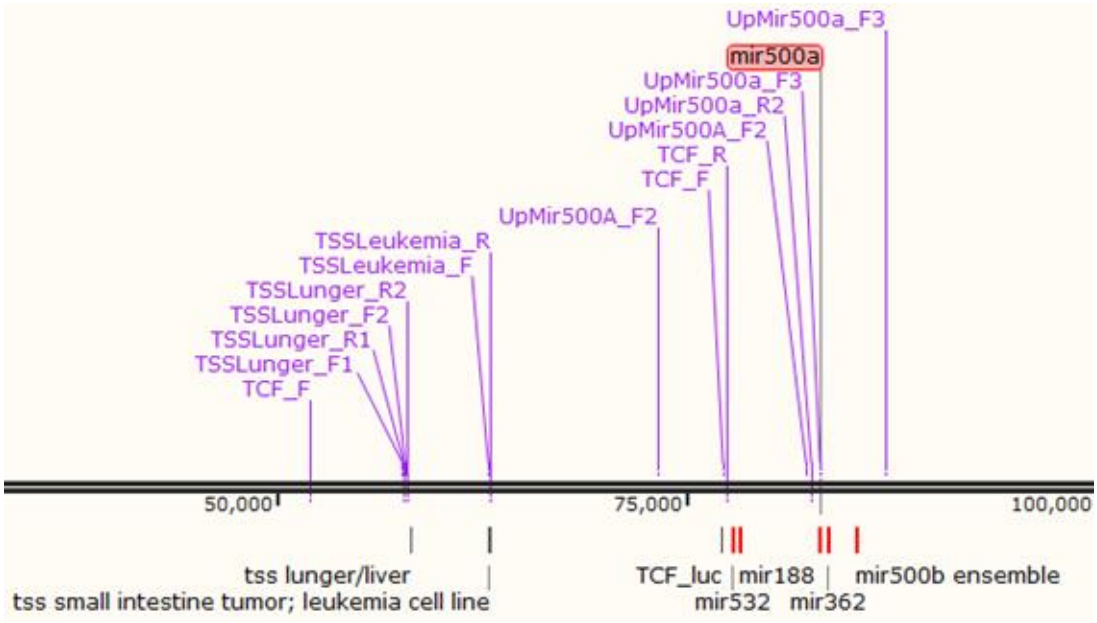

Figure 12: Binding site of oligos used as positive and negative controls to verify that ChIP experiments worked properly.

TERT became associated with promoter fragments containing the region upstream of hsa-*miR-500a*, including those within *Myc*, used as positive control. TERT binding to upstream elements was analyzed further through quantitative ChIP experiments using different primer pairs spanning upstream of hsa-*miR-500a*.

Due to the promoters or closest areas to hsa-*miR-500a* gene we are possibly regulated by hTERT, several results was observed (Fig 11a & Fig 11b):

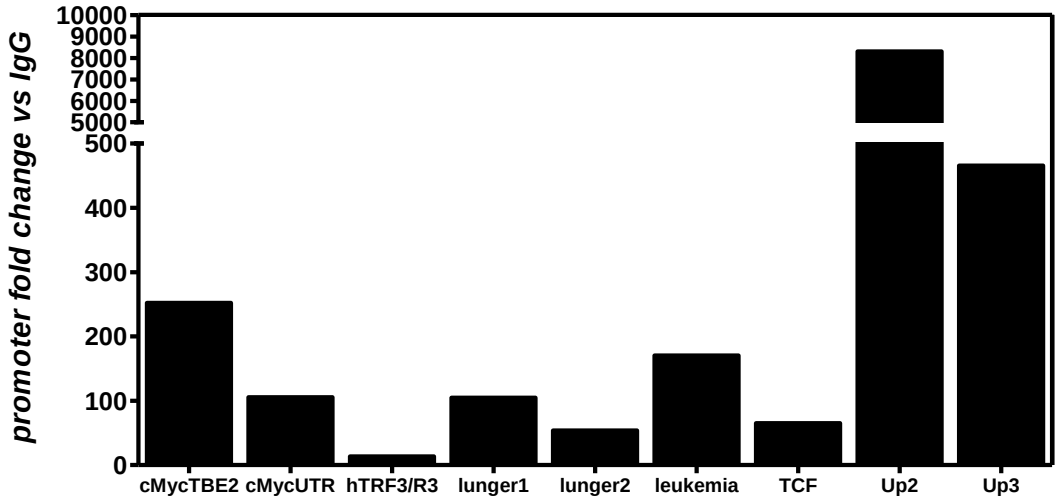

Figure 13: hTERT interact with the Up2-Up3 region that are at a few Kb of hsa-*miR-500a* gene. The results are representated by using a standardization method known as "Fold Enrichment". This method is based on two types of signals. On the one hand, a signal produced without the antibody a.k.a. background signal, and on the other hand, the representative signal of ChIP produced by increasing signal with regard the background

The amplification of ChIP in h*TERT* SAOS 2 shows that cMycTBE2 acts as a positive control and cMyc3'UTR acts as a negative control. hTRF3/R3 is an additional negative control more effective than the previous one (as a coding area, it can't be merging telomerase as a transcriptional factor). hTERT does not merge with TSSlung1, TSSlung2 and TCF (notice that the signals are at the same level as the negative controls). However, hTERT could be interacting with TSSLeukemia at an area which includes the start point of transcription in leukemia cell lines. If hTERT is interacting with this area, which is 20 Kb from hsa-*miR-500a*, then it is possible that hTERT regulates the expression of this miRNA and the rest of miRNAs located at the same cluster. Finally, hTERT would be merging to Up-mir-500a3 and with UpmiR-500a2 with a very efficient union, almost three times higher than the positive control. This area includes the hsa-*miR-500a* gene itself, so hTERT could be merging directly upstream or downstream of the gene and it could regulate its own transcription or processing. Fragments analyzed during the qPCR are 200-500 pb long, so the specific union area is unknown.

3.9. miR500 promoter characterization

Taking account the results from ChIP experiments, we next wanted to characterize the hsa-*miR-500a* promoter. For that, we cloned a 2 Kb fragment upstream hsa-miR-500a into luciferase reporters as described in materials & methods

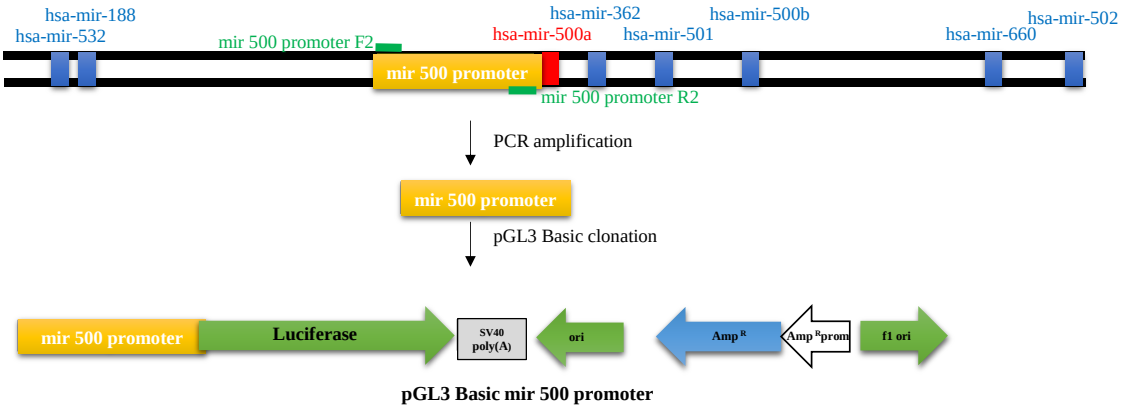

Figure 14: Schematic representation of hsa-miR-500a cloning into PGL3-Luc+ plasmid. We designed a pairs of oligos to amplify hsa-miR-500a promoter and the promoter was cloned in the PGL3 Luc+ plasmid as described in materials & methods.

In this sequence of hsa-miR-500a promotor there are two different zones, the TCF/LEF, which is a binding area of transcription factors and telomerase, and the E-box, which is a consensus sequence commonly found in the promoters. Transcription factors usually binds this E-box area too.

The luciferase activity results showed that this 2Kb fragment upstream of the hsa-miR-500a is able to drive the expression of the firefly luciferase reporter under hTERT overexpression conditions (figura 15).

