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Snail1 controls telomere integrity and transcription and telomerase expression

Dissertation presented by

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Work carried out under the supervision of Dr. Antonio García de Herreros and Sílvia Canudas Puig in the Epithelial to Mesenchymal Transition and Tumor Progression Group, the Cancer Research Program, Institut Hospital del Mar d'Investigacions Mèdiques (IMIM)

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Temps,

Només un moment,

D'abraçar la gent

És curt però és intens

És qüestió de temps.

Temps,

D'ensenyar les dents

De cantar el que sents

D'encendre el present

Val molt més

que l'or que tens

TOTHOM HO SAP

A la meva mare

A la maternitat,

per haver-me revolucionat la vida

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ABSTRACT

Snail1 transcription factor is the key inducer of epithelial-to-mesenchymal transition (EMT) and controls tumor invasion, resistance to apoptosis and the maintenance of cancer stem cell features. In this thesis we describe a novel role for Snail1 on the regulation of telomere integrity and transcription as well as telomerase expression. An analysis of telomere integrity by fluorescence in situ hybridization (FISH) indicates a dramatic increase of telomere alterations in Snail1-depleted mouse mesenchymal stem cells (MSC) and shorter telomeres. However, these cells present higher levels of TERT since Snail1 represses its expression, meaning that other mechanisms in telomere homeostasis are involved. In fact, telomeres are actively transcribed into a long non-coding RNA called telomeric repeat-containing RNA (TERRA). Although TERRA plays crucial a role in telomere homeostasis, little is known about how its transcription is regulated. Here we report that Snail1 transcription factor regulates TERRA transcription by repressing TERRA 2q, 11q and 18q. TERRA and TERT are transiently down-regulated during TGFβ-induced EMT in NMuMG cells correlating with Snail1 expression. Ectopic expression of TERRA affects the transcription of some genes induced during EMT such as fibronectin whereas TERT does not modify those genes. We propose that Snail1 control of TERRA besides being required for telomere maintenance is also necessary for the expression of a subset of mesenchymal genes.

RESUM

El factor de transcripció Snail1 és el principal inductor de la transició epiteli-mesènquima (EMT) i controla la invasió tumoral, la resistència a apoptosi i el manteniment de les característiques de les cancer stem cells. En aquesta tesi descrivim un nou paper de Snail1 en la reglulació de la integritat telomèrica i de la seva transcripció així com de l'expressió de la telomerasa. L'anàlisi de la intergritat telomèrica usant la tècnica d'hibridació fluorescent in situ (FISH) mostra un increment dramàtic d'aleracions telomèriques en en MSCs deficients de Snail1. En canvi, aquestes cèl·lules presenten nivells més elevats de TERT ja que Snail1 en reprimeix la seva expressió. Això significa que hi ha altres mecanismes que regulen l'homeòstasi telomèrica. De fet, els telòmers es transcriuen de forma activa en uns RNA no codificants llargs anomenats "telomeric repeat-containing RNA" (TERRA). Malgrat TERRA té un paper molt important en l'homeòstasi telomèrica, la regulació de la seva transcripció és un mecanisme força desconegut. Aquí demostrem que Snail1 regula la transcripció de TERRA reprimint el TERRA 2q, 11q i 18q. L'expressió de TERRA i TERT disminueix de forma transitòria durant EMT induïda per TGFβ en les cèl·lules NMuMG, correlacionant-se amb l'expressió de Snail1. L'expressió ectópica de TERRA afecta la transcripció d'alguns gens induïts durant l'EMT, com la fibronectina, mentres que TERT no modifica l'expressió d'aquests genes. Proposem que el control de TERRA per part de Snail1, no només és necessari pel manteniment telomèric sinó també per l'expressió d'un grup de gens mesenquimals.

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ACRONYMS AND ABREVIATIONS

53BP1: p53 binding protein 1
ALT: alternative lengthening of telomeres
ATM: Ataxia Telengiectasia Mutated
ATR: Ataxia Telengiectasia and rad3 related
Bp: Base pair
CAFs: Cancer-Associated Fibroblasts
Casp: Caspase
CCL: Chemokine (C-C motif) ligand
cDNA: Complementary Desoxyribonucleic Acid
ChIP: Chromatin immunoprecipitation
CHK2: Checkpoint Kinase 2
Cre: Circular Recombinase Especific
CSCs: Cancer Stem Cells
DAPI: 4',6-diamidino-2-phenylindole
DKC1: Dyskeratin 1
DMEM: Dulbecco's modified Eagle's medium
DNA: Desoxyribonucleic Acid
DDR: DNA Damage Response
DSB: Double Strand Break
E2F: Transcription factor family including E2F- and DP-like subunits
ECM: Extracellular Matrix
EDTA: Ethylenediaminetetra-acetic acid
EGF: Epidermal Growth Factor
EMT: Epithelial to Mesenchymal Transition
FBS: Fetal Bovine Serum
FC: Fold Change
FGF: Fibroblast Growth Factor
FISH: Fluorescence in situ Hybridization
gDNA: Genomic Desoxyribonucleic Acid
GFP: Green Fluorescent Protein
HGF: Hepatocyte Growth Factor
HIF: Hypoxia-Inducible Factor
HP1: Heterochromatin Protein 1
HR: Homologous Recombination
IgG: Immunoglobulin G
IL: Interleukin
Irf: Interferon
Kb: Kilo base
kDa: Kilo Dalton
KO: Knock-Down
LOXL1: Lysil Oxidase-like 1
LOXL2: Lysil Oxidase-like 2

LSD1: Lysine-specific histone demethylase 1A
MDC1: Mediator of DNA-damage Checkpoint 1
MEFs: Mouse Embryonic Fibroblasts
MET: Mesenchymal to Epithelial Transition
MMPs: Metalloproteinases
MSCs: Mesenchymal Stem Cells
NADPH: Nicotinamide adenine dinucleotide phospate
NES: Nuclear Export Signal
NF-κB: Nuclear Factor kappa B
NHEJ: Non-Homologous End-Joining
NHP2: Non-histone protein 2
NOP10: Nucleolar protein 10
PBS: Phosphate Buffer Saline-Tween
PCR: Polymerase Chain Reaction
PD: Population Doubling
PI3K: phosphatidylinositol -3-kinases
PNA: Peptid Nucleic Acid
POT1: Protection of Telomeres 1
PTEN: phosphatidylinositol -3, 4, 5- trisphosphate 3-phosphatase
Q-FISH: quantitative-Fluorescence in sito Hybridization
qPCR: Quantitative Polymerase Chain Reaction
Rap1: Repressor/activator Protein 1
Rb: Retinoblastoma protein
RNA: Ribonucleic Acid
RNA pol II: RNA polymerase II
RPMI: Roswell Park Memorial Institute
RT: Reverse Transcription
SA: Sister Association
SEM: Standard Error Medium
SFE: Signal Free end
SWI/SNF: SWItch/Sucrose NonFermentable
TBS-T: Tris Buffer Saline-Tween
TCAB1: Telomerase cajal body protein
TERC: Telomerase RNA component
TERRA: Telomeric Repeat-containing RNA
TERT: Telomerase Reverse Transcriptase
TGFβ: Transforming Growth Factor-β
TIN2: TRF1-Interacting Protein 2
TNF-α: Tumor Necrosis Factor-α
TPP1: POT-TIN2 organizing protein 1
TRAP: Telomeric Repeat Amplification Protocol
TRF: Telomeric Restriction Fragment
TRF1: Telomeric Repeat Binding Factor 1
TRF2: Telomeric Repeat Binding Factor 2
WB: Western Blot
WNT: Wingless-related integration site
WT: Wild Type
βCre: Cre Recombinases under βActin Promoter

INTRODUCTION

1. CANCER

1.1. Overview

According to the World Health Organization, cancer is a leading cause of morbidity and mortality worldwide. In 2012, 14 million of new cases were diagnosed and 8.2 million cancer related deaths were reported. The number of new cases is expected to rise by about 70% in the next two decades [1].

The regulation of cell division and multiplication is very important to ensure the correct response to the body, achieving the homeostasis of the organism [2]. When this balance is disrupted due to mutations in somatic cells that affect key genes involved in the regulation of cell proliferation and survival, cancer appears [3]. Consequently, cells begin to proliferate very rapidly and acquire malignant features. Tumorigenesis is a multistep process consequence of several somatic mutations. The most frequently mutated genes (onco-suppressors and oncogenes) in cancer cells are PI3K, Ras, p53, PTEN, Rb and $p16^{INK4a}$. Moreover, there is a large number of low-frequency changes that also contribute to tumorigenesis [4]. In the last decades, a lot of work has been put in studying these mutations and how them provide to cancer cells features that allow them initiate the tumor and make it progress [5].

Even though there are several types of cancer described until now, they share some characteristics. In the past years, several common hallmarks of cancer have been proposed. In fact, Hanahan and Weinberg described six essential characteristics in 2000, which are

the following: self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential, sustained angiogenesis, evasion to apoptosis, and tissue invasion and metastasis [6]. In the last years, four more features have been added: reprogramming of cellular energy metabolism, the evasion of the immune system, tumor-promoting inflammation and genomic instability and mutability [5,7,8] (Figure 1).

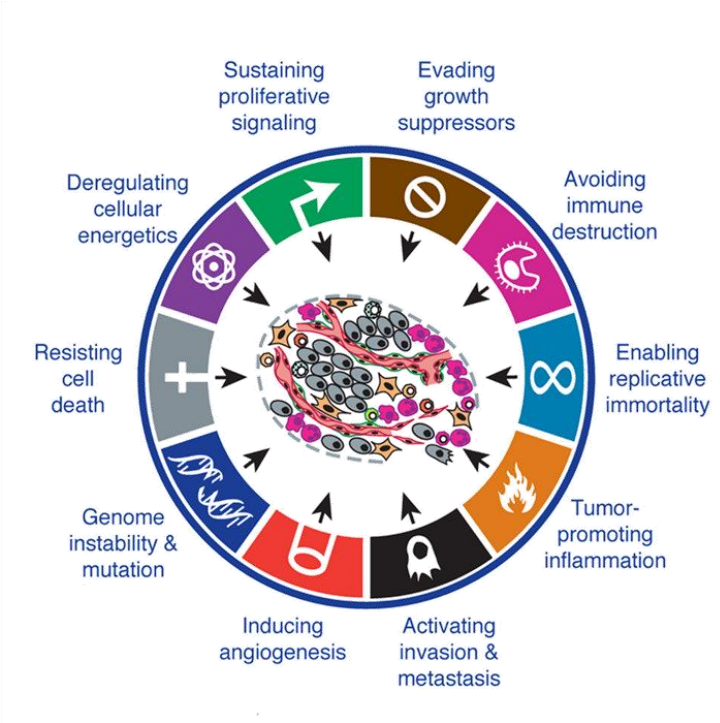


Figure 1. The hallmarks of cancer. Schematic illustration of the hallmarks of cancer proposed by Hanahan & Weinberg (2011) [5].

1.2. The Origin of Cancer

Although the effort to understand tumorigenesis, nowadays it is not clear the mechanism of tumor initiation and progression. There are

two main hypothesis, which are the clonal expansion model and the cancer stem cell hypothesis. Both models propose that tumors originate from a single cell that accumulate mutations and gain unlimited proliferative potential, but through different mechanisms [9]. The clonal evolution model postulates that when cancer cells acquire genetic alterations, their progeny is expanded as a neoplastic clone; afterwards, they acquire growth advantages and are selected and expanded to become the predominant subpopulation inside the tumor. Based in this model, any tumor cell that acquires the capacity of self-renew has the potential to contribute to tumor progression [10]. On the other hand, the cancer stem cell hypothesis postulates that mutations are accumulated in a subset of tumor cells with stem cell-like properties called “cancer stem cells” (CSCs), that give rise differentiated cells carrying the mutation, and also drive tumor initiation, progression and recurrence [11,12].

Moreover, another classification of cancer can be done considering the cell type where the mutation is originated. Most cancers (80-90%) originate from the epithelium and are called carcinomas, differentiating between the adenocarcinomas (developed from gland) and squamous cell carcinoma (developed in squamous epithelium) [13]. Cancer can also develop from mesenchymal tissue, differentiating into bone, muscle, fat and cartilage. It is postulated that in this case, cancer could originate from Mesenchymal Stem Cells (MSCs), which are multipotent cells that can lead or differentiate into these different mesenchymal tissues [14]. Some neoplasias also originate from blood cells precursors; among these

leukemia, originated in the immature leukocytes of the bone marrow; multiple myeloma, that arises from uncontrolled clonal growth of the plasma cells in the bone; and lymphoma that develops in the lymphatic system [13,15,16].

2. TELOMERES

2.1 Introduction

Telomeres are repetitive DNA elements that protect the ends of linear chromosomes from catastrophic damage. Telomeric repeats are assembled into a dynamic nucleoprotein complex and the 3' single-stranded overhang located at the end of chromosomes providing a protective cap (Figure 2).

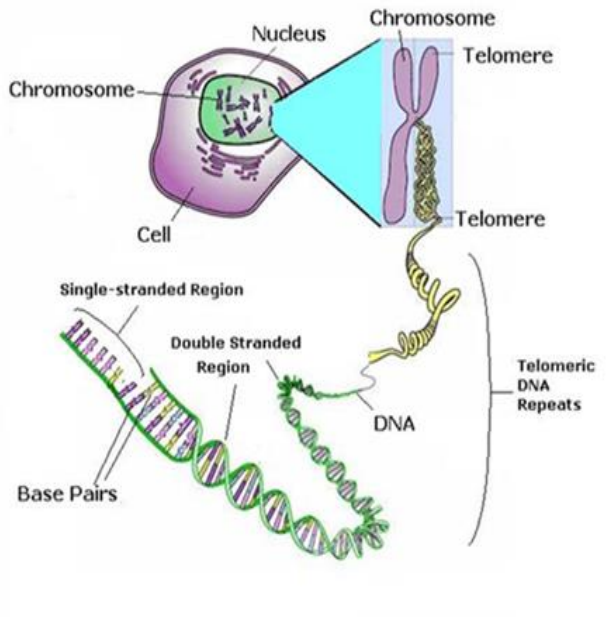


Figure 2. Telomeres are located at the ends of linear DNA. Telomeres are found at the termini of chromosomes, consisting of repetitive units of TTAGGG in vertebrates.