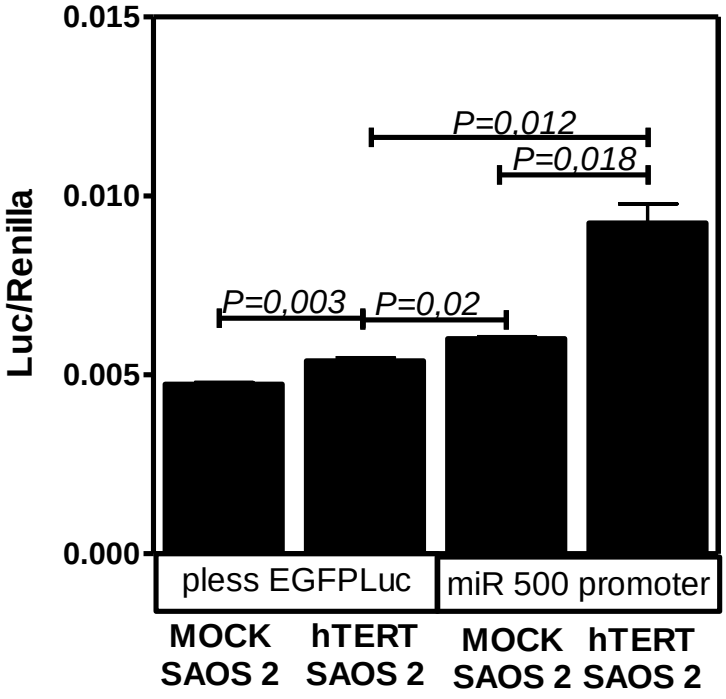

Figure 15: Analysis of hsa-miR-500a promoter activity in SAOS 2. pBABE SAOS 2 and h*TERT* SAOS 2 were tranfected with plessEGFPLuc (pless) or hsa-miR-500a promoter(2Kb)-EGFPLuc (pmiR-500a) and the pRL-CMV (10:1) reporter vectors. 24 hours after transfection, the firefly and *Renilla* luciferase activity was measured using the Dual-Luciferase Reporter Assay System. The results are expressed as the mean ± SEM of normalized luciferase activity relative to pless EGFPLuc transfected cells.

Next, to investigate wether telomerase activity is necessary for hsa-miR-500a up-regulation, we used a dominant-negative hTERT mutant, DN-hTERT, and examined whether this mutant protein can regulate the luciferase expression as hTERT. We performed an experiment where pmir500a–luciferase were cotransfected with hTERT or DN-hTERT, resulting that DNhTERT increased the luciferase expression and hsa-miR-500a expression in a similar level than hTERT

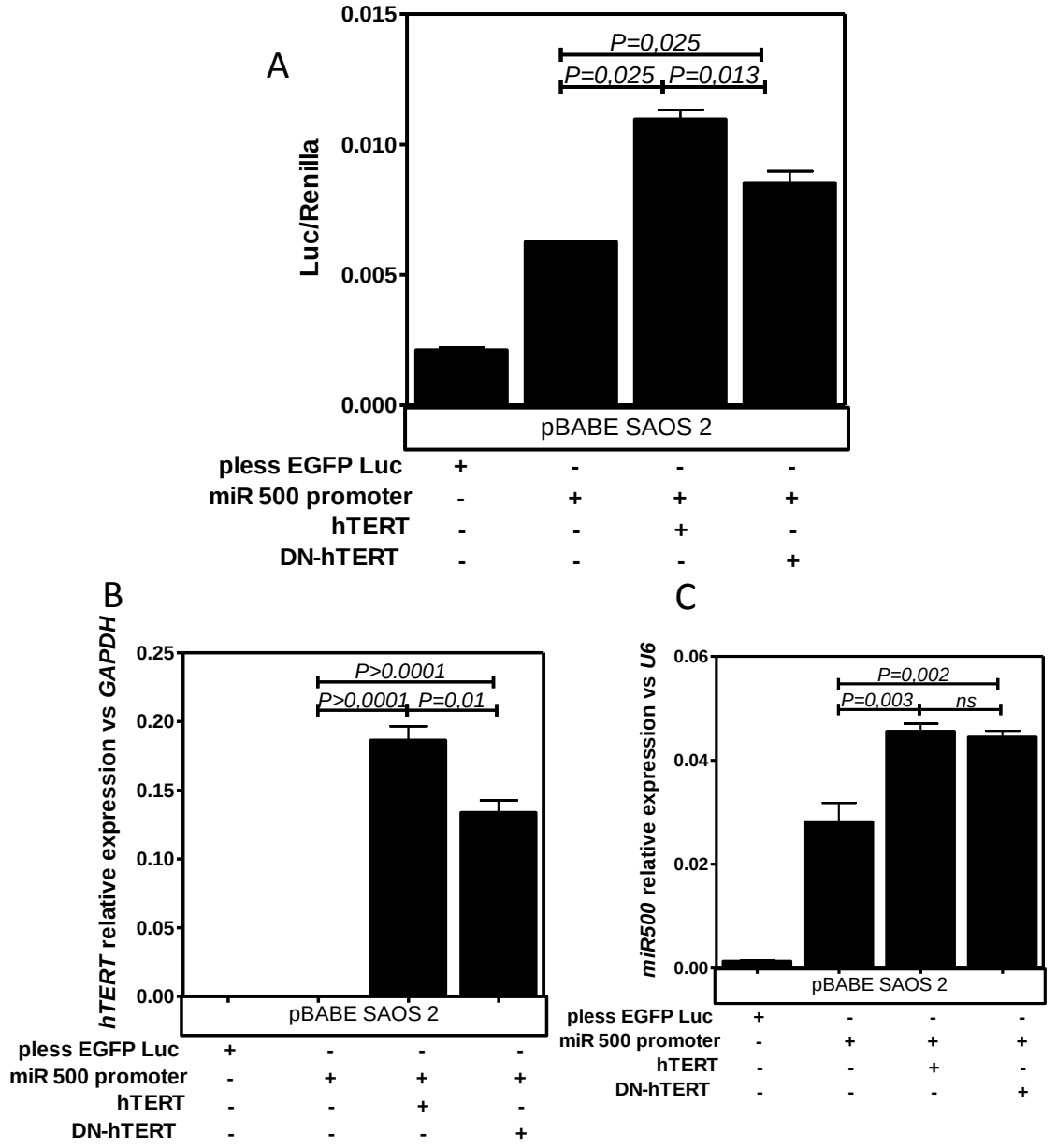

Figure 16: Analysis of hsa-miR-500a promoter activity in pBABE SAOS 2, hTERT SAOS 2 and DN-hTERT SAOS 2 . SAOS2 and SASO 2 hTERT were tranfected with plessEGFPLuc (pless) or hsa-miR-500a promoter(2Kb)-EGFPLuc (pmiR-500a) and the pRL-CMV (10:1) reporter vectors. 24 hours after transfection, the firefly and *Renilla* luciferase activity was measured using the Dual-Luciferase Reporter Assay System. The results are expressed as the mean ± SEM of normalized luciferase activity relative to plessEGFPLuc transfected cells.

These results support once again the hypothesis of an extracurricular role of hTERT in transcripcional regulation and its function in cancer progression and metastasis.

Therefore, we asked wether the decrease in h*TERT* expression could affect to luciferase expression drived by pmiR-500a. To check this hypothesis, siRNA engineered to target a sequence unrelated to hTERT was used to confirm whether the down-regulation of h*TERT* was able to decrease the promoter activity.

The results showed that, when we inhibit hTERT expression, we obtain a decrease in the activation of hsa-miR-500a promoter (figura 17 b), in agreement with the previous results obtained in hTERT overexpression conditions.

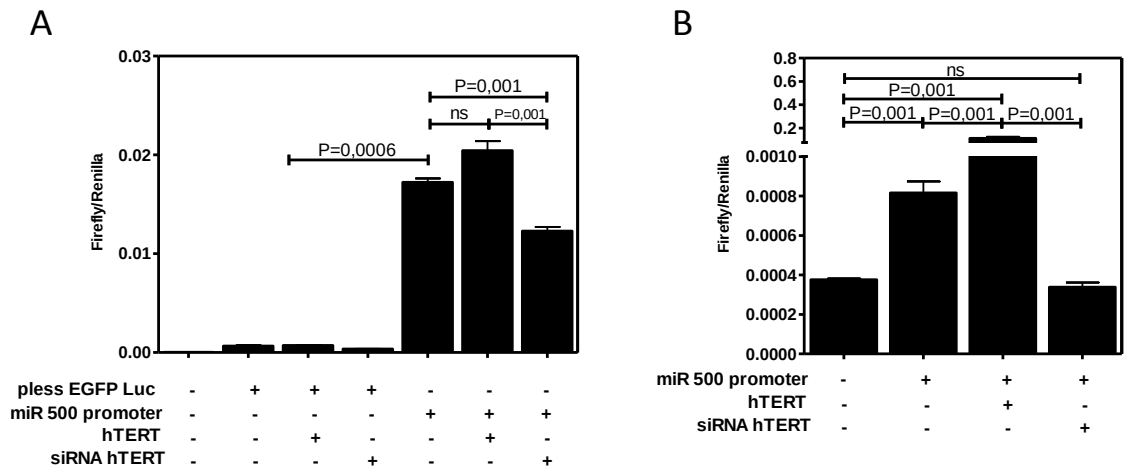

Figure 17: Analysis of hsa-miR-500a promoter activity in SAOS 2, SAOS 2 hTERT and SAOS 2 hTERT DN. SAOS2 and SASO 2 hTERT were tranfected with plessEGFPLuc (pless) or miR 500 promoter(2Kb)-EGFPLuc (pmiR 500) and the pRL-CMV (10:1) reporter vectors. Twenty-four hours after transfection, the firefly and *Renilla* luciferase activity was measured using the Dual-Luciferase.