Telomeres are essential in normal cells for the genomic integrity maintenance and to protect chromosome ends to be recognized as DNA double-strand-breaks (DSB) [17]. Due to the linear structure of mammalian chromosomes, cell division and proliferation progressively shortens telomere length; stress conditions can increase the shortening rate [18-20]. When telomeres get to a critical length, the protective function cannot be provided, and cells get in a process of senescence that can be bypassed in some pathologies such as cancer among others [21-24]. Therefore, telomere shortening induces chromosomal instability that, in the absence of functional tumor suppressor genes, contribute to tumorogenesis.

2.2. Telomere Structure

Mammalian telomeres consist of TTAGGG DNA tandem repeats [25, 26] and a six-subunit complex (TRF1, TRF2, TIN2, TPP1, POT1, Rap1) known as shelterin. These telomere sequence specific binding proteins bind to both the single and the double telomere stranded forms, enabling the formation of higher specialized structures. Shelterin regulates the maintenance of telomere length and protects the ends to be recognized as damaged DNA [27,28]. Both the sequence DNA motif and shelterin are required for genomic protection and stability [29].

The telomere repeat length shows individual variation between species: in human is considered to be around 10-15 kilobases (kb) for each chromosome [25], but in mice they are longer, achieving up to 80 kb [30-32].

Short telomeres block the proliferative capacity of stem cells, affecting their potential to regenerate tissues, and trigger the development of age-associated diseases including cancer. Some evidence suggest that telomere length is inherited: mutations in telomere maintenance genes are associated with pathologies referred to as telomere syndromes, including Hoyeraal-Hreidarsson syndrome, Dyskeratosis Congenita, pulmonary fibrosis, aplastic anemia, and liver fibrosis. Dyskeratosis Congenita is a rare progressive congenital disease characterized by short telomeres, where the offspring of patients with this disease have increasingly short telomeres in next generations [33,34]. Moreover, in some mice models it has been demonstrated that telomere length is genetically determined for a given species [35]. Furthermore, studies in twins, have shown that telomere-size variation is genetically determined to a large extent [36]. However, nutrition and lifestyle are known to modulate aging process and age-related diseases affecting telomere length with a great impact on healthspan [37,38].

2.2.1 T-loop

Eukaryotic telomeres normally terminate with 3'-G rich single-stranded-DNA overhang, which is essential for telomere maintenance and capping since it prevents from the recognition of the chromosome ends as DSB. In order to be protected, telomeres form large loop structures called telomere loops or T-loops, in which the single-stranded DNA curls around in a long circle, and is stabilized by telomere-binding proteins. At the very end of the T-loop, the single stranded telomere DNA invades the double-stranded region, thus creating an internal D-loop [39] (Figure 3). This "cap" structure has a dynamic behavior during the cell cycle, particularly during S-phase, when telomeres undergo replication [40]. T-loop

size is proportional to telomere length [41]. In fact, human T-loop mice is 3 kb long, whereas in mice is 18 kb [29], reflecting their different telomere length [30-32].

2.2.2. Shelterin

As it has been commented before, telomeres are further stabilized by a protein complex called shelterin [41], which is necessary for telomere integrity. Shelterin comprises DNA binding proteins as well as protein-protein components that interact to telomeric sequences. It is composed of six proteins:TRF1 and TRF2 (telomeric repeat binding factor 1 and 2), POT1 (protection of telomeres 1), TIN2 (TRF1-interacting protein 2), TPP1 (POT1-TIN2 organizing protein, also referred to as TINT1, PIP1 and PTOP), and Rap1 (repressor/activator protein 1). TRF1, TRF2 and POT1 bind directly to telomeric repeats: TRF1 and TRF2 bind to the double-stranded portion of telomeric DNA and enable the formation of the T-loop [41,42], while POT1 binds to the single-stranded overhang, which is important for the formation of the D-loop [39,43-45] (Figure 3).

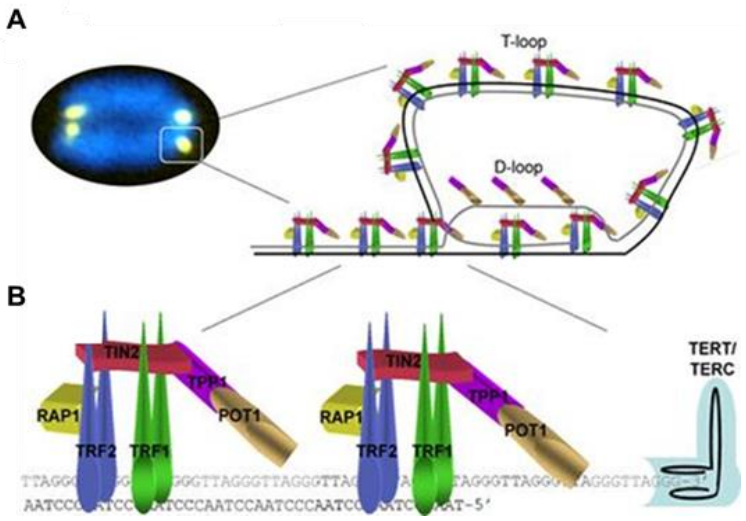


Figure 3. Secondary structure of telomeres. A) Formation of the T-loop and internal D-loop secondary structures of telomeres. **B)** Shelterin complex is involved in the formation and stability of T-loop and D-loop.

Even though human cells have only one POT1 gene, mice have two variants that are highly homologous (POT1a and POT1b) although the role at telomeres is distinct [46,47]. These three proteins recruit three additional proteins: TRF2 recruits Rap1, while TIN2 associates with both TRF1 and TRF2 as well as with the TPP1, which in turn is a POT1-binding partner, required for POT1 binding to the telomere overhang [45]. Overall, the shelterin complex is involved in telomere stability and telomere length regulation [43]. Specifically, TRF1 plays a role in telomere length and TRF in telomere protection. Mutations in shelterin components have also been found in cancer. Several studies have reported up-regulation of the shelterin complex TRF1 and TRF2 in lung, gastric, breast and renal cancers, suggesting that their expression might confer proliferative advantages to tumor cells.

However, the role of TRF1 and TRF2 in cancer development and progression is still unknown [48]

2.3. Telomere Function

Telomeres (TTAGGG DNA repeats + shelterin) are considered as a telomeric “cap” and they play a fundamental role due to the linear nature of human chromosomes. Over the years, it has been described the important role that telomeres have in the stability and mobility of the genome and how telomeres prevent the erosion of coding DNA. In addition to its protective role, the telomere has also been hypothesized to serve as a molecular clock that counts the number of cell divisions and limits further divisions at a predetermined point.

2.3.1. Genomic Stability

Linear DNA fragments are a problem to mammalian cells, and effective mechanisms have evolved to deal with them. Therefore, telomere structure needs to be distinguished from DSBs and do not activate the activation of DNA damage response (DDR). This mechanism provides protection from homologous recombination (HR) and non-homologous end-joining (NHEJ). Therefore, when telomeres are unmasked, genome instability arises [49]. Critically shortened telomeres or missing shelterin components can activate the DDR [50] and the up-regulation of cell cycle checkpoints such as p53 [51-53]. In some cells, cell cycle checkpoints can be by-passed increasing the risk of neoplastic transformation.

2.3.2. End-replication Problem

Cells go through a finite number of cell divisions before undergoing replicative cellular senescence, and it is known as the Hayflick limit [54,55]. Olovnikov and Watson proposed that this limited number was due to the fact that linear chromosomes cannot replicate their ends, termed as the “end-replication problem” [56,57], since the DNA polymerase can only replicate in the 5’ to 3’ direction and requires a short RNA primer for initiation [58]. As DNA is double stranded, one strand will be synthetized in short “okazaki” fragments, but at the 5’ end of the lagging strand, there will be a gap due to removal of the last RNA primer [58] (Figure 4). Successive cellular proliferation leads to progressive shorter telomeres, and as somatic cells are unable to correct for this shortening, telomere reduces to a critical length that leads to cell senescence and apoptosis [59]. It is known that cells lose telomeres at a constant rate [60]. However, it has been demonstrated the heterogeneity in the number of cell divisions that a cell can undergo [61,62] and in the rate of telomere shortening [63].

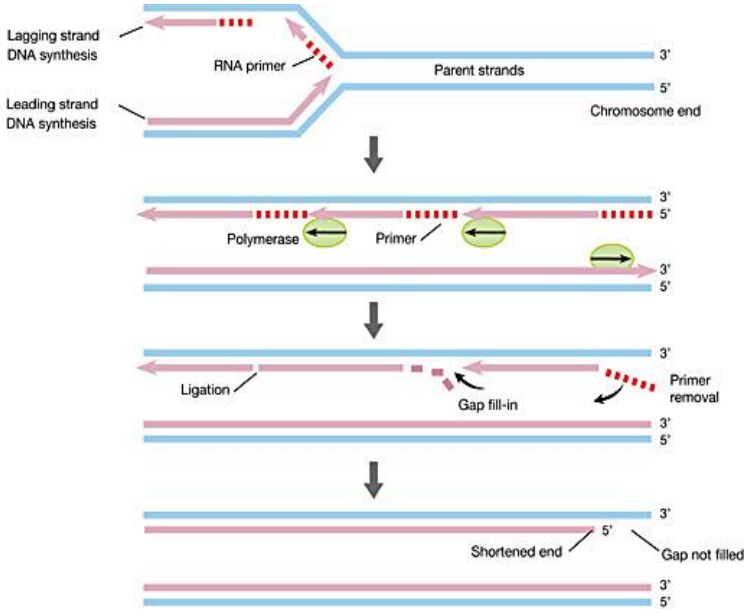


Figure 4. The end-replication problem. Schematic representation of the end-replication problem, where the DNA polymerase can only replicate in the 5' to 3' direction and requires a short RNA primer for initiation.

2.4. Telomeres and Cell Senescence

In some age-related diseases and also with age, senescent cells have been shown to accumulate in mammalian tissue, suggesting that they might contribute to the loss of tissue function observed with age. This phenomenon is characterized not only by a loss in replicative capacity, but also by a series of dramatic changes in cell morphology, gene expression, metabolism, epigenetics and others [64]. One of the main mechanisms by which cells go to senescence is telomere shortening and dysfunction. During each cell division, telomeres shorten by~ 50-200 base pairs (bp) due to the end

replication problem [65,66], which leads telomeres to reach a critical length that activates a DDR. The subsequent processing of dysfunctional telomeres is almost identical to the canonical DDR at intra-chromosomal break sites. In that response, protein kinases (ATM, CHK2), adaptor proteins (53BP1 and MDC1) and chromatin modifiers (ɣ-H2AX), a variant of histone H2A that localizes to sites of DNA damage, are involved [67]. This telomere-dependent activation of DDR leads to the activation of p53 and up-regulation of its downstream target gene p21 [65,67], that at last inhibits cell cycle progression through the activation of pRB that in turn, inactivates the transcriptional factor E2F. E2F induces the expression of genes responsible for cell-cycle progression. Thus, the repression of E2F induces cells to enter to a state of permanent cell cycle arrest (senescence) [65,67]. Dysfunctional telomeres appear to function as a potent tumor suppressor by involving cellular pathways that activate replicative senescence and/or apoptosis to inhibit tumor formation. Therefore, it is not surprising that if any of these cell cycle checkpoints are by-passed normally due to mutations (most frequently p53), the cell will continue to divide and telomeres will shorten until a crisis phase is reached. p53 loss results in a permissive environment that favors proliferation and survival of genomically damaged cells and the eventual progression to cancer.

Here, chromosome fusions occur due to the activation of the NHEJ pathway [68]. It has been described that a key factor involved in senescence and apoptosis is that cells with shortened telomeres cannot form a closed T-loop. In this direction, TRF2 inhibition *in vitro* causes the activation of DDR and senescence [69]. Moreover, it is not known whether the telomere length limit that induces senescence in humans and in mice is different. It is thought that

mice, with an average T-loop size 6-7 times greater than in humans [43], require longer telomeres to maintain a closed T-loop structure.

2.5. Telomerase

2.5.1 Introduction

In order to overcome the problem of telomere shortening, cells have developed different mechanisms. The most common one depends on telomerase, a reverse transcriptase discovered by Dr. Elizabeth Blackburn and Dr. Carole Greider [70,71]. Another less common mechanism to overpass telomere shortening is the alternative lengthening of telomeres (ALT), a recombination-based mechanism for telomere elongation that was discovered in telomerase negative cancer cells [72,73].

2.5.2. Structure and Function

Telomerase is a reverse transcriptase that synthesizes telomeric DNA de novo using integral RNA as the template. The core components of telomerase are the reverse transcriptase (TERT) and telomerase RNA component (TERC). Telomerase is associated with a set of accessory proteins including dyskeratin (DKC1), nucleolar protein 10 (NOP10), non-histone protein 2 (NHP2), GAR1 and telomerase Cajal body protein 1 (TCAB1), that contribute to the biogenesis and trafficking of telomerase inside the nucleus [65] (Figure 5).

Many proteins function in order to direct recruitment of telomerase. In that direction, TERT prefers to elongate shorter telomeres [74] due to the fact that longer telomeres contain more shelterin proteins that

may repress the access of telomerase to telomere ends.