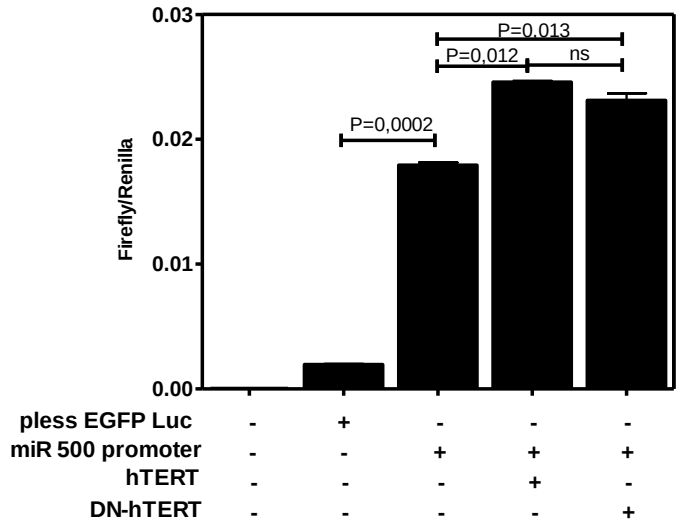

Figure 18: Analysis of miR 500 promoter activity in HEK 293. HEK were tranfected with plessEGFPLuc (pless) or miR 500 promoter (2Kb)-EGFPLuc (pmiR 500) plus hTERT, miR 500 promoter(2Kb)-EGFPLuc plus hTERT Dominant negative and the pRL-CMV (10:1) reporter vectors. Twenty-four hours after transfection, the firefly and *Renilla* luciferase activity was measured using the Dual-Luciferase

3.10. The hsa-miR-500 cluster is regulated by hTERT

The previous results of the ChIP experiments suggest that the whole hsa-miR-500 cluster could also be regulated by hTERT. We have shown that hTERT binds to a possible promotor sequence in cell lines of leukemia (TSSLeukemia), and that could be affecting the expression of the whole cluster (Fig. ?). Moreover, it is already published that there are some signs of another miRNAs (hsa-miR-532, hsa-miR-362, hsa-miR-502) that could be involved on some types of specific cancer (Janssen et al. 2010). For all of these reasons, we wonder wether hTERT also regulates these miRNAs. o study wether hTERT controls the expression of the different miRNAs of the cluster, we obtained total RNA from pBABE SAOS 2 and hTERT SAOS 2 cell lines, and we generated cDNA from all present miRNAs for their subsequent quantification by qPCR

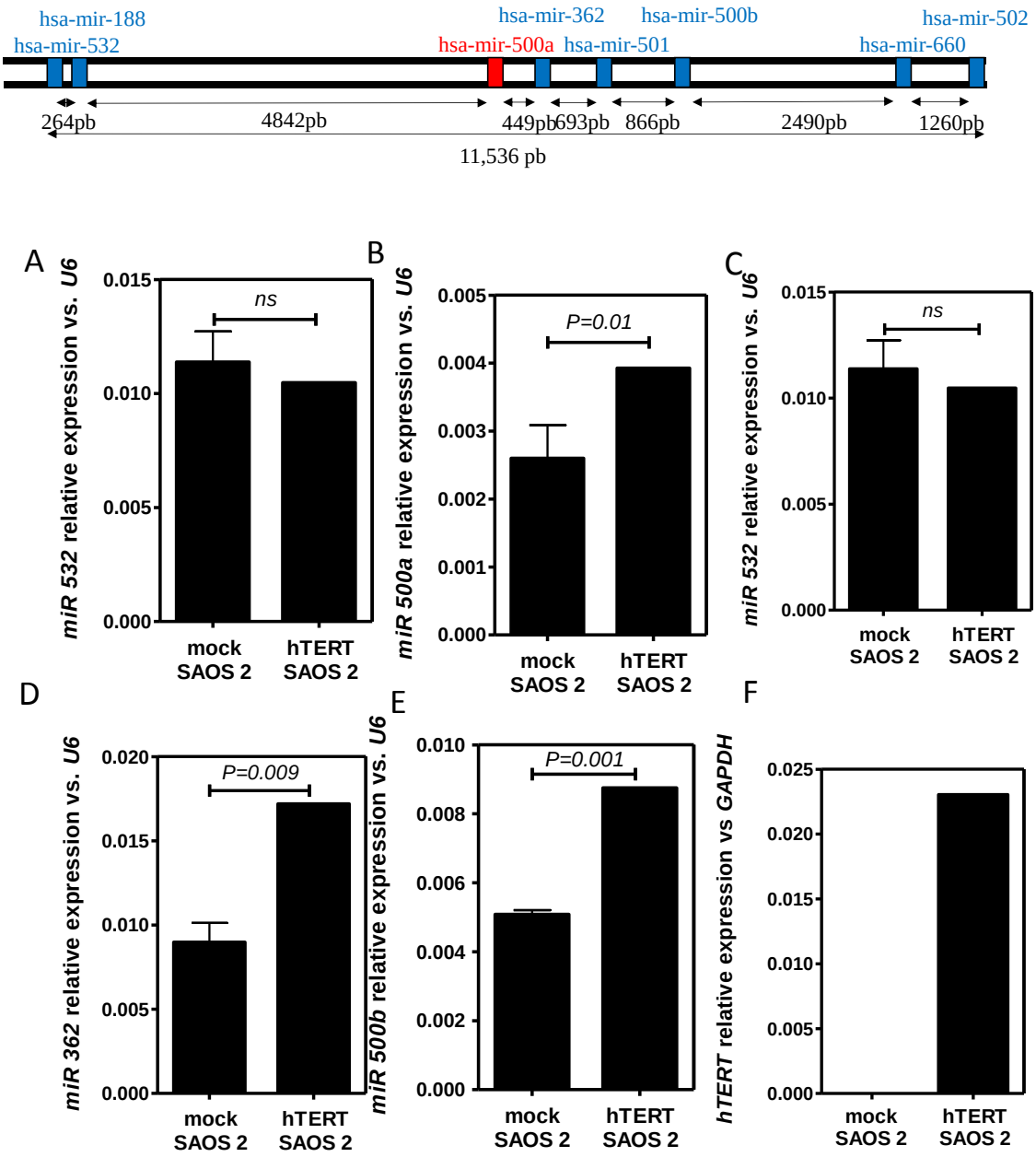

Fig 19: The hsa-*miR-500* cluster is regulated by hTERT A) Schematic representation of the hsa-miR-500 cluster position map in the genome. B) Validation of hTERT overexpression and study of miRNAs expression under h*TERT* overexpression conditions by RT-qPCR using as signification level a p value < 0.05.

These results verified that hsa-*miR-500a* has a significant higher expression on cells with h*TERT* overexpression compared to control. Furthermore, we observed that hsa-*miR-362*, hsa-*mir-500b* and hsa-*miR-502* have a higher expression under h*TERT* overexpression conditions, indicating that telomerase could be regulating their expression. However, the expression level of hsa-*miR-532* is not affected by hTERT levels, so telomerase could not regulate this one.

To summarize, hTERT could be acting like a transcriptional factor that up-regulates the expression of hsa-*miR-500a* and the miRNAs located downstream this one.

According to the ChIP experiment, telomerase binds to two areas: the predicted promoter on leukemia cell lines and a close area of hsa-*miR-500a* gene.

A hypothesis to explain this result and the overexpression of the different miRNAs of the cluster, excluding that one located upstream of hsa-*miR-500a*, could be the existence of a DNA loop created by hTERT through simultaneous binding to the predicted promoter area of leukemia cells and to the upstream area of hsa-*miR-500a*. The formation of this loop would assist the up-regulation of the miRNA cluster excluding that ones located upstream of hsa-*miR-500a*, like hsa-*miR-532*. However, further experiments are necessary to demonstrate this hypothesis.

But, the fact that hTERT up-regulates hsa-*miR-500a* to a level higher than the rest of miRNAs, telomerase could be regulating either way the transcription of this concrete miRNA or its stability.

3.11. Identification of possible hsa-miR-500a targets.

Because the experimental identification of miRNA targets is difficult, there have been a huge development in bioinformatic methods to predict targets. The initial prediction programs evaluated the degree of complementarity with the target mRNA and predicted the free energy of the mRNA/miRNA complex. But one of the most important contributions to the recognition of new targets was discovering that nucleotides between positions 2 and 8 in the miRNA bind to the target mRNA with perfect complementarity. This binding zone composed of 7 nucleotides is usually found in the 5' region of the miRNA, and is known as the seed sequence. The complementarity between the seed sequence of the miRNA and its target mRNA was the basis used for the development of multiple target prediction algorithms. Nowadays, there are numerous bioinformatic tools available for the prediction of target genes of the miRNAs, and although they all have some bases in common, small differences in prediction algorithms provoke great diversity of results, so is difficult to judge which of the algorithms produce the best prediction.

All the targets predicted by the algorithm were analyzed in the MetaCore program, which shows the genetic routes enriched by the potential targets of the hsa-miR-500a (Fig. 20).