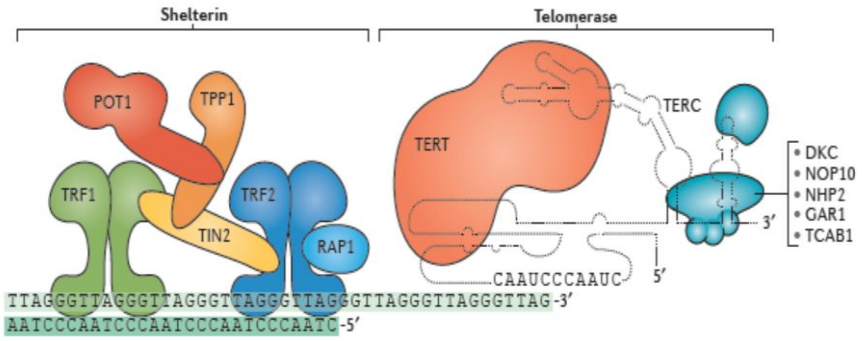


Figure 5. Telomerase structure. Telomerase is a ribonucleoprotein complex formed of a catalytic reverse transcriptase core protein (TERT) and a RNA molecule that acts as a template for the addition of telomeric repeats “de novo” to the 3’ end of telomeres. Adapted from [75].

It is important to consider that the expression and activity of TERT is tightly regulated at different levels: transcription, splicing, post-translational modifications and subcellular localization [76,77], and it is the rate limiting component of telomerase activity. In that direction, transcriptional control of TERT is supposed to play an important role in the regulation of telomerase activity. Ten different splice variants of TERT have been described [78-81]. Telomerase activation during tumorigenesis is often accomplished through mutations in the TERT promoter [82]

Telomerase is ubiquitously expressed in the developing embryo but its expression is down-regulated after embryogenesis in most somatic cells [83]. Thus, somatic cells do not have the capacity to divide indefinitely. Telomerase is not expressed homogenously in all

the cells: highly proliferative tissues such as activated lymphocytes, germ cells as well as some stem cell populations have higher telomerase activity; however, telomeres still shorten in these cell types meaning that it is insufficient to solve the end-replication problem [84]. Moreover, telomerase expression in somatic cells is different between species. Telomerase is still expressed to some degree in mice somatic cells whereas not in somatic human cells [85,86].

2.5.3. Non-canonical Telomerase Functions

It is well established that telomere length maintenance is necessary for malignant cells to achieve infinite proliferative potential during oncogenesis, and reactivation of telomerase expression is a critical step in transformation. It is important to notice that TERT expression is highly associated with risk of cancer. It has been described that telomerase is expressed in over 85% of human tumors [86].

In addition to its requirement in cancer development by maintaining telomere length, in the last years, accumulating evidence indicates that telomerase is involved in some other biological functions, some of which are independent of the enzymatic activity. These are considered non-canonical functions of telomerase. In oncongenesis, telomerase has been reported to act as a transcriptional factor [87], protecting cells from apoptosis and from DNA damage [87-89], regulating cell survival [90] and in providing stem cell properties independent of telomere elongation [91]

Some of these non-canonical functions of TERT are involved in signaling cascades that influence cancer development and

progression, including the NF-κB and Wnt/β-catenin pathways [87]. Deregulation of the transcription factor NF-κB is implicated in the pathogenesis of inflammation and cancer [92]. Ectopic expression of telomerase results in increased cancer cell proliferation and protection from cell death, which could be mitigated by repressing NF-κB signaling [93]. Moreover, telomerase overexpression promotes enhanced expression of NF-κB target genes. Interestingly, TERT was found to bind to p65 and localize to promoters of a subset of NF-κB target genes, including interleukin IL-6, TNF-α and IL-8, cytokines that promote inflammation and cancer [93]. These findings support a functional interplay between telomerase and NF-κB signaling [94].

Besides NF-κB, telomerase has also been described to regulate the transcriptional activity of the Wnt/β-catenin complex. TERT acts as a co-factor in the β-catenin transcriptional complex through its interaction with Brg1, a SWI/SNF-related chromatin remodeling factor [95].

2.6. Telomeric Transcription (TERRA)

Telomere structure resembles constitutive heterochromatin. In fact, telomeric chromatin contains DNA and histone modifications that are typically associated with constitutive heterochromatin, such as H3K9me3 and HP1 proteins that control chromatin structure and are also important in telomere length maintenance and function [96-98]. Precisely, due to this heterochromatic structure, telomeres can silent expression of genes located to subtelomeric regions [99]. Telomeres are transcribed into telomeric repeat-containing RNA (TERRA) molecules [100,101]. This RNA remains partially associated with telomeres playing important functions [102].

TERRA is a long non-coding RNA that is heterogeneous in length, from 100 bases up to 9 kb in human cells. TERRA is conserved through evolution and is found in all eukaryotes [100,103]. Its transcription is directed by RNA polymerase II (RNA pol II) [104] and initiates in the subtelomeric region, from CpG island-containing subtelomeric promoters, located on average 1kb upstream of TTAGGG repeats [101]. Telomere transcription can be activated in response to developmental changes and cellular stress conditions [100,104].

Even telomere transcription has been intensively studied, TERRA functions are still not clear. It has been suggested that TERRAs act as a scaffold recruiting different factors to chromosome ends and impacting telomeric functions in different ways. TERRA is involved in heterochromatin establishment and maintenance by promoting H3K9 trimethylation [105,106]. Moreover, some *in vitro* as well as *in vivo* studies suggest that TERRA contributes to telomere length regulation: TERRA transcription promotes exonuclease 1-dependent resection at chromosome ends, and inhibits TERT activity [103,104,107-109]. Another proposed TERRA function is the regulation of telomeric replication [106,110-112]. Finally, TERRA has also been proposed to be implicated in promoting telomere end protection [113] and enhances the recruitment of chromatin modifiers to damaged telomeres [114,115].

2.7. Telomeres and Cancer

Telomeres have been studied for a long time, but its role in disease is a relatively new area of research. In 1990 it was proposed that

telomere shortening was related to aging [116], and age related diseases were the first association between telomeres and disease. Some studies among years suggest that short telomeres have an impact on human health [23,117-121]. Nowadays, telomere length has been related to a variety of diseases that include chronic inflammation and infection, cancer, cardiovascular diseases and premature age related syndromes [21,32,122,123]. However, more research is necessary to further elucidate the telomeres mechanisms involved in diseases.

The down-regulation of telomerase in somatic cells and the progressive shortening of telomeres are tumor suppressor mechanisms due to the limitation of cell division [124,125]. Consequently, the telomere tumor suppressor pathway may be a powerful mechanism to limit cancer development. As stated in section 2.4, dysfunction of p53 pathways causes cells to continue to divide until they reach a state of crisis. Dysfunctional telomeres causes genomic rearrangements that can induce up-regulation of oncogenes and finally, promote tumorigenesis [126-128]. Moreover, cancer cells in order to overcome the end-replication problem, reactivate telomerase by up-regulation of its transcription, or use the ALT pathway (Figure 6).

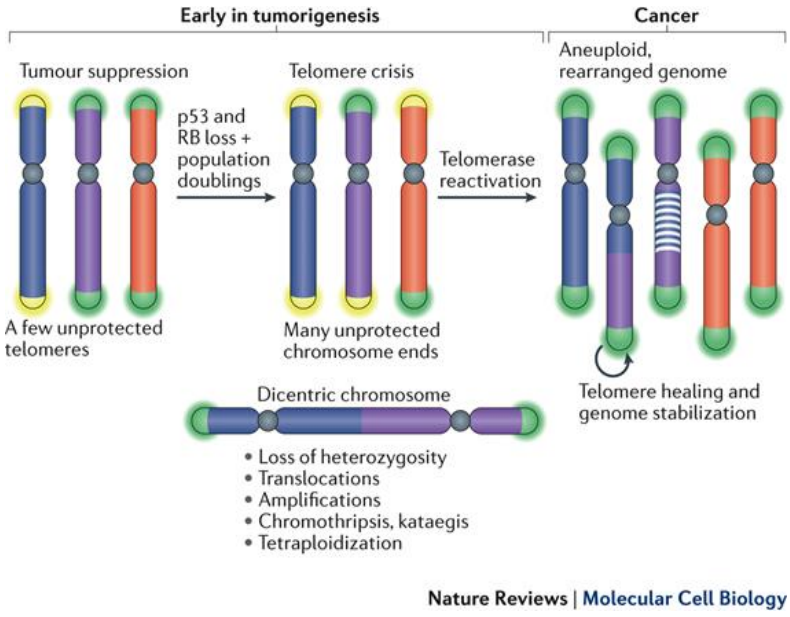


Figure 6. Telomere crisis. Schematic representation of the molecular basis of telomere crisis. Adapted from [75].

Therefore, activation of telomerase provides a path out of telomere crisis, ultimately leading to the formation of cancer clone with a heavily rearranged genome. Noteworthy, telomeres in human cancer cells are often shorten than in normal tissues. It is possible that this setting of short telomere length reflects selection for a telomere length distribution that affords a low level of genome instability without disminishing cell viability.

As stated before, telomerase is highly expressed in around 85% of tumors [86] allowing cancer cells to skip cellular checkpoints and acquire limitless replicative potential giving rise to uncontrollable proliferating cells [86]. For this reason, some research has focused

on telomerase as a target treatment for cancer [129,130]. Moreover, as mentioned in state 2.5.2, it is postulated that non-canonical telomerase functions are involved in cancer progression.

Interestingly, TERRA levels are altered in cancer cells compared to normal cells, and the type of telomere elongation mechanism used also has an influence on the amount of TERRA [131]. Thus, targeting TERRA-mediated regulation of TERT would be a promising therapeutic strategy against cancer and age-associated diseases.

3. THE TRANSCRIPTION FACTOR SNAIL1

3.1. General Characteristics

Snail1 is a zinc-finger transcription factor that belongs to the Snail superfamily of repressors, which is subdivided into the Snail and Scratch families. To date, three members of the Snail family have been described in vertebrates: Snail1 (Snail), Snail2 (Slug) and Snail3 (Smuc). These three transcription factors share a common organization: a highly conserved C-terminal region that contains from four to six zinc fingers of the C_2H_2 type, and a divergent N-terminal regulatory region [132].

Snail1 has four zinc fingers in its C-terminal domain, which act as a DNA binding domain and bind to specific sequences called E-boxes: 5'-CACCTG-3' or 5'-CAGGTG-3' [133,134], located in the promoters of its target genes. Upon binding to DNA, Snail1 (and Snail family members) are thought to act as transcriptional repressors [135,136] (Figure 7).

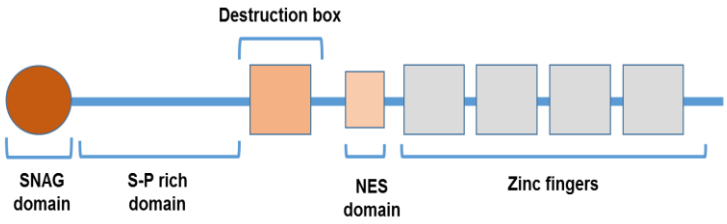


Figure 7. Scheme of Snail1 protein. Graphic representation of Snail1 protein domains. Adapted [137]

The central region of Snail1 is composed by a nuclear export signal (NES), a destruction box domain and a serine-proline rich region [137], and is involved in the protein stability and localization.

Finally, in its N-terminal domain, Snail1 has a SNAG (Snail/Gfi-1) domain, which is responsible for the interaction with co-repressors and repressive activity [133,138]. Through this domain, Snail1 recruits histone deacetylases family members [139], mSin3A, Ajuba LIM proteins [140,141], Polycomb repressive complex 2 [142], and LSD1 [143] among others.

Snail1 expression is regulated through a complex signaling network that acts at the transcriptional and post-transcriptional level. The most studied activators of Snail1 expression are: TGFβ, NOTCH, FGF/EGF, WNT, NF-κB. Moreover, some stress insults such as γ-radiation and hypoxia also up-regulate Snail1 [144-146].

Snail1 is highly and ubiquitously expressed during embryo development. In contrast, in adult tissues its expression is very restrictive, and it has been only described during wound healing [147], fibrosis [148,149] and in some tumors [147,150] such as

ovarian [151], colorectal [147], breast [152,153], gastric [154], sarcoma [155] among others.

3.2. Snail1 and Epithelial to Mesenchymal Transition

EMT is a biological and reversible process by which epithelial cells lose their characteristics and acquire a mesenchymal phenotype. The changes that epithelial cells undergo during this process include: loss of cell tight, adherents and gap junctions as well as desmosomes and cytokeratins; loss of apical-basal polarity; and a rearrangement in their cytoskeleton, in which intermediate filaments are reverted to vimentin from keratins [144,156-159]. The resulting cells are spindle-shaped, motile, more invasive and more resistant to apoptosis (Figure 8). The reverse process, known as a mesenchymal-to-epithelial transition (MET), has also been described [160].

Epithelial and mesenchymal cells differ in various functional and phenotypic features. Epithelial cells have adhesive structures between them, such as adherens junctions, desmosomes, hemi-desmosomes and tight junctions, to establish intercellular adhesions and facilitate intercellular communication. Thus, cellular motility is restricted and individual cells function as a cohesive unit [161]. Epithelial cells have an apico-basal polarization, and the apical and basal surfaces perform different functions. E-cadherin, a transmembrane protein localized to the adherens junctions, is the best characterized molecular marker that epithelial cells express.