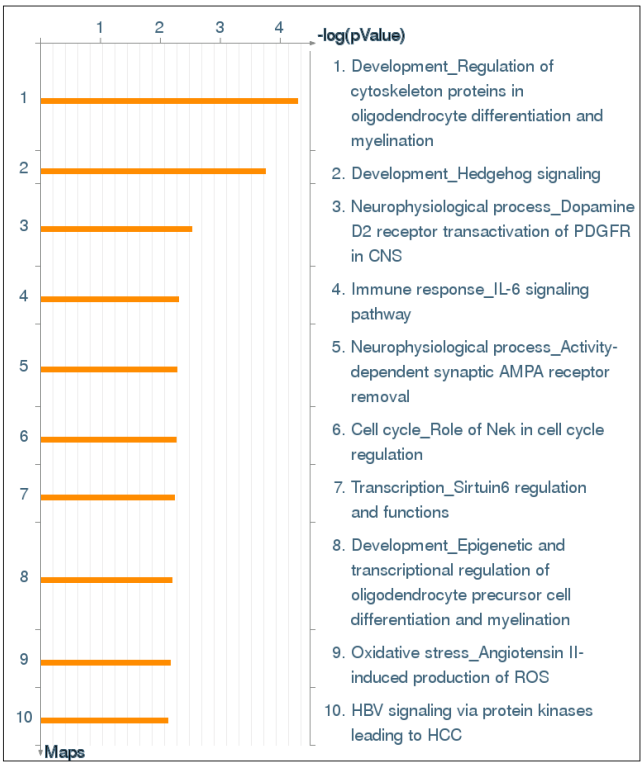

Figure 20: Gene pathways enriched by the potential targets of the miR-500a ordered by significance according to MetaCore.

Among all routes, special attention was given to those related to cancer, such as the Hedgehog, Wnt / β-catenin, and IL6 (interleukin 6) signaling pathways.

After analyzing these three routes, we decided to select some of the target genes of miR-500a that, according to MetaCore, were significantly overexpressed, namely: *CUL3* (cullin 3), *GLI3* (GLI family zinc finger 3) and *PTCH1* (Patched 1) of the Hedgehog signaling pathway (Figure ?); *SKP1* (S-phase kinase-associated protein 1) and *TLE4* (transducin-like enhancer of split 4) of the Wnt / β-catenin signaling pathway (Figure ?); *IL6R* (interleukin 6 receptor) and *NR3C1* (nuclear receptor subfamily 3, group C, member 1) of the IL6 signaling pathway (Figure ?).

Therefore, our hypothesis would be that if the miR-500a actually inhibits these genes, there would be a deregulation of the Hedgehog, Wnt / β-catenin and IL6 pathways, leading to cancer progression. We also selected the target genes *OCLN* (occludin) and

MTDH (metadherin) (Figure ?), which although were not directly related to the above routes, they encoded proteins involved in cell adhesion and cell-cell contact. So, based on the function of these genes, we hypothesized that if the miR-500a would be inhibiting their expression, these cell adhesion proteins would not be present, the tumor cells could detach from each other and colonize other tissues.

3.12. miR 500a expression in breast cancer tumor.

Our data from xenograft experiments with zebrafish show that h*TERT* SAOS 2 cells have higher percentage of metastasis than pBabe SAOS 2, and those from the microarray show that hsa-*miR-500a* was overexpressed on h*TERT* SAOS 2 compared to control. It was thought that the cause of this metastasis increase could be the overexpression of this miRNA because of telomerase. The telomerase could be very important in this procedure due to its role in the regulation of routes, which is on study. Furthermore, previously studies mentioned in the introduction about liver cancer (Yamamoto et al. 2009) and gastric cancer (Zhang et al. 2015) indicates that hsa-miR-500 is related with this kind of diseases.

To verify wether hsa-*miR-500a* is overexpressed in tumors, we analyzed tumor tissue samples from 18 patients and tissue from healthy people. The tumor tissue samples were classified in metastatic and no metastatic following Pathological Anatomy criteria according to sentinel ganglion data. The sentinel ganglion is the first lymphatic ganglion found by tumor cells when they try to spread through the lymph. So, if this test is positive, tumor cells are able to spread to another tissues creating metastasis.

Then, we studied the hsa-*miR-500a* expression in different kind of tissues by RT-qPCR.

The results of the relative expression of hsa-*miR-500a* (Fig 15a & 15b) shows that it is significantly higher in metastatic tumor tissue (fig 15c) than in no metastatic tumor tissue and the healthy one, using as significance level $p\ value < 0.05$. Both control tissues have an expression average similar, being a bit higher in healthy tissue. This could be due to, although healthy tissue was removed from patients with metastatic and

no metastatic tumors, they were selected areas without tumor cells through histological tissue observation, and contaminations could be possible.

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Then, we studied the hsa-*miR-500a* expression in different kind of tissues by RT-qPCR. The results of the RT-PCR were based on the use of housekeeping gene a.k.a constitutive gene, as well as was used at the quantification experiment of different miRNAs of the cluster, which has a constant expression of cells and is able to compare its expression with the analyzed gene. The constitutive gene used was U6. Mat&met

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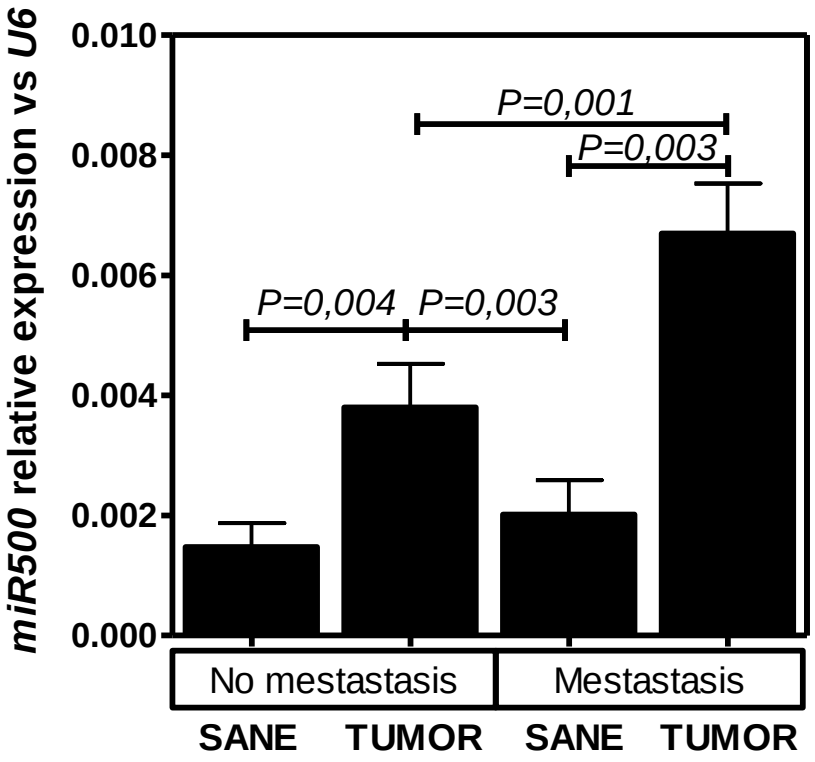

Figure 29: hsa-*miR-500a* expression in different tissue types. The representation shows the average and the standard deviation for every type of tissue.(A) The representation shows the relative expression of each sample.(B) Statistical analysis of different tissue types, using as significant level a p value < 0,05 (C)

Therefore, it is possible to relate hTERT, hsa-miR-500a and metastasis due to hTERT direct binding with closer miRNA areas and the metastatic tumor tissue shows a higher expression of miRNA. However, more studies are needed to demonstrate how hTERT and hsa-miR-500a are involved in the metastasis process.

These results could be used to have a better knowledge of the metastasis process, which is responsible of the most mortality in patients with cancer. Furthermore, the analysis of hsa-miR-500a could be used as an additional marker of metastasis in breast cancer, due to its overexpression related to no metastatic tumor tissue, because the sentinel ganglion test is not effective 100%.

4. Discussion

Human telomerase reverse transcriptase (hTERT), which regulates telomere length, can promote tumor development. Telomerase is reactivated in 90% of all cancers (Harley, 2008). In approximately 15% of human cancers, telomere length is maintained independently of telomerase by the homologous recombination (HR)-mediated alternative lengthening of telomeres (ALT) pathway (Zheng *et al.*, 2017). Most research on hTERT has been focused on its crucial function of telomere maintenance. However, there are many phenomena that cannot be explained by its reverse transcriptase activity. Accumulating evidence suggests that hTERT has functions independent of its protective function at the telomere ends, such as increasing the anti-apoptotic capacity of cells, enhancing DNA repair, maintaining stem cells and regulating gene expression.

In human cancer, high expression of telomerase is correlated with tumor aggressiveness and metastatic potential. Patients with metastatic cancer suffer the highest rate of cancer-related death, but the existing animal models of metastasis have disadvantages that limit our ability to understand this process. The zebrafish is increasingly used for cancer modelling, particularly xenografting of human cancer cell lines and drug discovery, and may provide novel scientific and therapeutic insights.

Taking advantage of the xenograft assay in zebrafish, we wanted to check wether hTERT overexpression in the SAOS 2 cell line (a **primary osteogenic sarcoma** cell line which uses ALT system to maintain the telomere length and do not express endogenous hTERT), confers higher invasion capacity. We have demonstrated that h-TERT increased the invasiveness in hTERT-SAOS 2 compared with a control cell line (pBABE-SAOS 2).

MicroRNAs play key roles in tumor metastasis[17]. Several studies have shown that microRNAs can regulate a number of tumor-related transcription factors that are involved in tumor invasion and proliferation.[18] We used a microRNA array approach to analyze gene expression changes in hTERT-SAOS 2 and pBABE-SAOS 2 cells. The analysis showed an upregulated microRNA called miR-500a.

Interestingly, the overexpression of miR-500a in both pBABE-SAOS 2 and hTERT-SAOS 2 increased the invasion in xenografted zebrafish larvae, indicating the implication of this microRNA in metastasis *per se*. On the other hand, the inhibition of miR-500a

produced a decrease in invasion *in vivo*, but only in hTERT-SAOS 2 cells, in hTERT overexpression conditions. These results have been shown to have clinical implications as they may be therapeutic approaches using miRNAs (Hung et al., 2014, Shah and Chen 2014, Ye and Cao 2014)

It has been reported that miR-500a is up-regulated in both prostate cancer cells and primary tumors. In prostate cancer patients, high miR-500 expression (Cai B et al, 2017) is associated with poor prognosis and overall survival. Importantly, miR-500 was upregulated in gastric cancer, regulating a novel mechanism for constitutive NF-κB activation and was highly correlated with malignant progression and poor survival.

Although much has been published about miR-500 targets, nothing is known about how hTERT regulates the expression of miR-500.

It has been demonstrated that telomerase directly regulates the expression of specific genes belonging to the NF-κB signaling pathway or the Wnt/β-catenin pathway (Choi *et al.*, 2008; Ghosh *et al.*, 2012), participates in DNA damage repair, and promotes cell survival under oxidative stress or endoplasmic reticulum stress conditions. Additionally, it has been shown that hTERT protects developing neurons from DNA damage-induced cell death (Fu *et al.*, 2000). TERT also regulates mitochondrial function and cell metabolism (Haendeler *et al.*, 2009). Among the telomere-independent activities of hTERT, the role of hTERT in gene transcription has been investigated in detail. Several studies have shown a direct implication of hTERT in gene transcription.

The detailed mechanisms of TERT-mediated miRNA regulation will be covered in this studies: Lassmann et al. (2015) have described that TERT is involved in the regulation of microRNA expression. This study reveals that deletion of TERT leads to the descent of microRNAs expressed in THP-1 cells (human monocytic leukemia cell line) and HeLa, so the authors conclude that TERT appears to act as a positive regulator of the expression of microRNAs. In the same way, Drevytska et al. (2014) described a positive correlation between TERT expression levels and levels of several microRNAs. However, to date it is unknown whether TERT binds directly to the promoters of the microRNAs genes or whether it regulates their transcription indirectly, for example through the induction of transcriptional factors, as proposed by Lassmann et al . (2015). In addition, since 60% of the promoters of microRNAs are formed by CpG islands and

have patterns of modification of specific histones, another theory proposed is that TERT could contribute to the epigenetic regulation of the promoters of microRNAs.

We performed a ChIP assay to check the ability of TERT to bind to miR-500 DNA upstream region. Interestingly, TERT antibody could inmunoprecipitate a region very near of miR-500 localization. The clonation of this region driving a luciferase reported gene showed that this fragment was regulated by TERT, hence its overexpression increased the luciferase expression and however telomerase inhibition by siRNA dicreassed the luciferase activity. Surprisingly, the same activation was obtained when we used a dominant-negative telomerase mutant, supporting definitively that hTERT regulatory function on miR-500 is independent of its enzymatic activity.

miR-500 is located in a chromosome region together a microRNA cluster and this could be affecting the expression of the whole cluster. (Figure 6c) Although there are published some signs of another miRNAs (hsa-miR-532, hsa-miR-362, hsa-miR-502), miR-500 could be involved on some types of specific cancer (Janssen et al. 2010). For all these reasons, we wonder wether telomerase also regulates this miRNAs.

Effectively, we observed that *hsa-miR-362*, *hsa-mir-500b* and *hsa-miR-502* have a higher expression in *hTERT* SAOS 2 cells, which means that telomerase could be regulating their expression. However, the expression level on both cell types for *hsa-miR-532* does not show significant differences, so telomerase couldn't regulate this one.

Following the chromatin immunoprecipitation experiment, telomerase binds to a close area of *hsa-mir-500a* gene. A hypothesis to explain this result and the overexpression of the different miRNAs of the cluster, excluding that one located upstream of hsa-*miR-500a*, could be the existence of a DNA loop created by hTERT through simultaneous binding to the predicted promoter area of leukemia cells and to the upstream area of hsa-*miR-500a*. The formation of this loop would assist the up-regulation of the miRNA cluster excluding that ones located upstream of hsa-*miR-500a*, like hsa-*miR-532*. However, further experiments are necessary to demonstrate this hypothesis.

But, the fact that hTERT up-regulates hsa-*miR-500a* to a level higher than the rest of miRNAs, telomerase could be regulating either way the transcription of this concrete miRNA or its stability.

It has been reported that TERT extensively affects the expression levels of mature microRNAs. The suppression of TERT resulted in the downregulation of microRNAs expressed in THP-1 cells and HeLa cells (Lassmann et al.2015). Primary and precursor microRNA levels were also reduced under the suppression of TERT. Similar results were obtained with the suppression of either BRG1 (also called SMARCA4) or nucleostemin, which are proteins interacting with TERT and functioning beyond telomeres. These results suggest that TERT regulates microRNAs at the very early phases in their biogenesis, presumably through non-telomerase mechanism(s). This could give us a key to understand how *hsa-mir-500a* has a significant higher expression on cells with hTERT overexpression compared to the control.

microRNA (miRNA) regulates diverse biological mechanisms and metabolisms in plants and animals. Thus, the discoveries of miRNA has revolutionized the life sciences and medical research. The miRNA represses and cleaves the targeted mRNA by binding perfect or near perfect or imperfect complementary base pairs by RNA-induced silencing complex (RISC) formation during biogenesis process. One miRNA interacts with one or more mRNA genes and vice versa, hence takes part in causing various diseases. We use different microRNA target databases to predict and to study functional annotations of predicted targets after subsequent validation of the chosen target by RT-qPCR.

It has been reported that miR-500 overexpression plays important roles in gastric cancer progression and that miR-500 is a critical activator of NF-κB signaling. Understanding the precise role of miR-500 in the pathogenesis of gastric cancer and activation of the NF-κB signaling pathway will increase our knowledge of the biological basis of cancer progression.

Among all routes, we gave special attention to those that were related to cancer, such as the Hedgehog, Wnt / β-catenin, and IL6 (interleukin 6) signaling pathways. After analyzing the three previously mentioned routes, we decided to select the following target genes: CUL3 (cullin 3), GLI3 (GLI family zinc finger 3) and PTCH1 (Patched 1) of the

Hedgehog signaling pathway; SKP1 (S-phase kinase-associated protein 1) and TLE4 (transducin-like enhancer of split 4) of the Wnt / β-catenin signaling pathway; IL6R (interleukin 6 receptor) and NR3C1 (nuclear receptor subfamily 3, group C, member 1) of the IL6 signaling pathway. Therefore, if the miR-500 actually inhibits these genes, then there would be a deregulation of the Hedgehog, Wnt / β-catenin and IL6 pathways, leading to cancer progression.

We also selected the target genes *OCLN* (occludin) and *MTDH* (metadherin) ,which although were not directly related to the above routes, they encoded proteins involved in cell adhesion and cell-cell contact. So, based on the function of these genes, we hypothesized that if the miR-500a would be inhibiting their expression, these cell adhesion proteins would not be present, the tumor cells could detach from each other and colonize other tissues.

Finally, the results of the relative expression of hsa-*miR-500a* (Fig 15a & 15b) shows that it is significantly higher in metastatic tumor tissue (fig 15c) than in no metastatic tumor tissue and the healthy one. Both control tissues have an expression average similar, being a bit higher in healthy tissue. This could be due to, although healthy tissue was removed from patients with metastatic and no metastatic tumors, they were selected areas without tumor cells through histological tissue observation, and contaminations could be possible.

Our results could be use to have a better knowledge of the metastasis process, which is responsible of the most mortality in patients with cancer. Furthermore, the analysis of hsa-miR-500a could be used as an additional marker of metastasis in breast cancer, due to its overexpression related to no metastatic tumor tissue, because the sentinel ganglion test is not effective 100%.

Zhang and colleagues (2015) showed that an antagomiR-500 inhibited the tumorigenicity of the gastric cancer cells. Importantly, they also found that miR-500 expression was markedly upregulated in gastric cancer tissues but remained comparatively low in normal gastric tissues. Their results together with our results suggest that miR-500 may represent a promising therapeutic target in cancer.

Therefore, it is possible to relate hTERT, hsa-miR-500a and metastasis due to hTERT direct binding with closer miRNA areas and the metastatic tumor tissue shows a higher expression of miRNA. However, more studies are needed to demonstrate how hTERT and hsa-miR-500a are involved in the metastasis process.