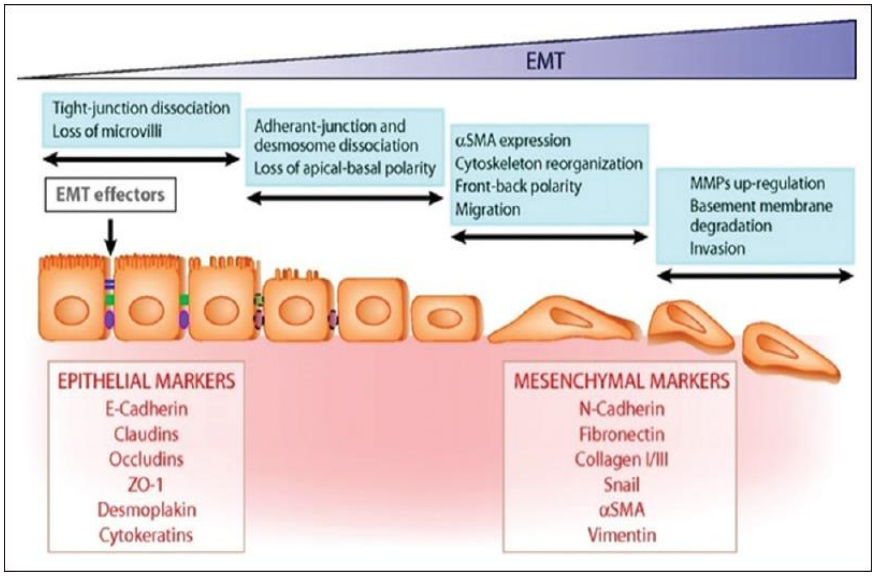


Figure 8. Epithelial and mesenchymal cell traits. The figure summarizes the changes that epithelial cells undergo during EMT.

In contrast with epithelial cells, mesenchymal cells do not have stable intercellular junctions; so they do not form an organized cell layer, nor do they have the apico-basal polarization and the actin cytoskeleton. The contact with its neighboring cells is only focal. Other mesenchymal features are front-to-back asymmetry that facilitates motility and locomotion [159], filipodia at the leading edge, and the expression of matrix metalloproteinases (MMPs) that digest basement membranes and promote invasion [162,163]. Intermediate filaments, such as vimentin, and extracellular components, such as fibronectin and collagen precursors, are increased in mesenchymal cells [164].

From a molecular point of view, the hallmark of EMT is the down-regulation of E-cadherin. Moreover, many other epithelial markers, such as claudins and occludins located in tight junctions, are also down-regulated during EMT [165,166]. These changes in protein expression are associated with changes in transcription [164,167]. Thus, activation and repression of specific genes during EMT are tightly regulated. Several transcription factors have been implicated in the transcriptional repression of E-cadherin, including zinc-finger proteins such as Snail1 [135,136], Snail2 [168], Zeb1 [169] and Zeb2 [170]; Twist and the basic helix-loop-helix factor E12/E47 [146,171]. Among these, Snail1 has been described as the most important inductor of EMT [135,136], since it is rapidly induced by cytokines or stress conditions triggering EMT, and binds and represses the expression of E-cadherin and other epithelial genes [135,172]. Besides its action as transcriptional repressor, Snail1 is also involved in the activation of mesenchymal genes such as Fibronectin [174,175].

Interestingly, recent work in our laboratory has shown that Snail1 and the co-repressor LOXL2 play a role in regulating major satellite transcription and heterochromatin reorganization during EMT [173]. In that direction, HP1α is transiently released from heterochromatin foci concomitantly with a down-regulation of major satellite transcription during EMT; the regulation of this heterochromatin transcription is regulated by Snail1 through LOXL2 creating a favorable transcriptional scenario necessary for a complete EMT. This result is a piece of evidence that chromatin reorganization occurs during EMT.

3.2.1 Physiological EMT

Embryonic development

EMT has been widely studied in embryonic development, where this program is a crucial process for the generation of tissues and organs during embryogenesis of both vertebrates and invertebrates [176]. The earliest example of EMT during embryonic development is the generation of the mesoderm, which marks the beginning of gastrulation. The process of gastrulation is a crucial step in the formation of the vertebrate body plan. The induction of mesoderm begins in a specific area of the primitive ectoderm (primitive streak). After invagination of the epithelial cells, the basement membrane breaches locally and cells lose their tight cell-cell adhesions and remain attached to neighboring cells only by focal contacts. Subsequently, these cells undergo mesenchymal differentiation and migrate along the narrow extracellular space underneath the ectoderm to form the mesoderm [161,177].

Another example of EMT is the neural crest delamination. After gastrulation, neural crest is developed at the boundary between the neural plate and the epidermal ectoderm, with the presence of a specific sub-population of cells with rounded and pleomorphic shape, in contrast with those of the polarized neural tube cells. These cells lose cell-cell adhesion [178] and invade through the basal lamina to migrate away from the neural tube. These cells are a transient, multipotent, migratory cell population that will give rise to different cell lineages including bone, smooth muscle, melanocytes, some endocrine cells and most of the peripheral nervous system [176,179].

3.2.2 Pathological EMT

As mentioned before, EMT not only occurs during embryonic development or as a physiological response to injury, but it is also important is some pathologic situations such as fibrosis or cancer progression. At a cellular level, pathological EMT is similar to physiological one, as they share similar signaling pathways and effector molecules. Here we focus on the role of EMT in cancer.

EMT has been studied in many *in vitro* cancer models, which have evidenced that EMT promotes dissemination of a single carcinoma cell from the primary tumor to distant sites by intravasation into lymph or blood vessels due to the acquisition of mesenchymal gene-expression profiles and properties. Indeed, the induction of EMT is the first step in the metastatic process [146,180]. Moreover, the activation of EMT during tumorigenesis also requires signaling between cancer cells and stromal cells [156]. Cancer cells in advanced primary carcinomas are thought to recruit different cell types into the neighboring stroma (fibroblasts, macrophages, mesenchymal stem cells, etc…). These recruited cells create an inflammatory microenvironment that is crucial for the release of EMT-inducing signals, promoting survival, growth and invasiveness of the tumor. Carcinoma cells in direct contact to the reactive stroma respon to these signals by activating transcription factors that will direct EMT programs in them and consequently, secret cytokines and proteases that promote angiogenesis and activate non-neoplastic cells [181,182]. Moreover, cells having undergone EMT present more resistance to cell death and senescence as well as more resistance to chemotherapy and immunotherapy since they escape immune surveillance [176]. In addition, TGFβ1-induced EMT

promotes metabolic reprogramming by reducing the expression of enzymes necessary to convert glucose into fatty acids and concomitant triggered respiration [183]. In that sense, cancer cells reprogram their metabolism by increasing glycolysis in the presence of oxygen (the Warburg effect). Elevated glucose metabolism is used for energy production, provision of macromolecular precursors and establishment of an NADPH pool to enable cells to resist oxidative stress [184,185]. In addition, cancer cells also have increases in de novo fatty acid synthesis for lipogenesis and membrane production [186].

In resume, the *in vivo* model for EMT-mediated metastasis postulates that the primary tumor contains some cells closer to the stroma that undergo EMT and become mesenchymal, losing cell-cell junctions and acquiring motility, thus being able to migrate from the primary tumor. The invasive front can intravasate in the bloodstream. Cytokines and other factors released from the stroma cells, help cancer cells to maintain their mesenchymal phenotype. From the bloodstream, cancer cells can reach organs with a microenvironment similar to that of the tumor where cells will be able to form a metastasis [137,187] (Figure 9).

It has been described that cancer cells that undergo EMT acquire stem-like characteristics due to the fact that some of the signals that control normal stem cell homeostasis are inducers of EMT, and are important to the generation and maintenance of cancer stem cells (CSCs). It means that EMT by itself can induce non-CSCs to enter into a CSC-like state [188,189]. CSCs have self-renewal and migratory capacities, which are important both for the formation of

new tumor masses and also for the genesis of metastasis. Since anti-cancer treatments are addressed against characteristics of the tumor bulk, CSCs are not affected, and it is thought to be a cause of recurrence and resistance to treatments [190]. Moreover, some studies have revealed that CSCs can be early detected not only in the tumor but also disseminate to other organs, and it could be an explanation of why complete surgical removal of a tumor is sometimes not enough to avoid metastasis [191,192].

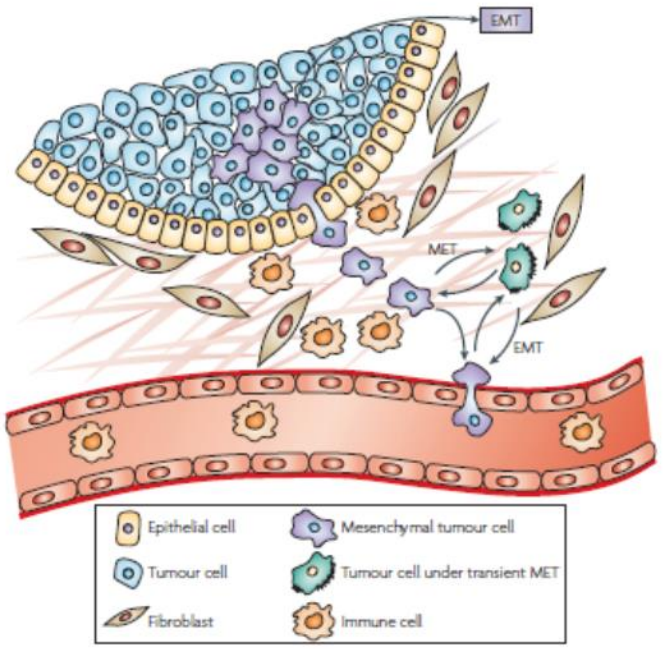


Figure 9. EMT and MET in tumor progression and metastasis. Cells in the primary tumor undergo EMT, migrate and intravasate into the blood stream where they can migrate to distant organs, where they can form a metastasis undergoing MET and growing. Adapted from [137].

Interestingly, it has been proposed that cancer cells do not undergo a complete EMT, thus, cells retain some characteristics of epithelial

cells but also show mesenchymal markers. This intermediate phenotype, also known as “partial EMT” or “metastable phenotype” [193] is important because gives consistency to the CSC theory. The features of these cells allow them to migrate in group since they partially maintain cell-cell contacts. Moreover, expressing factors of both phenotypes, allows them to adapt better and to fast transcriptional reprogramming [144,193]. Since metastasis have similar histology from the primary tumor from which they have arised suggest that migrated cells may undergo MET when they reach an organ to establish micrometastasis. Therefore, the acquisition of mesenchymal features and properties may be a transitory event to facilitate invasion and intravasation of cancer cells, but may be reversible to allow metastasis establishment and growth at distant organs.

3.3. Snail1 in Mesenchymal Stem Cells

MSCs are multipotent progenitor cells with self-renewal capacity that are mostly found in the bone marrow, but also in other tissues such as connective and adipose tissue. MSCs are important for the bone marrow homeostasis maintenance and differentiate into chondrocytes, osteoblast or adipocytes [194-196].

MSC are involved in tumor progression since they are able to secrete some factors such as IL-6, IL-8, CCL5 and EGF that trigger cancer cell proliferation and invasion [197]. Moreover, MSCs can promote angiogenesis [198] and protect the tumor of being recognized by the immune system [199]. Furthermore, MSCs contribute to the pool of

cancer-associated fibroblasts that participate in tumor growth and progression.

TGF-β promotes MSCs proliferation and controls MSCs differentiation. TGF-β is necessary for MSCs differentiation to chondrocyte but prevents the differentiation to adipocytes and osteoblasts [200] Furthermore, Snail1 is necessary for MSCs maintenance. It has been demonstrated that in Snail1-condicional adult mice, upon Snail1 depletion, the number of bone marrow MSCs decreases. In culture, Snail1-deficient MSCs triggers prematurely differentiation to adipocytes or osteoblasts and are resistant to the TGFβ1-induced differentiation block. By contrast, ectopic expression of Snail1 prevents its differentiation [201].

OBJECTIVES

Because Snail1 is involved in some other hallmarks of cancer besides activating invasion and metastasis, the main objective of this thesis was:

To study the role of Snail1 in regulating replicative immortality mechanisms by investigating the role of Snail1 in telomere maintenance and integrity.

RESULTS

1. SNAIL1 HAS A ROLE IN TELOMERE INTEGRITY AND MAINTENANCE

1.1. Snail1 Depletion Leads to Telomere Alterations

Snail1 is the key inducer of epithelial-to-mesenchymal transition (EMT) [135,136] and controls tumor invasion, resistance to apoptosis and the maintenance of cancer stem cell features [176,188,189]. It has also been shown that Snail1 is required for the maintenance of MSCs [201]. Therefore, we asked whether Snail1 could have a role in telomere maintenance and integrity since its dysregulation is a key mechanism involved in cancer [126-128].

In this thesis, we have mainly worked with MSCs, as a model of stem cell line, where Snail1 has an important role [201]. These cells were obtained from mice that carried one allele of Snail1 deleted and another one floxed. Upon transduction with Cre recombinase (pMx-Cre) or with empty vector, we obtained Snail1-KO cells or Snail1-CT cells (holding one wild type allele of Snail1). After 24 h, cells were selected with puromycin (See Materials & Methods, 2.1.1). Depletion of Snail1 was confirmed by RT-qPCR and Western Blot (WB) analysis (Figure 10).