5. Conclusions

The results obtained in this work lead to the following conclusions:

1. hTERT overexpression in Sarcoma osteogenic cells (hTERT-SAOS 2), which maintain their telomere length through the alternative mechanism ALT, increases their aggressiveness in zebrafish xenograft experiments compared to control (pBABE-SAOS 2).

2. hTERT overexpression affects the expression of a microRNAs cluster that includes miR-500a.
3. miR-500a plays an essential role in the invasion capacity of osteosarcoma cells, since its overexpression or inhibition increases or decreases the invasion, respectively, in zebrafish xenograft experiments.

4. miR-500a expression is regulated by hTERT through the direct binding to an upstream region of miR-500a. This region regulates the expression of all the cluster genes (*hsa-miR500a, hsa-miR-362, hsa-mir-500b* and *hsa-miR-502)* except for *hsa-miR532*, which is upstream of this region.

5. A 2 Kb regulatory region upstream the microRNA-500a has promoter activity and is regulated by hTERT.

6. We have identified some mRNA targets through which miR-500a could act in cancer. These targets are involved in important cancer pathways, such as Hedgehog and Wnt/β- Catenin pathways.

7. miR-500a constitutes an aggressiveness marker in breast cancer and can be used for prognosis and as a therapeutic target.

8. The zebrafish is an exceptional model for predicting the degree of invasiveness of tumor cells.

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Resumen en español

1. Introducción

Los cromosomas eucariotas consisten en grandes moléculas de ADN lineal y proteínas asociadas que almacenan la información genética. Los extremos de los cromosomas se denominan **telómeros** y consisten en una secuencia corta que se repite en tándem cientos de veces. La longitud de los telómeros es variable en función de la especie y la secuencia telomérica TTAGGG está conservada desde eucariotas inferiores hasta mamíferos [Greider, 1998]. Además, los telómeros tienen una estructura nucleoproteica especial que "camufla" los extremos libres y evita que sean reconocidos como roturas de ADN de doble cadena, asegurando así la integridad de los cromosomas [Greider, 1996].

Debido al "problema de la replicación de los extremos", los telómeros se acortan en cada división celular. Las ADN polimerasas sintetizan ADN en la dirección 5'→3' y requieren la presencia de un oligonucleótido de ARN como punto de partida. Como resultado, los cromosomas lineales se acortan con cada ronda de replicación [Watson, 1972; Olovnikov, 1973]. En ausencia de un mecanismo compensatorio, tras un número de divisiones celulares las células acabarían perdiendo material génico y desembocando en la muerte celular programada o en una senescencia replicativa, lo que pondría en peligro la supervivencia no sólo del individuo sino también de las especies.

Los organismos eucariotas han solventado este problema mediante la **telomerasa**, que es una DNA polimerasa dependiente de RNA con actividad retrotranscriptasa que añade repeticiones teloméricas al final de los cromosomas, haciendo posible que la longitud de los telómeros sea restaurada y mantenida [Blackburn, 2005]. La telomerasa es un complejo ribonucleoproteico compuesto por una subunidad catalítica con actividad retrotranscriptasa (*TElomerase RetroTranscriptase*, TERT), un componente

de RNA (*TElomerase RNA Component*, TERC o TR) que actúa como molde para la síntesis de telómeros y una serie de proteínas accesorias específicas de especie encargadas de regular la biogénesis, de la localización subcelular y de otra funciones *in vivo* de la telomerasa [Wyatt *et al.*, 2010]. En humanos, se han identificado 7 proteínas: Dyskerin, NHP2, NOP10, GAR1, TCAB1 [Fu & Collins, 2007], Pontin y Reptin [Veinteicher *et al*., 2008].

Una longitud telomérica mínima es un prerrequisito para que haya replicación celular [Blackburn *et al*., 2006]. La telomerasa es activa durante la embriogénesis y el desarrollo mientras que, en adultos, está limitada a los tipos celulares con una alta tasa de proliferación (epitelios, linfocitos activados), a la línea germinal y a las células madre [Ulaner *et al.*, 1997]. En el resto de tipos celulares, los telómeros se acortan de manera progresiva con cada división hasta que alcanzan una longitud crítica y, entonces como se ha comentado con anterioridad, se activan los mecanismos de respuesta de daño en el ADN y las células entran en senescencia o en apoptosis [de Jesús & Blasco, 2012; Galati *et al.*, 2013]. Sin embargo, a veces las células pueden sufrir alteraciones en los mecanismos de control de daño en el ADN y, en vez de entrar en senescencia, continúan proliferando de manera ilimitada, dando lugar a la aparición de tumores que, en combinación con otras mutaciones, pueden acabar desarrollando un cáncer. En estos casos, las células re-establecen la estabilidad genómica y también la longitud telomérica ya sea mediante la re-expresión de la telomerasa o por un mecanismo de mantenimiento de los telómeros independiente de la telomerasa y basado en la recombinación homóloga, el denominado "mecanismo ALT" (por sus siglas en inglés, *alternative lengthening of telomeres*) [Lundblad & Blackburn, 1993; Teng &. Zakian, 1999; Teng *et al*., 2000].

Al margen de su función en la síntesis de los telómeros, en los modelos humano y murino se han descrito otras funciones del complejo de la

telomerasa que se han denominado **funciones "extracurriculares"**. En mamiferos, TERT puede funcionar como un modulador transcripcional de la ruta de señalización de Wnt-β-catenina [Choi *et al*., 2008; Park *et al.*, 2009]. y de NFKB. Además de la interación con el complejo RMRP (RNA component of mitochondrial RNA processing endoribonuclease) actuando como una polimerasa dependiente de RNA. Sorprendentemente la acción reguladora sobre la ruta de señalización de Wnt-β-catenina lo hace uniéndose directamente al DNA y activando la regulación de loa genes. Por último la telomerasa también está implicada en la regulación de la apoptosis de manera independiente al mantenimiento de los telómeros [Cong & Shay, 2008] y a través de la mitocondria [Santos *et al*., 2006]. Se sabe que la contribución de TERT a la proliferación epitelial, a la tumorogénesis y al envejecimiento también está mediada por un mecanismo independiente del alargamiento telomérico [Choi *et al.*, 2008; Sarin *et al.*, 2005; Stewart *et al.*, 2002; Flores *et al*., 2005; Geserick & Blasco, 2006].

Es de destacar que recientemente se ha publicado que TERT tiene una function fundamental regulando las homeostasis de pequeños RNAs incluidos los denominado microRNAS. MicroRNAs tienen una function en la metástasis del tumor[17]. Un gran número de studios han mostrado que los microRNAs pueden regular factores de transcripción inplicado en la tumorogénesi, invasión y proliferación[18]

Los micro RNAs son pequeñas moléculas de ARN no codificantes altamente conservadas (NcRNAs) que no se traducen en proteínas. NcRNAs juegan papeles críticos en la regulación de la expresión génica en la transcripción, procesamiento de ARN, y los niveles de traducción (Macfarlane et al 2010) en diversos procesos biológicos. Dependiendo de la longitud y las funciones, ncRNAs se pueden clasificar en tres grupos: muy pequeños RNAs (18 - 25 nucleótidos) - microRNAs (miRNAs) y pequeños ARN interferentes

(siRNAs); Pequeños ARNs (smRNAs, 20-200 nucleótidos); Y ARNs medianos y grandes (piRNAs, 200-10000 nucleótidos) (Esteller 2011). Los microARN son moléculas de ARN no codificantes monocatenarias y de aproximadamente 21-25 nucleótidos de longitud, que desempeñan un papel crucial en la regulación posttranscripcional de la expresión génica (Bartel 2004). MicroRNAs son parcial o totalmente secuencia complementaria a los ARNm objetivos, y su principal función es reducir la estabilidad, expresión y / o traducción de mRNAs en una variedad de formas, incluyendo mRNA de clivaje, la represión traslacional y deadenylation (Bartel 2009).

En el cáncer humano, la alta expresión de telomerasa está correlacionada con la agresividad tumoral y el potencial metastásico. Los pacientes con cáncer metastásico sufren la mayor tasa de muerte relacionada con el cáncer, pero los modelos animales existentes de metástasis tienen desventajas que limitan nuestra capacidad de entender este proceso.

El pez cebra se utiliza cada vez más para el modelado del cáncer, particularmente el xenotrasplantes de líneas celulares de cáncer humano, y el descubrimiento de fármacos, y puede proporcionar nuevas ideas científicas y terapéuticas.

El modelo de pez cebra proporciona herramientas únicas para la visualización del comportamiento de las células tumorales y la interacción con las células huésped. Los embriones de pez cebra se desarrollan ex utero y hasta un mes de edad, sus larvas son transparentes, lo que permite una imagen directa del desarrollo, la organogénesis y la progresión del cáncer. En los adultos, las líneas transparentes del pez cebra permiten monitorizar las células trasplantadas hasta el nivel de célula única .

. Las ventajas del sistema modelo, como una buena accesibilidad, fácil manejo, bajos costos y tiempos cortos de incubación, lo convierten en un sistema prometedor para futuros estudios funcionales en tumores primarios.