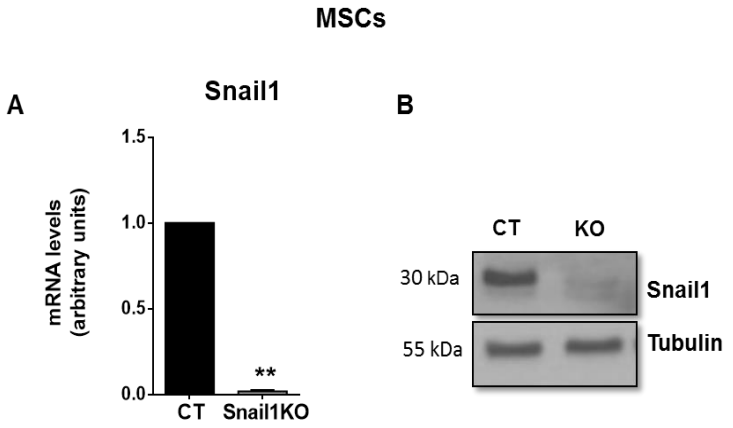


Figure 10. Generation of MSCs Snail1-KO. Expression of Snail1 in MSCs control (CT) and depleted Snail1 gene (Snail1-KO) is shown by RT-qPCR **(A)** and Western Blot **(B)** probed with anti-mSnail1 antibody.

First of all, we wanted to know whether Snail1 could affect telomere maintenance and integrity. For that aim, metaphase spreads of CT and Snail1-KO MSCs were analyzed by fluorescence in situ hybridization using peptide nucleic acid probes PNA - FISH, using a specific probe for telomere TTAGGG repeat (see Materials & Methods). We observed that Snail1-KO MSCs presented chromatids lacking telomeric signal (signal free ends-SFE) as well as chromatids with fused sister telomeres (sister association-SA) (Figure 11). Specifically, we observed that Snail1-KO MSCs presented 24% of SFE and 15% of SA of the analyzed chromosomes. In contrast, only a small part of CT MSCs presented these telomere alterations (2.4 % of SFE and 5.8% of SA respectively) (Figure 12). These results suggest that Snail1 is important for telomere integrity.

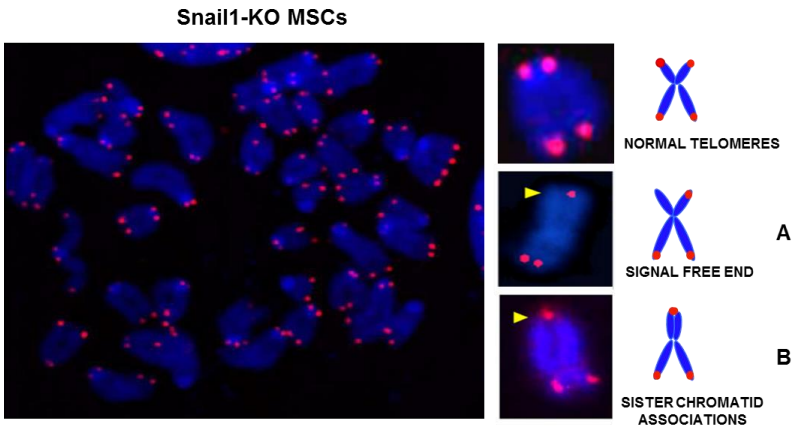


Figure 11. PNA-FISH analysis of metaphase spreads in MSCs. Telomere repeats were detected by using a Cy3-(CCCTAA) $)_3$ PNA probe (red). DNA was stained with DAPI (4, 6-diamidino-2-phenylindole) (blue). A representative metaphase spread from Snail1-KO MSCs is observed, showing some telomere alterations: **A)** signal free ends (SFE) and **B)** sister chromatid associations (SA) (both indicated with yellow arrowheads).

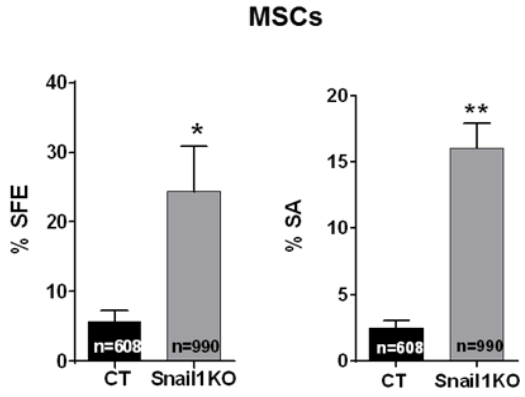


Figure 12. Snail1 loss causes telomere dysfunctions. The graph shows the distribution of the number of telomere atlerations (SFE and SA) observed in CT and Snail1-KO MSCs. N indicate the number of chromosomes analyzed. Error bars show mean ± SEM of at least three independent experiments. *p<0.05. **p<0.01

1.2 Snail1 Regulates Telomere Length

We then asked whether these telomere alterations observed in Snail1-KO MSCs could have an impact on the normal function of telomeres. Thus, we decided to analyze the telomere length in these cells. Metaphases of CT and Snail1-KO MSCs were analyzed by quantitative fluorescence in situ hybridization (Q-FISH) at late passages upon Snail1 depletion (See Material & Methods). Analysis of fluorescence intensities showed that Snail1-KO MSCs presented abnormally short telomeres compared to CT cells (Figure 13).

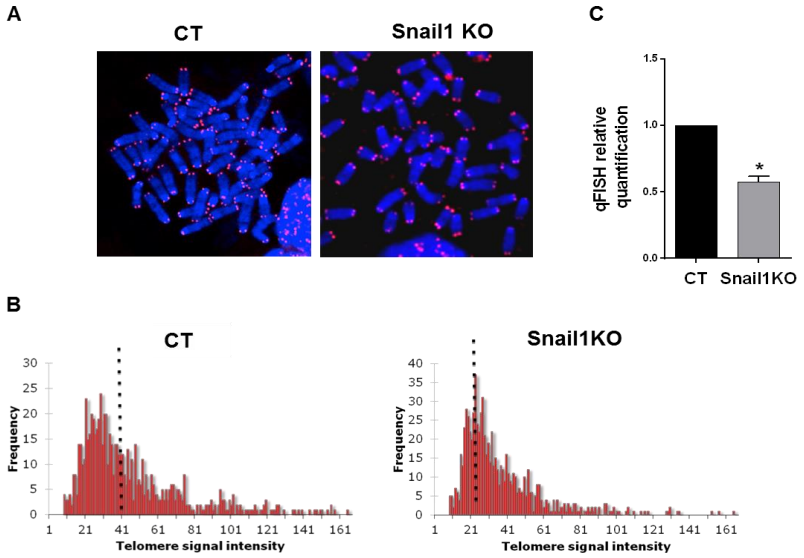


Figure 13. Snail1 regulates telomere length in MSCs. A) Images of representative metaphase spreads from MSCs of CT and Snail1-KO mice. DNA was stained with DAPI (blue), and telomeres were stained with a Cy3-labeled PNA telomere probe (red). **B)** Q-FISH analysis of individual metaphase preparations from MSCs was used to measure telomere length. 40 metaphases were analyzed for each histogram. Average telomere length is indicated for each histogram**. C)** Graph of the mean telomere lengths from Q-FISH analysis (as shown in B) for the indicated MSCs. Error bars show mean ± SEM of at least three independent experiments. * $p < 0.5$

This result suggests that Snail1 has a positive role on telomere length regulation.

In order to confirm these results in an alternative model, telomeres were studied by telomere restriction fragment (TRF) analysis, which is the gold standard assay to analyze telomere length. TRF analysis was performed in a human cell line, the HTC75 cell line, which express high levels of telomerase (TERT) and maintains telomeres at a constant length. We generated stable HTC75 sub-lines by expressing wild-type (WT) Snail1 or the transcriptional inactive P2A mutant [135] by infecting cells with pBABE-mSnail1HA (Snail1), pBABE-mSnail1-P2AHA (Snail1-P2A) and pBABE-empty (CT) (Figure 14). Snail1P2A is a mutated form of Snail1 that exhibits a point mutation in the N-terminal SNAG domain (a proline in position 2 is changed to alanine) that is definitive in repression [135]. SNAG domain is a short domain common to all members of vertebrate Snail family [202,203] that mediates transcriptional-repressor characteristics of Snail [134,138]. Therefore, we decided to use this Snail1-P2A mutant in order to elucidate whether the changes we observed in telomere length were due to a transcriptional regulation of Snail1.

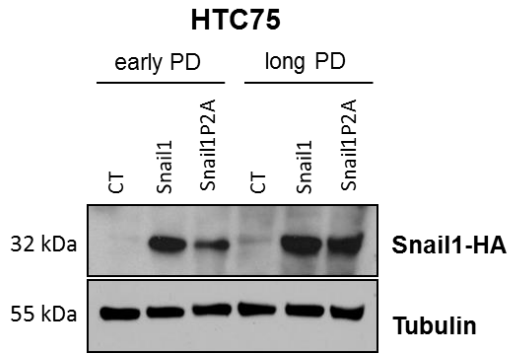


Figure 14. Generation of stable HTC75 sublines. Immunoblot analysis of extracts from stable HTC75 cell lines expressing pBabe empty (CT), pBabe-mSnail1HA (Snail1), and pBabe-mSnail1-P2A-HA (Snail1P2A), at early (E) population doubling (PD 2) or late (L) population doubling (PD 68) probed with anti-HA.

TRF analysis was performed at early and late population doubling (PD 2 vs PD 68). This assay showed that cells overexpressing Snail1 had an increase in telomere length that was not observed in CT cells or in cells overexpressing the mutant Snail1-P2A (Figure 15 A and B). This result confirmed that the transcriptional repressive activity of Snail1 is necessary for effective telomere elongation.

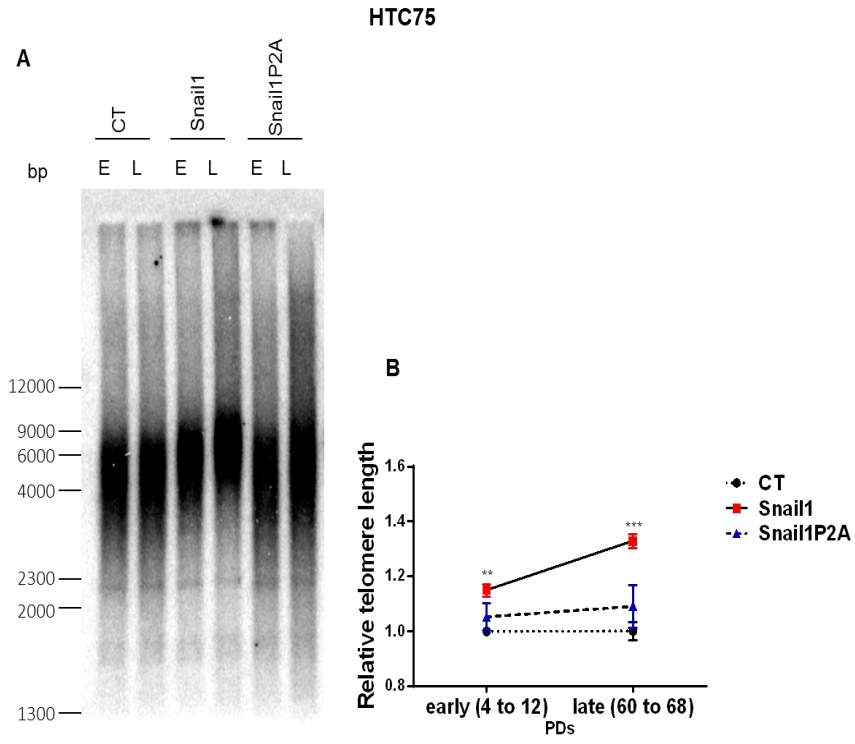


Figure 15. **Snail1 regulates telomere length in HTC75 cell line. A)** A representative TRF assay from HTC75 cells at early (E; PD2) or late (L; PD68) population doubling. Analysis of restriction enzymes-digested gDNAs were run on agarose gel and hybridized to a ^{32}P-labeled $(CCCTAA)_4$ oligonucleotide probe. **B)** Graphical representation of quantification of relative telomere length assessed by three independent TRF assays on HTC75 expressing the indicated alleles at different passages. **p<0.001. Plots represent the mean telomere length values derived from the Southern blots in (A).

With these results we can conclude that Snail1 is necessary for telomere integrity since its depletion leads to abnormal telomeres. Moreover, Snail1 is necessary for effective telomere length by its transcriptional repressive activity.

2. SNAIL1 IS INVOLVED IN TELOMERASE REGULATION

We have obseved that Snail1 is necessary for the integrity and maintenance of telomeres since its absence leads to aberrant and abnormal short telomeres. Therefore, our next step was to study whether Snail1 regulated any mechanism involved in telomere maintenance and integrity.

2.1 Snail1 Has no Effect on Shelterin Protein Expression

As mentioned in the introduction, telomeres consist in TTGGGA repeats and shelterin complex [25,26,43]. We first focused on analyzing the expression of some shelterin and other telomeric associated proteins in CT and Snail1-KO MSCs, since they are involved in the maintenance of telomere length [27,28] and telomeric stability [39]

In Figure 16, we can observe that Snail1 has no effect in the expression of TIN2, TRF1 nor Rad51, some of the components of shelterin and transiently associated proteins as Rad51. Therefore, it seems that Snail1 regulates telomere integrity and length by other mechanisms rather than regulating the expression of shelterin protein expression.

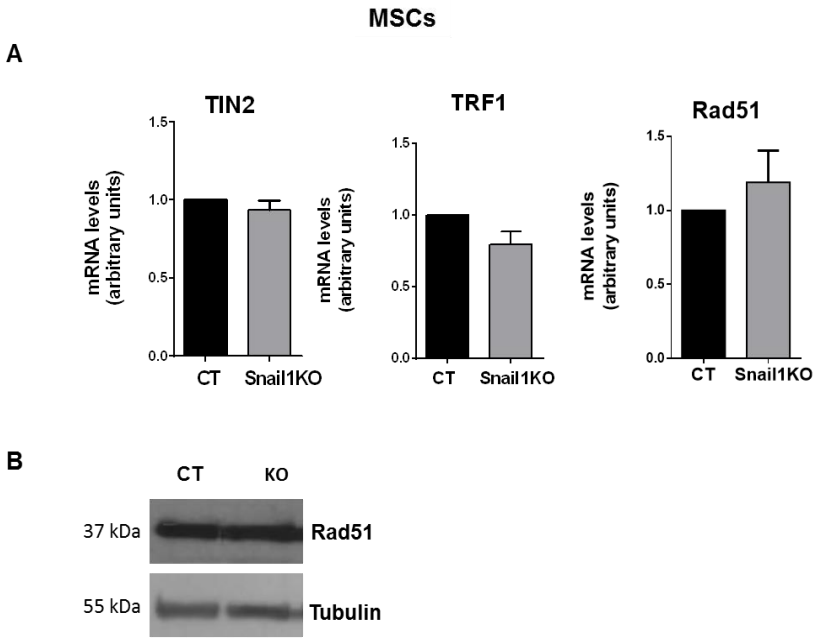


Figure 16. Snail1 has no effect on telomeric associated protein expression. A) TIN2, TRF1 and Rad51 expression were analyzed by RT-qPCR in MSCs. Gene expression was normalized against an endogenous control (*Pumilio*) and presented as RNA levels over those obtained in CT MSCs, which was set as 1. Error bars show mean ± SEM of at least three independent experiments. **B)** Rad51 expression was analyzed by WB, without any significant differences between CT and Snail1-KO MSCs.

2.2. Snail1 Controls TERT Expression

It is well established that the main mechanism involved in telomere maintenance is the enzyme telomerase (TERT) [86]. Since Snail1 is involved in the regulation of telomere integrity and length and does not seem to be involved in the regulation of shelterin or other telomeric associated proteins, we then asked whether Snail1 could regulate TERT expression in MSCs. Although Snail1-KO MSCs presented shorter and abnormal telomeres compared to CT MSCs (Figures 11, 12, 13), these cells showed a two-fold increase in

mRNA TERT levels compared to CT cells (Figure 17), suggesting that Snail1 is involved in the regulation of TERT expression.

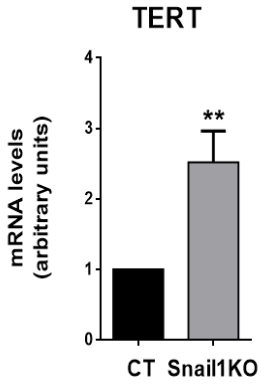


Figure 17. Snail1 controls TERT mRNA expression in MSCs. RT-qPCR shows the change in the expression of Tert in CT and Snail1-KO MSCs. Gene expression was normalized against an endogenous control (*Pumilio)*. Results are shown as relative RNA quantification (RNA levels over those obtained in CT cells, which was set as 1). Error bars show mean ± SEM of at least three independent experiments. **p<0,01

To further confirm the role of Snail1 in regulating TERT, Snail1 was overexpressed in the Snail1-KO MSCs by stable transfection. In Figure 18 A, the Snail1 overexpression in Snail1-KO MSCs is observed by WB (left) and RT-qPCR (right). Upon Snail1 overexpression, TERT mRNA levels were down-regulated (Figure 18 B), thus, recovering partially the expression observed in CT MSCs, and suggesting that Snail1 is involved in TERT repression.

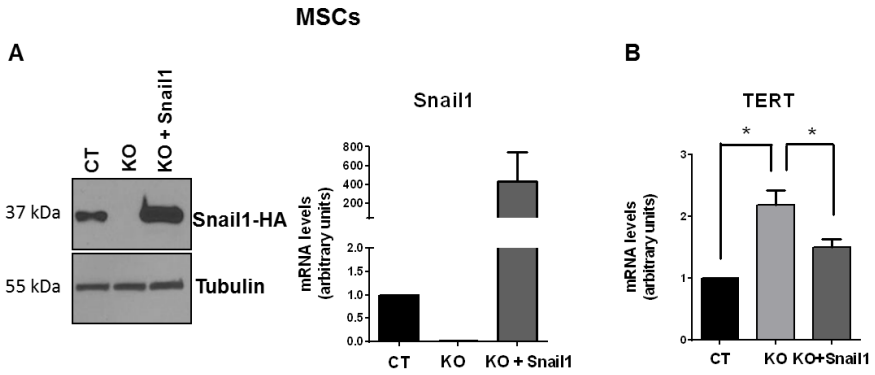


Figure 18. **Snail1 regulates TERT mRNA expression. A)** Western Blot (left) and RT-qPCR (right) for Snail1 expression in CT MSCs, Snail1-KO MSCs and after Snail1 transfection in Snail1 depleted MSCs (KO+Snail1). **B)** Analysis of TERT expression by RT-qPCR in CT, Snail1-KO and KO+ Snail1 MSCs. In all experiments, gene expression was normalized against an endogenous control (*Pumilio)*. Results are shown as relative RNA quantification (RNA levels over those obtained in CT cells, which was set as 1). Error bars show mean ± SEM of at least three independent experiments. *p<0.5

These results were also reproduced in another cell line, mouse embryonic fibroblasts (MEFs), where the absence of Snail1 induced an increase on TERT levels (Figure 19).

We further confirmed the role of Snail1 in the regulation of TERT expression in two epithelial cell lines, RWP-1 and NMuMG, in which Snail1 is not normally expressed. Stable RWP-1 cells expressing pcDNA3-Snail1-HA were generated in our laboratory [204]. Snail1 levels are observed in Figure 20 A. In Figure 20 B, we can observe that human TERT mRNA levels were down-regulated in RWP-1 cell line overexpressing Snail1 compared to RWP-1 CT cells in about 60%.

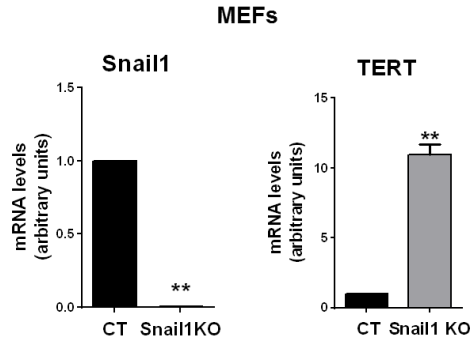


Figure 19. Snail1 represses TERT mRNA expression in MEFs. RT-qPCR shows the change in the expression of Snail1 and Tert in CT and Snail1-KO MEFs. In all experiments, gene expression was normalized against an endogenous control (*Pumilio)*. Results are shown as relative RNA quantification (RNA levels over those obtained in CT cells, which was set as 1). Error bars show mean ± SEM of at least three independent experiments. **p<0,01.

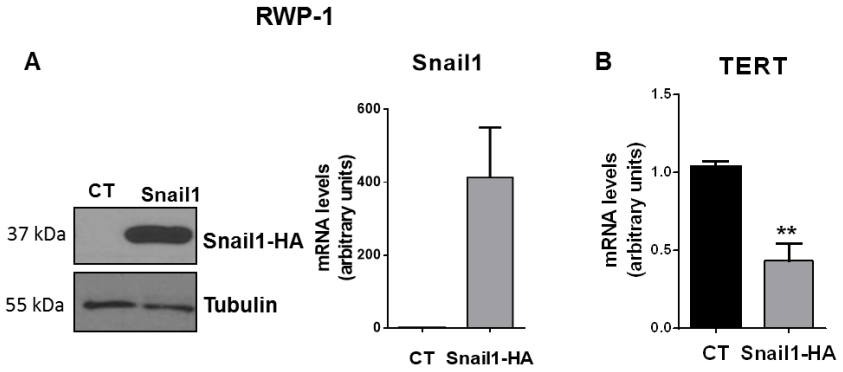


Figure 20. Snail1 overexpression represses TERT expression in RWP-1 cell line. A) Analysis of Snail1 expression by WB (left) and RT-qPCR (right) in RWP-1 cell line with (mSnail1) or without (CT) Snail1 overexpression. **B)** Analysis of TERT mRNA levels by RT-qPCR with (Snail1-HA) and without (CT) Snail1 overexpression. Gene expression was normalized against P*umilio* endogenous control. Results are shown as relative RNA quantification (RNA levels over those obtained in CT cells, which was set as 1). Error bars show mean ± SEM of at least three independent experiments. **p<0,01.

Next, we analyzed TERT expression in NMuMG cell line. As we can observe, upon transient Snail1 overexpression, TERT levels were down-regulated about 50 % compared to CT cells (Figure 21).

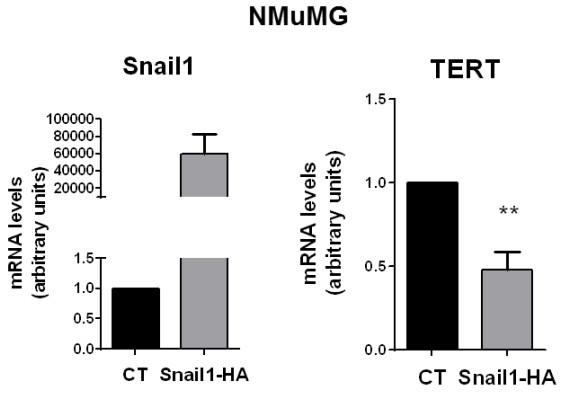


Figure 21. Snail1 overexpression represses TERT expression in NMuMG cell line. Analysis of Snail1 and TERT expression by RT-qPCR in NMuMG cell line upon Snail1 overexpression (Snail1-HA). Gene expression was normalized against P*umilio* endogenous control. Results are shown as relative RNA quantification (RNA levels over those obtained in CT cells, which was set as 1). Error bars show mean ± SEM of at least three independent experiments. **p<0,01.

Taken together, all these results demonstrate that Snail1 represses TERT expression.

We then asked whether this repression correlated with a change in the enzymatic activity of TERT, which is responsible for telomere elongation. For this aim, the catalytic activity of TERT was assessed by using the Telomeric Repeat Amplification Protocol assay (TRAP) (See Materials & Methods). As shown in Figure 22 A, CT MSCs showed lower levels of TERT activity compared to Snail1-KO MSCs. Quantification of PCR products indicated a four-fold increase in TERT activity in Snail1-KO MSCs compared to CT cells (Figure 22

B), demonstrating that, as expected, Snail1 represses the enzymatic activity of TERT.

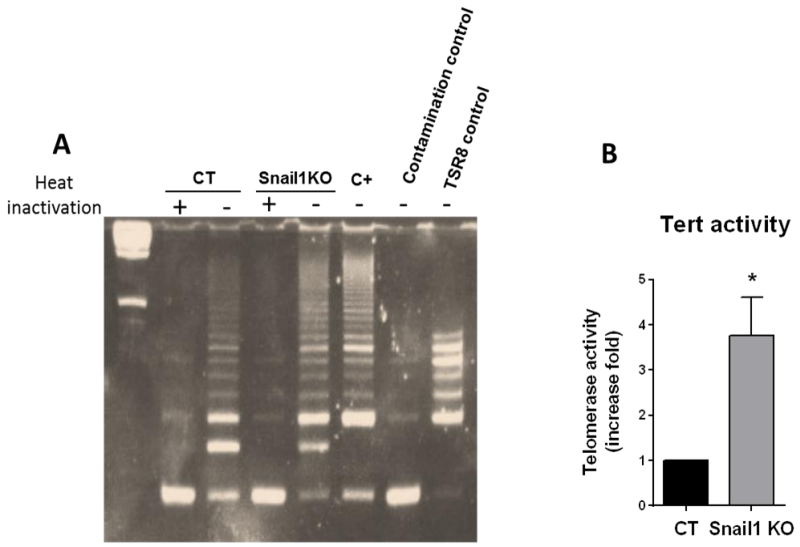


Figure 22. Snail1 represses TERT activity. A) Representative image of a TRAP assay in MSCs. The PCR products were separated by electrophoresis in a 12.5% non-denaturing polyacrylamide gel in CT and Snail1-KO MSCS extracts with or without heat inactivation. Positive telomerase activity cell extract (C+), contamination control and TSR8 control are shown. **B)** Quantification of telomerase activity, in CT and Snail1-KO MSCs extracts subjected to the TRAP assay. Errors bars show mean ± SEM of at least three independent experiments. *p<0.05.

2.3. Snail1 is a Transcriptional Repressor of TERT Promoter Activity

As we have demonstrated that Snail1 is involved in the regulation of the expression and activity of TERT, we wanted to go one step further and study whether Snail1 directly repressed TERT expression.

To search for Snail1 putative binding sites in TERT promoter, we performed a Bioinformatic analysis using a Consensus Sequence programs TFSEARCH ver 1.3 ** (c) 1995 Yutaka Akiyama (Kyoto Univ.), and TF_BIND. These rutine highly correlated sequence fragments versus TFMATRIX transcription factor binding site profile data base TRANSFACT and TFMATRIX. Non of these data bases analysis gave us any putative Snail1 binding site in TERT promoter. However, we searched for E-boxes in TERT promoter sequence. This manual analysis indicated that TERT promoter contained several E-boxes (CACCTG or CAGGTG) corresponding to putative Snail1 binding sites at -253, -123, with respect to the transcription start and more 3' downstream, at +301, +324 and +426 (Figure 23 and 26 A).

Figure 23. Putative Snail1 binding sites in mTERT promoter. Representation of mTERT promoter from -1161 to +874 from the transcriptional start site TSS indicated in red "A". In yellow, exons are showed. In blue boxes, the Snail1 binding sites (E-Boxes) are localized.

In order to verify whether Snail1 repressed mTERT promoter activity, luciferase assays were performed by transient transfection of pGL3-E-cad (178/92) and using a -599/+53 fragment of the mouse TERT promoter cloned in pGL3 plasmid (See Materials & Methods). Luciferase experiments were done first in MSCs, where we observed that the expression of Snail1 significantly decreased the activity of the TERT promoter. Moreover, E-cadherin promoter was used as a control because it is a well-known Snail1 repressed gene (Figure 24). Luciferase assays were measured 24 h after transfection.