2. Objetivos

En el presente trabajo se proponen los siguientes objetivos concretos:

1. Caracterización del mecanismo por el que la sobre-expresión de TERT influye en el aumento de la invasión y metástasis

2. Validación del modelo de pez cebra para el estudio de la invasividad de células humana mediante experimentos de xenotrasplante.

3. Materiales y Métodos

Animales

Peces cebra (*Danio rerio*) fueron cedidos por el centro internacional de recursos del pez cebra (ZIRC, Oregón, EEUU) y mantenidos como se describe en el manual del pez cebra [Westerfield, 2000]. La línea mutante para *tert* (alelo hu3430) fue obtenida del Instituto Sanger y ha sido previamente descrita [Anchelin *et al.*, 2013; Henriques *et al*., 2013]. La línea mutante *casper* [White *et al.*, 2008] y la línea transgénica Tg(*gata1*:DsRed) [Traver *et al.*, 2003] también fueron descritas previamente. Las líneas transgénicas Tg(*lyz*::DsRed) [Hall *et al.*,2007], Tg(*mpx*::eGFP) [Renshaw *et al.*,2006], Tg(*mpeg1*::GAL4/*uas*::NTRmCherry) (en el texto se usa *mpeg1*::mCherry para mayor simplicidad) [Ellett *et al*., 2011] y Tg(*cd41*::eGFP) [Ma *et al*., 2011] fueron amablemente proporcionadas por los Drs. P. Crosier, SA. Renshaw, G. Lieschke y RI. Handin, respectivamente.

Los experimentos desarrollados cumplen con la directiva de la Unión Europea (86/609/EU) y han sido aprobados por el Comité de Bioética del Hospital Clínico Universitario "Virgen de la Arrixaca" (España) y por el Comité Institucional del Cuidado y Uso Animal del Hospital Infantil de Boston (EEUU).

Microinyección

ADN, ARN, PAMPs (*Pathogen Associated Molecular Patterns*) o morfolinos (MOs), solos o en combinación, se prepararon en la mezcla de microinyección (0.5x de tampón Tango y rojo fenol al 0.05 %) y se microinyectaron en el saco vitelino de embriones de pez cebra en el estadío de

desarrollo de 1-8 células usando un microinyector Narishige IM300 (0.5-1 nL por embrión). Las dosis están indicadas en cada figura. Una vez microinyectados, los embriones se incubaron en *egg water* a 28.5 ºC.

Análisis bioinformático

La búsqueda de los sitios de unión de los factores de transcripción putativos en el promotor de la telomerasa del pez cebra se llevó a cabo usando el *software* TESS (*Transcription Element Search System*): http://www.cbil.upenn.edu/cgi-bin/tess/tess. Los sitios de unión de los factores de transcripción putativos en el promotor de la telomerasa humana fueron descritos previamente por Pericuesta y colaboradores en 2006.

Análisis del desarrollo

El efecto de los morfolinos en el desarrollo fue evaluado como se publicó anteriormente [Kimmel *et al.*, 1995]. Brevemente, se analizó la presencia de flexiones de lado a lado a las 22 horas de vida (estadío de 25-26 somitos); el latido cardiaco, la presencia de eritrocitos en el saco vitelino y la pigmentación primaria de la retina y de la piel a las 24 horas (estadío Prim 5); el ángulo formado por la cabeza y el tronco (HTA), la pigmentación de la retina, el inicio de la circulación, el reflejo al tacto, la completa extensión de la cola y el desarrollo de la arteria caudal hasta la mitad de la cola a las 30 horas (estadío Prim 15); y el inicio de la movilidad, la pigmentación de la cola, la circulación fuerte, y el desarrollo de la arteria caudal hasta las ¾ de la cola a las 36 horas (estadío Prim 30).

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Ensayo de actividad telomerasa

El análisis de la actividad telomerasa se llevó a cabo mediante el protocol de amplificación de las repeticiones teloméricas cuantitativo (Q-TRAP). Brevemente, extractos proteicos de embriones/larvas fueron obtenidos usando tampón de lisis NP-40 frío. El Q-TRAP a tiempo real se hizo a partir de 0,1 y 1 □g de dicho extracto. Para hacer la recta patrón se usó una dilución seriada 1:10 de un extracto proteico de una muestra con telomerasa (células HeLa). Las muestras control se obtuvieron tratando los extractos proteicos con 1□□g de RNasa a 37 ºC durante 20 min. Los datos fueron convertidos a unidades de actividad telomerase relativa (RTA) con el siguiente cálculo: RTA de la muestra=$10^{(Ct\ muestra-\gamma int)/pendiente}$. La curva estándar obtenida fue: y= -3.2295x + 23.802.

Análisis de la expression génica

El ARN total fue extraído de embriones/larvas o de suspensions celulares con TRIzol (Invitrogen) siguiendo las instrucciones del fabricante y posteriormente fue tratado con DNasa I libre de RNasa (Invitrogen). La retrotranscriptasa Superscript III RNasa H^- (Invitrogen) se usó para sintetizar el cDNA con un cebador oligo-dT_{18} (para todos los genes, excepto para *TR*) o R1 (para *TR*) a partir de 1 μg de RNA total a 50ºC durante 50 minutos. La PCR a tiempo real fue realizada en un aparato MyiQ™ (BIO-RAD) usando SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara). Las mezclas de reacción fueron incubadas durante 10 min a 95°C, seguido de 40 ciclos de 15 s a 95°C, 1 min a 60°C, y finalmente 15 s a 95°C, 1 min a 60°C, y 15 s a 95°C. Para cada ARN, la expression génica fue corregida por la protein ribosónica

S11 (*rps11*) contenida en cada muestra usando el método *Ct* comparativo ($2^{-\Delta\Delta}$*Ct*). En todos los casos, cada PCR se llevó a cabo con cada muestra por triplicado y fue repetida al menos con dos muestras independientes.

Análisis estadístico

Los datos fueron analizados mediante el análisis de la varianza (ANOVA). Las diferencias entre dos muestras se analizaron mediante el Test-*t* de Student. El Test Log-Rank fue usado para determinar las diferencias estadísticas en las curvas de supervivencia de distintos grupos experimentales.

4. Resultados y discusión

La telomerasa humana, una la transcriptasa inversa (hTERT), que regula la longitud de los telómeros, puede promover el desarrollo del tumor. La telomerasa se reactiva en el 90% de todos los cánceres (Harley, 2008). En aproximadamente el 15% de los cánceres humanos, la longitud de los telómeros se mantiene independientemente de la telomerasa por el alargamiento alternativo mediado por la recombinación homóloga de los telómeros (ALT) (Zheng et al., 2017) La mayoría de las investigaciones sobre hTERT se han centrado en su crucial función del mantenimiento de los telómeros. Sin embargo, hay muchos fenómenos que no pueden ser explicados por su actividad de transcriptasa inversa. Muchos son los datos que sugieren que hTERT tiene funciones independientes de su función protectora en los extremos telómero, como el aumento de la capacidad anti-apoptótica de las células, mejorar la reparación del ADN, el mantenimiento de las células madre y la regulación de la expresión génica.

En el cáncer humano, la alta expresión de telomerasa está correlacionada con la agresividad tumoral y el potencial metastásico. Los pacientes con cáncer metastásico sufren la mayor tasa de muerte relacionada con el cáncer, pero los modelos animales existentes de metástasis tienen desventajas que limitan nuestra capacidad de entender este proceso. El pez cebra se utiliza cada vez más para el modelado de cáncer, particularmente el xenotrasplante de líneas celulares de cáncer humano, y el descubrimiento de fármacos, y puede aportar nuevos conocimientos científicos y terapéuticos

Tomando ventaja del ensayo de xenotrasplante en el pez cebra, queremos comprobar si la sobreexpresión de hTERT en la célula SAOS 2, (una línea celular de sarcoma osteogénica primaria que utiliza el sistema ALT para mantener el telómero y no expresa hTERT endógena), confieren más invasividad. Hemos demostrado que h-TERT aumentó la invasividad en HTERT-SAOS en comparación con la línea celular de control, pBABE-SAOS

MicroRNAs desempeñar papeles clave en la metástasis tumoral. Varios estudios han demostrado que los microRNAs pueden regular una serie de factores de transcripción relacionados con el tumor que están involucrados en la invasión y proliferación de tumores. Utilizamos el análisis de los microARN diferencialmente expresados para analizar los cambios de expresión génica y rutas moleculares en células hTERT-SAOS y

pBABE-SAOS. El análisis ha demostrado la sobre-expresión de un microRNA llamado mir500a.