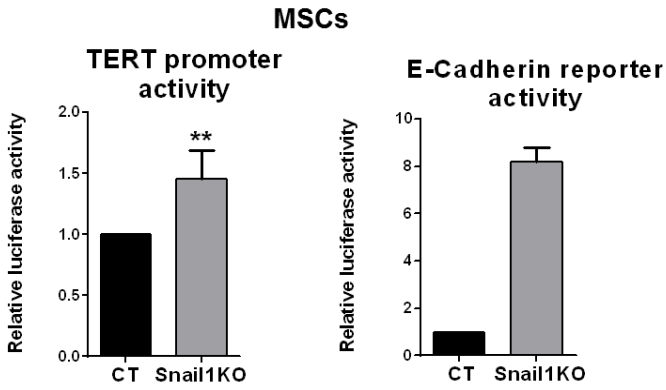


Figure 24. Snail1 regulates mTERT activity in MSCs. Luciferase assays showing the activity of TERT promoter (-599 to +53) and E-cadherin promoter (178/92) in CT and Snail1-KO MSCs. Errors bars show mean ± SEM in at least three independent experiments (except for E-cadherin promoter activity in MSCs, n=2). **p<0.01.

These results were confirmed by luciferase analysis in two more cell lines: 293T and RWP-1. Luciferase activites were measured 24h after transfection. The promoter activity was determined by transient transfection of the pGL3-E-cad (178/92) promoter or pGL3-mTERT promoter upon Snail1 transfection (pcDNA3-Snail1 or pcDNA3 empty). In both cell lines, overexpression of Snail1 (transiently in

293T cell line; stably in RWP1 cells) produced a significant decrease in mTERT promoter activity. Again, E-cadherin promoter was used as a positive control (Figure 25 A and B).

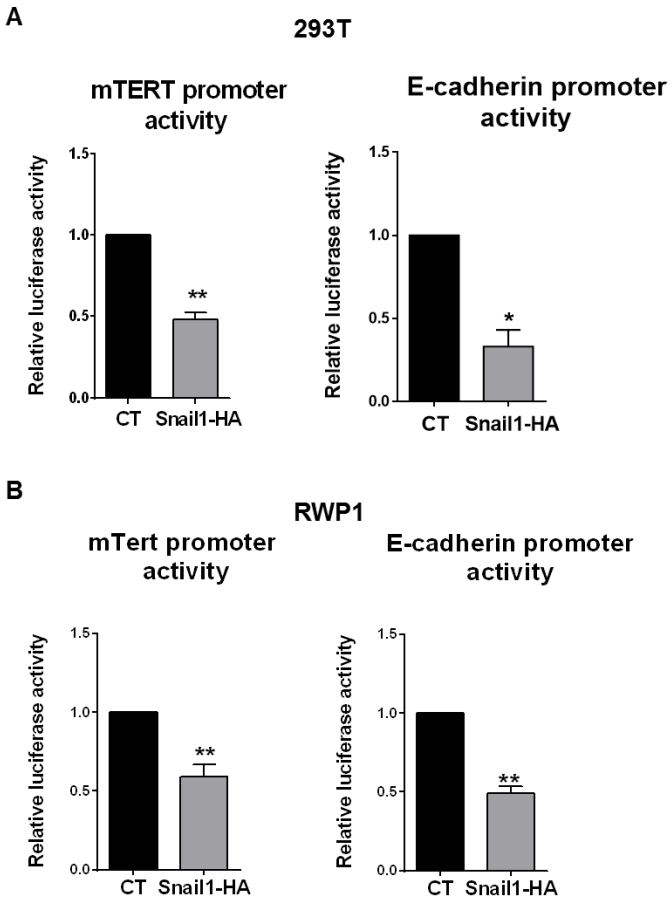


Figure 25. Snail1 regulates mTERT activity in 293T and RWP-1 cell lines. Activity of mTERT promoter and E-cadherin promoter after Snail1 overexpression in 293T **(A)** and RWP-1 cells **(B).** Errors bars show mean ± SEM in at least three independent experiments. *p<0.05, **p<0.01.

After confirming that Snail1 represses mTERT promoter, and due to the fact that mTERT promoter contains Snail1-binding sites (E-boxes), we asked whether Snail1 caused this repression by directly binding to the promoter. For that aim, ChIP assays were performed, and we confirmed that Snail1 directly binds to mTERT promoter in CT MSCs, while no binding was observed when an irrelevant IgG was used as a negative control or in Snail1-KO MSCs (Figure 26 B).

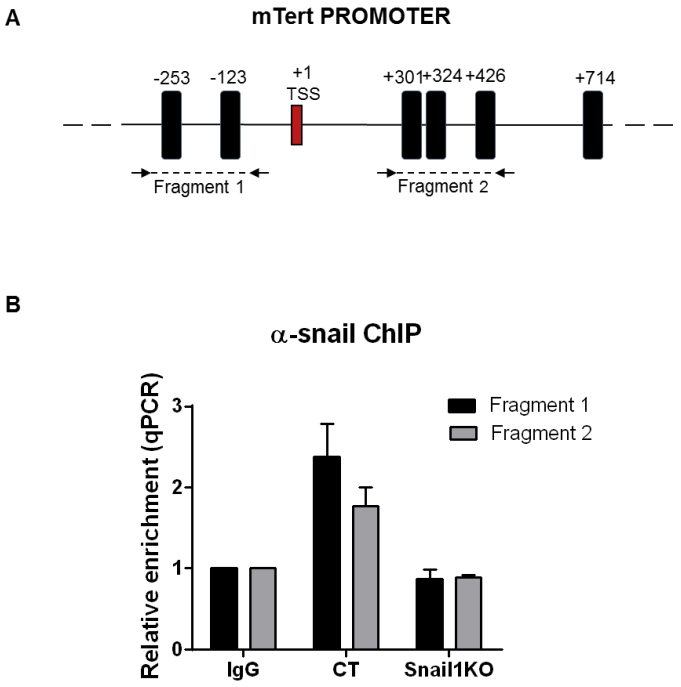


Figure 26. Snail1 binds to TERT promoter. A) Schematic representation of mTERT proximal promoter. E-boxes are represented in black. Arrows indicate the primers used in the ChIP experiment. **B)** Snail1 ChIPs in CT and Snail1-KO MSCs corresponding to the mTERT promoter. An irrelevant IgG was used as a negative control in the same regions. The value given for the IgG sample was set as 1. n=2

Taken together, these data demonstrate that Snail1 is a transcriptional repressor of the murine TERT mRNA.

3. TERT IS DOWNREGULATED DURING EMT

Since Snail1 is a transcriptional factor that plays a key role in EMT, we wonder whether it also regulates mTERT during this process.

For this reason, we used the well-established model of mouse mammary epithelial NMuMG cells, which undergo EMT after TGFβ1 treatment. Addition of this cytokine induces Snail1 up-regulation (Figure 27 A and B) that precedes the up-regulation of mesenchymal genes such as Fibronectin (FN) and ZEB1, and the down-regulation of E-cadherin (Figure 27 A and B). Moreover, we observed that mTERT transcription significantly decreased upon 2 h of TGFβ1 exposure (Figure 27 C) concomitantly with Snail1 up-regulation.

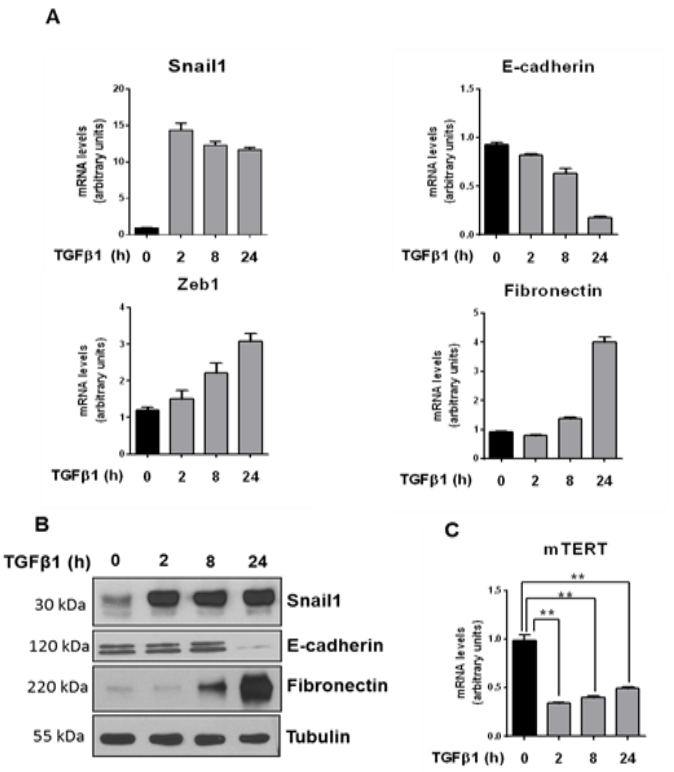


Figure 27. TERT is downregulated during EMT. Snail1 induction upon TGFβ1 (5ng/mL) is shown by RT-qPCR **(A)** and WB **(B)** in NMuMG cells at different time points (0, 2, 8, 24h). **A)** RT-qCR shows CADH1 (E-cadherin gene) down-regulation and up-regulation of FN1 (Fibronectin) and ZEB1 mRNA levels upon Snail1 induction. **B)** Immunoblot showing E-cadherin repression and FN up-regulation upon Snail1 induction. **C)** RT-qPCR shows the down-regulation of mTERT mRNA levels in NMuMG cells upon TGFβ1 treatment at different time points (0, 2, 8, 24h). Gene expression was normalized against an endogenous control (*Pumilio*) and presented as RNA levels over those obtained without TGFβ1 treatment. Error bars indicate SD ± SEM in at least three independent experiments. **p<0.01.

In order to study the biological relevance of TERT repression during EMT, mTERT was overexpressed in NMuMG cells by stably infection with pBABE-mTERT and pBABE-empty as a control (Figure 28 A).

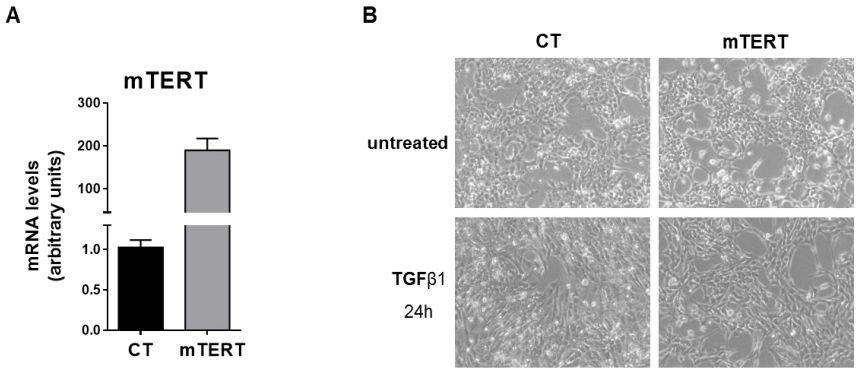


Figure 28. TERT expression does not affect TGFβ1-induced changes in cell morphology. A) RT-qPCR shows the overexpression of mTERT on NMuMG cells. **B)** Representative images of phase contrast microscopy of mTERT or CT NMuMG cells upon TGFβ1 treatment (5ng/mL). No phenotypic differences were observed upon TGFβ1 treatment between NMuMG-CT and NMuMG-mTert cells.

mTERT overexpression did not appear to significantly modify the cell morphology of NMuMG cells after TGFβ1-mediated EMT (Figure 28 B). Moreover, no changes were observed at the protein level (Figure 29) nor in mRNA expression (Figure 30) of Snail1, E-cadherin, Fibronectin and Zeb1 after mTERT overexpression during EMT.

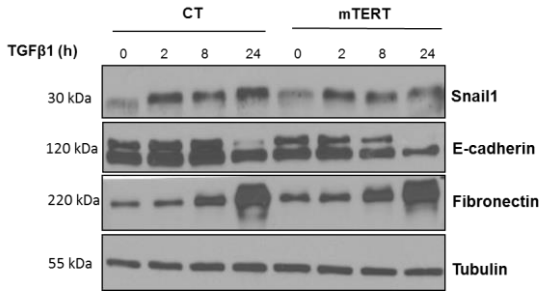


Figure 29. Overexpression of mTERT has no effect on EMT at protein level. The Inmunoblot shows Snail1, E-cadherin and Fibronectin protein levels in NMuMG-CT and NMuMG-mTERT cells upon TGFβ1 treatment at the indicated time points (0, 2, 8, 24 h). No differences at the protein level of these proteins was observed upon Snail1 induction.