Curiosamente, la sobreexpresión de mir 500 en ambos SAOS y HTERT-SAOS aumentó la invasión en xenotrasplante de larvas de pez cebra, lo que indica que la participación de este microRNA en metástasis per se. Por otro lado, la inhibición de mir 500 produjo una disminución en la invasión in vivo sólo en células hTERT-SAOS que la sobreexpresaron. No tiene efecto sobre el pBABE-SAOS. Estos resultados han demostrado tener implicaciones clínicas, ya que existen enfoques terapéuticos utilizando miRNAs (Hung et al., 2014, Shah y Chen 2014, Ye y Cao 2014)

Ha sido publicado que MiR-500 está regulado positivamente tanto en las células de cáncer de próstata como en los tumores primarios. En pacientes con cáncer de próstata, la alta expresión de miR-500 (Cai B1, et 2017) se asocia con mal pronóstico y supervivencia global. Es importante destacar que miR-500 es sobre-expresado en el cáncer gástrico y que que regula un mecanismo novedoso para la activación constitutiva NF-κ B y estaba altamente correlacionada con la progresión maligna y la pobre supervivencia

Aunque mucho se ha publicado sobre mir500, nada se ha hecho acerca de cómo la regulación de mir500 expresión por hTERT. Se ha demostrado que la telomerasa regula directamente la expresión de genes específicos pertenecientes a la vía de señalización de NF-κB oa la vía Wnt / β-catenina (Choi et al, 2008, Ghosh et al., 2012), participa en la reparación del daño del ADN , Y promueve la supervivencia celular bajo condiciones de estrés oxidativo o de estrés endoplásmico. Adicionalmente, se ha demostrado que hTERT protege a las neuronas en desarrollo de la muerte celular inducida por el daño del ADN (Fu et al., 2000). TERT también regula la función mitocondrial y el metabolismo celular (Haendeler et al., 2009). Entre las actividades independientes de los telómeros de hTERT, el papel de hTERT en la transcripción de genes se ha investigado en detalle. Varios estudios han demostrado una participación directa de hTERT en la transcripción de genes

Los mecanismos detallados de la regulación de miRNA mediada por TERT serán estudiados en esta Tesis

Lassmann et al. (2015) han descrito que TERT está implicado en la regulación de la expresión de microARN. Este estudio revela que la deleción de TERT conduce al descenso de microRNAs expresados en células THP-1 (línea celular de leucemia

monocítica humana) y HeLa, por lo que los autores concluyen que TERT parece actuar como un regulador positivo de la expresión de microRNAs. De la misma manera, Drevytska et al. (2014) describieron una correlación positiva entre los niveles de expresión de TERT y los niveles de varios microRNAs. Sin embargo, hasta la fecha se desconoce si TERT se une directamente a los promotores de los genes de microRNAs o si regula la transcripción de estos indirectamente, por ejemplo a través de la inducción de factores transcripcionales, según lo propuesto por Lassmann et al. (2015). Además, dado que el 60% de los promotores de microRNAs están formados por islas CpG y tienen patrones de modificación de histonas específicas, otra teoría se ha propuesto es que TERT podría contribuir a la regulación epigenética de los promotores de microRNAs. Se realizó un experimento de ChIP para comprobar la capacidad de TERT para unirse a la región de DNA aguas arriba de mir500. Interesantemente el anticuerpo de TERT podría inmunoprecipitar una región muy cerca de la localización de mir500 La clonación de esta región que dirige la expresión del gen de la luciferasa mostró que este fragmento estaba regulado por TERT, por lo tanto su sobreexpresión aumentó la expresión de la luciferasa y sin embargo la inhibición de la telomerasa por el siRNA disminuyó la actividad luciferasa. . Sorprendentemente, la misma activación se obtuvo cuando se utilizó una versión mutante, dominante negativo , de la telomerasa que carece de actividad enzimática. Apoyando definitivamente que Telomerase actúa su función reguladora en mir 500 independientemente de su actividad enzimática

Debido a que hay un mayor aumento de hsa-mir-500a en células con alta expresión de hTERT, relacionado con el resto de miRNAs, podría ser que la telomerasa que está regulando de alguna manera la transcripción de este miARN concreto o su estabilidad. Se ha informado de que TERTafecta ampliamente los niveles de expresión de microRNAs maduros. La supresión de TERT resultó en la regulación negativa de microRNAs expresados en células THP-1 y células HeLa (Lassmann et al., 2015). Los niveles de microARN primarios y precursores también se redujeron bajo la supresión de TERT. Se obtuvieron resultados similares con la supresión de BRG1 (también llamada SMARCA4) o nucleostemin, que son proteínas interactuando con TERT y funcionando más allá de los telómeros. Estos resultados sugieren que TERT regula microRNAs en las fases muy tempranas en su biogénesis, presumiblemente a través de mecanismo (s) no dependientes de la actividad telomerasa.

MicroRNA (miRNA) regula diversos mecanismos biológicos y metabolismos en plantas y animales. Por lo tanto, los descubrimientos de miRNA ha revolucionado las ciencias de la vida y la investigación médica. El miRNA reprime y escinde el ARNm dirigido vinculando pares de bases complementarios perfectos o casi perfectos o imperfectos por la formación de RIC inducida por RNA durante el proceso de biogénesis. Un miRNA interactúa con uno o más genes mRNA y viceversa, por lo tanto, participa en la causa de diversas enfermedades. Utilizamos diferentes bases de datos de microARN para predecir y estudiar las anotaciones funcionales del objetivo previsto., posteriores validadcions fueron realizadas mediante PCR cuantitativa

Se ha informado que la sobreexpresión de miR-500 juega un papel importante en la progresión del cáncer gástrico y que miR-500 es un activador crítico de la señalización de NF-κB.

Entre todas las rutas, se prestó especial atención a las que estaban relacionadas con el cáncer, como las vías de señalización Hedgehog, Wnt / β-catenina y IL6 (interleuquina 6). Después de analizar las tres rutas mencionadas anteriormente, decidimos que los siguientes genes objetivo fueron seleccionados, a saber: CUL3 (cullin 3), GLI3 (dedo de zinc de la familia GLI 3) y PTCH1 (Patched 1) de la ruta de señalización de Hedgehog; SKP1 (proteína 1 asociada a la quinasa en fase S) y TLE4 (potenciador del tipo 4 de la transducina de la división 4) de la vía de señalización Wnt / β-catenina; Por lo tanto, si el miR-500 realmente inhibe estos genes, habrá una desregulación de las rutas moleculares de Hedgehog, Wnt / β- Catenina y IL6, lo que conduce a la progresión del cáncer.

Tras nuestro análisis, pudimos observar que todos los genes inicialmente seleccionados, que finalmente resultaron ser directos de los miR-500 fueron *CUL3, GLI3* y *PTCH1*, del Via Hedgehog; *SKP1* y *TLE4*, de la vía Wnt / β-catenina; *NR3C1*, de la vía *IL6;* y *MTDH* y *OCLN*, que participan en la adhesión celular, ya que los niveles de expresión de estos genes disminuyó cuando el miR-500 fue overexpressed. Sin embargo, nuestros resultados demuestran que el gen IL6R no era un objetivo directo de miR-500, ya que la sobreexpresión de este mirRNA se correlaciona con una disminución en la expresión de dicho gen. Estos resultados demuestran que miR-500 desempeña un papel oncogénico en la progresión del cáncer a través de la regulación de Hedgehog y Wnt / β-catenina vías, y la adhesión celular, ya que la regulación

Finalmente, los resultados de la expresión relativa de hsa-mir-500a (Fig. 15a y 15b) muestran que el tejido tumoral metastático tiene una expresión significativa de mir500 (figura 15c) mayor que el tejido de tumor no metastático y el sano

AntagomiR-500 inhibió la tumorigenicidad de las células de cáncer gástrico. Es importante destacar que también encontraron que la expresión de miR-500 fue marcadamente sobreexpresada en tejidos de cáncer gástrico, pero se mantuvo comparativamente bajo en los tejidos gástricos normales.

Por lo tanto, nuestros resultados junto con otros resultados de los laboratorios sugieren que miR-500 puede representar un prometedor objetivo terapéutico en el cáncer.

5. Conclusiones

Los resultados de este trabajo conducen a las siguientes conclusiones:

1. La sobre-expresión de hTERT en células de osteosarcoma hTERT-SAOS ,que mantienen su telomeros por medio del mecanismo alternativo, incrementa su agresividad en los experimentos de xenotransplante de pez cebra con respecto a un control pBABE-SAOS
2. La sobre-expresion de hTERT cambia la expresión de un set de microRNAS, entre ellos miR500a

3. miR-500a tiene un papel fundamental en invasión, ya que su sobre-expresión o ihbición aumenta la invasión o la disminuye respectivamente en experimentos de xenotrasplante en pez cebra

hTERT regula la expresión de mir500 a través de la unión directa a una región aguas arriba del micro RNA 500a . Esa región regula a todo el cluster .(*hsa-miR500a,hsa-miR-362, hsa-mir-500b* & *hsa-miR-502)* excepto a *hsa-miR532* que se encuentra aguas arriba de la región

4. La región reguladora aguas arriba del cluster a la que se une hTERT tiene actividad

5. Se han identifcado algunas de las dianas de mRNA por las que miR500aª actuaría en cácncer. Estas dianas están implicadas en las rutas moleculares tan importantes y decisivas en cáncer como Hedgehog y Wnt / β- Catenina.

6. mir500a constituye un marcado de agresividad y puede ser utilizado tanto para pronóstico como diana terapética

7. El pez cebra es un modelo excepcional para predicir grado de invasividad de las células