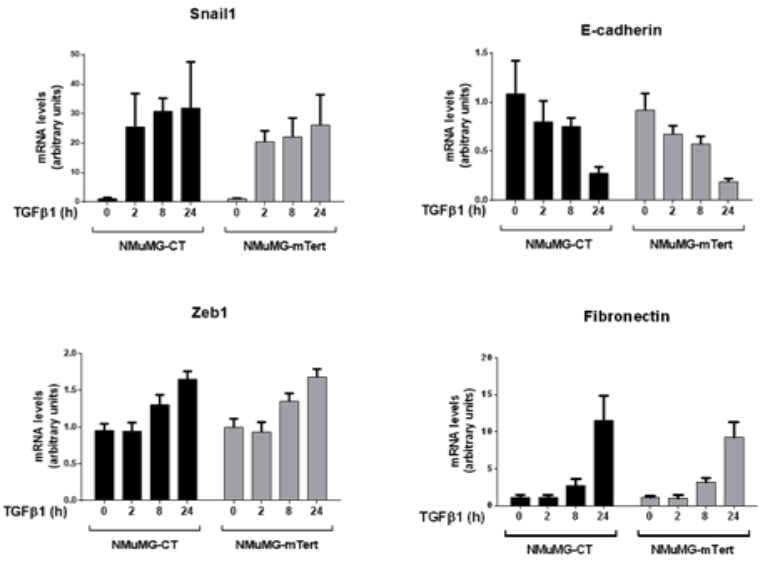


Figure 30. Overexpression of mTERT has no effect on EMT at a transcriptional level. RT-qPCR shows mRNA levels of Snail1, E-cadherin, ZEB1 and Fibronectin in NMuMG-CT and NMuMG-mTERT cells upon TGFβ1 treatment (5ng/mL) at the indicated time points (0, 2, 8, 24h). Gene expression was normalized against an endogenous control (*Pumilio*) and presented as RNA levels over those obtained in NMuMG cells at 0 h, which was set as 1. Error bars indicate SD±SEM in at least three independent experiments.

With these results we can conclude that mTERT overexpression has no effect on EMT, suggesting that the repression on mTERT mRNA expression is not essential for the full EMT progression. This issue is being currently investigated.

4. BIOLOGICAL EFFECTS OF mTERT IN MSCs

We have demonstrated that Snail1 plays an important role in telomere maintenance and integrity. Moreover, Snail1 is a repressor of mTERT expression and activity. Since non-canonical functions of TERT has been described apart from its role in elongating telomeres, we asked whether this TERT repression by Snail1 had a role in the Snail-dependent acquisition of functional changes that are important in cancer.

4.1. Glucose Consumption

As commented in the Introduction, Snail1 is involved in promoting metabolic reprogramming by reducing the expression of enzymes necessary to convert glucose in fatty acids in cancer cell lines [183-185]. In cancer cells, elevated glucose metabolism is used for energy production. Therefore, as Snail1 is a TERT repressor, we wanted to know whether TERT was involved in that regulation. For that aim, CT and Snail1-KO MSCs were stably infected with pBABE-empty and pBABE-mTert (Figure 31 A). Afterwards, glucose consumption was measured (see Materials & Methods). As it can be observed in Figure 31 B, no changes in glucose consumption were observed between CT and Snail1-KO MSCs. Moreover, glucose uptake was

not significantly modified upon mTERT overexpression in CT and Snail1-KO cells.

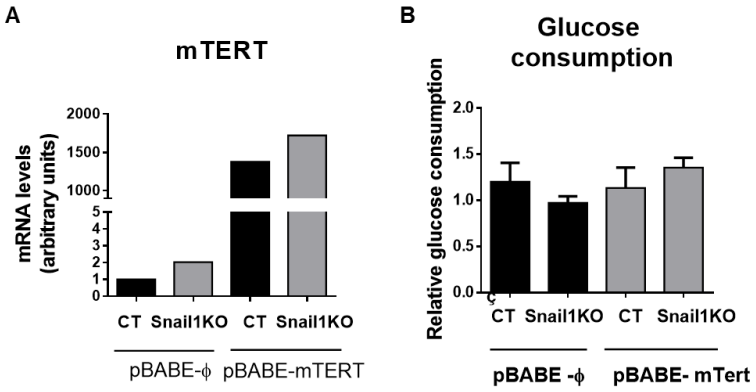


Figure 31. mTERT overexpression has no effect on glucose consumption. A) mTERT expression levels were analyzed by RT-qPCR in CT and Snail1-KO MSCs stably infected with pBABE empty and pBABE-mTERT. **B)** Relative glucose consumption analysis in CT and Snail1-KO MSCs after transfection with mTERT. Error bars show mean ± SEM of at least three independent experiments.

Since the metabolic reprogramming in cancer has been widely studied in EMT [183-186], we analyzed glucose consumption in NMuMG cell line upon TGFβ1 treatment. For that, cells were stably infected with pBABE-mTERT and pBABE-empty (Figure 32 A). Upon TGFβ1 treatment, glucose consumption was analyzed. Figure 32 B shows that, as expected, upon TGFβ1 treatment glucose consumption was approximately 1.8 fold-change increased, but was not significantly altered by mTERT overexpression.

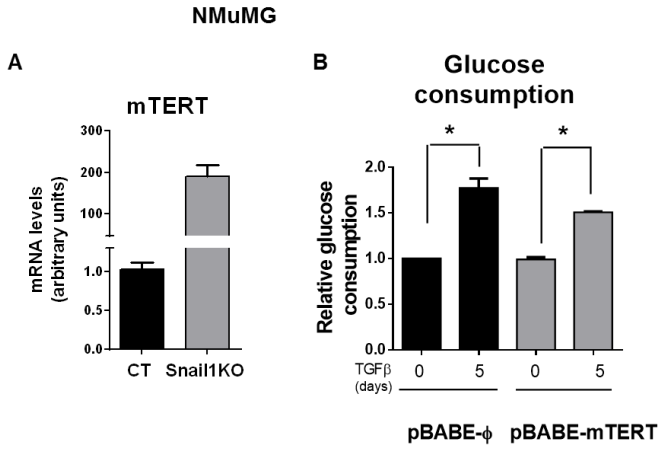


Figure 32. mTERT overexpression has no effect on glucose consumption in NMuMG cell line. **A)** mTERT expression levels analyzed by RT-qPCR in NMuMG cells stably infected with pBABE-empty and pBABE-mTERT. **B)** Relative glucose consumption analysis in NMuMG cells after 24 h TGFβ1 treatment (5ng/mL). Error bars show mean ± SEM of at least three independent experiments. *p <0.05

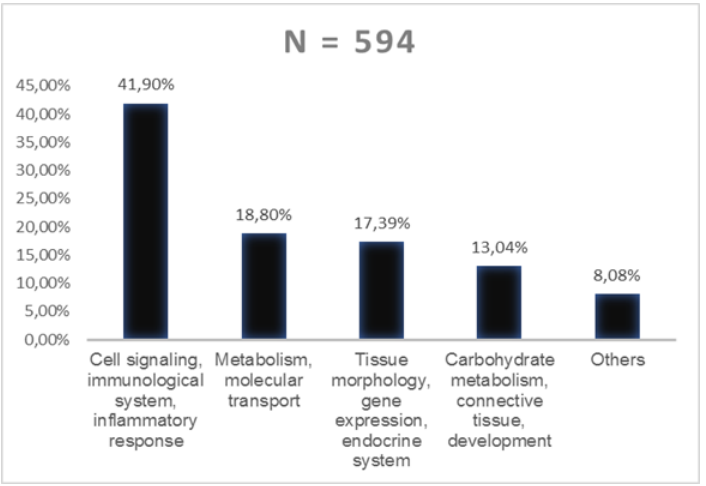
With these results we can conclude that during EMT, Snail1 increases glucose consumption upon TGFβ1 treatment, but glucose consumption is not affected by Snail1 in MSCs nor by mTERT.

4.2. Global Gene Expression Analysis

In order to ascertain which genes were differently expressed by mTERT expression, we decided to compare the global transcriptome of Snail1-CT and Snail1-KO MSCs upon mTERT overexpression by microarray expression analysis. Gene ontology analysis revealed that 594 genes were differentially regulated by TERT in Snail1-CT cells respect to Snail1-KO MSCs. When studying that list in more detail, we observed that most of these genes were mainly related to

those pathways associated to cell signaling, inflammatory response and immunological signal (41,9%) (Figure 33 A). From these genes, we decided to select those that were differentially regulated also in Snail1-CT cells upon mTERT overexpression compared to Snail1-CT cells without mTERT overexpression, such as *Casp1, Casp4, Casp12, IL8, Irf9 and Mmp9* (Figure 33 B).

A



B

Symbol	Description	CT vs KO	CT+TERT vs CT
Casp1	Caspase 1	-1.6	1.85
Casp4	Caspase 4	-0.77	1.11
Casp12	Caspase 12	-0.93	1.24
IL8	Interleukin 8	-0.68	0.35
Irf9	Interferon 9	-0.52	0.53
Mmp9	Matrix metallopeptidase	0.82	-1

Figure 33. Genes differently regulated by mTERT in Snail1-CT cells compared with Snail1KO MSCs. **A)** Schematic representation of the genes differently expressed upon TERT overexpression. Classification of genes into families. **B**) List of the main genes related to immune and antiinflamatory pathways differently expressed between CT and Snail1 MSCs or upon TERT overexpression.

As shown in Figure 34, the observed changes for selected genes were validated by qRT-PCR for Casp1, Casp4 and Casp12, with no significant differences in the other ones. Snail1 depletion produced a two-fold increase Casp1 and Casp4 expression, and a 1.6 fold increase in Casp12 expression compared to CT MSCs. Moreover, when TERT was overexpressed in CT MSCs the increase in the expression of these three genes was also observed, validating that Snail1 and also TERT are involved in the expression of these genes

.

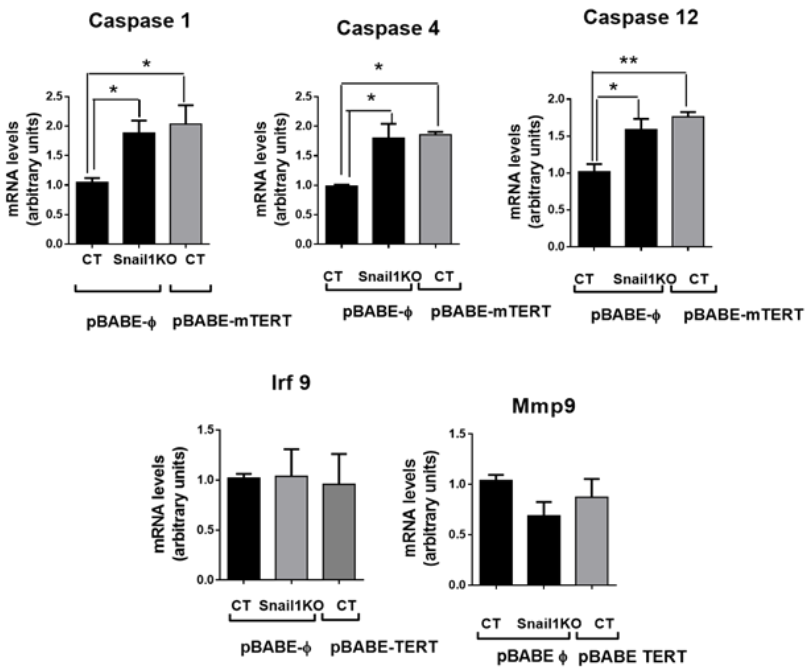


Figure 34. Casp1, Casp4 and Casp12 but not Irf9 and Mmp9 are differently regulated by TERT. Validation by RT-qPCR of selected genes in CT, Snail1-KO MSCs, and also between CT MSCs with or without TERT overexpression. Gene expression was normalized against an endogenous control (*Pumilio*). Results are shown as relative RNA quantification (RNA levels over those obtained in CT cells, which was set as 1). Error bars show mean± SEM of at least three independent experiments. *p<0.05 **p<0.01

This group of caspases are involved in inflammation and immune response rather than in apoptosis [205-207]. In particular, they are responsible for the processing of pro-inflammatory cytokines from an inactive precursor to an active, secreted molecule, such as IL-1β and IL-18. In order to study whether Snail1 and TERT were involved in monocyte recruitment, monocyte migration assays were performed (see Materials & Methods) using the HTP cell line. As it can be observed in Figure 35, Snail1 depletion produced a nearly three fold increase in the monocyte recruitment compared to CT MSCs. Moreover, mTERT overexpression also significantly increased monocyte migration.

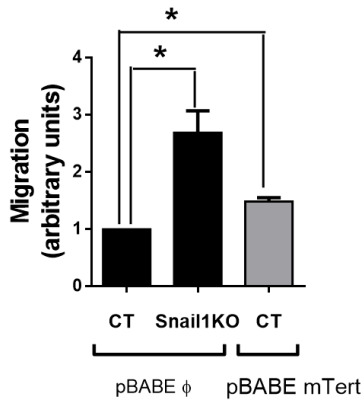


Figure 35. Snail1 and mTERT regulate monocyte migration. 10^5 MSCs (either CT and Snail1-KO) were seeded in a Boyden chamber (lower chamber). HTP1 cells were seeded in the upper chamber. Migration of HTP1 cells was quantified after 18h by the measure of cells in the bottom side of the membrane. Error bars show mean± SEM of at least three independent experiments. *p<0.05 **p<0.01

With this result we can conclude that both Snail1 and TERT are involved in the recruitment of monocytes, probably due to a different secreted cytokine pattern expression induced by Snail1 or TERT.

5. TELOMERIC TRANSCRIPTION IS MODIFIED DURING EMT

5.1. Snail1 Regulates Telomeric Transcription

Although we observed that Snail1 deficient cells presented shorter telomeres and telomere abnormalities, Snail1 did not increase but repressed telomerase transcription and activity (see above). These results suggest that there are additional mechanisms by which Snail1 regulates telomere length and integrity. Therefore, we investigated whether Snail1 could have a role in telomere transcription. As it has been explained in the introduction, telomeres are transcribed into telomeric repeat-containing RNAs (TERRA), large non-coding RNAs that form integral part of telomeric heterochromatin. TERRA up-regulation causes telomere shortening [108,208].

TERRA transcription was analyzed in CT and Snail1-KO MSCs by RT-qPCR using sets of primers mapping to the subtelomeric region of different mouse chromosomes (chr2q, chr11q, chr18q). We observed a significant increase in TERRA transcription in the Snail1-KO MSCs compared to CT MSCs, being the chr18q TERRA the one presenting the higher Snail1-depending expression (Figure 36).

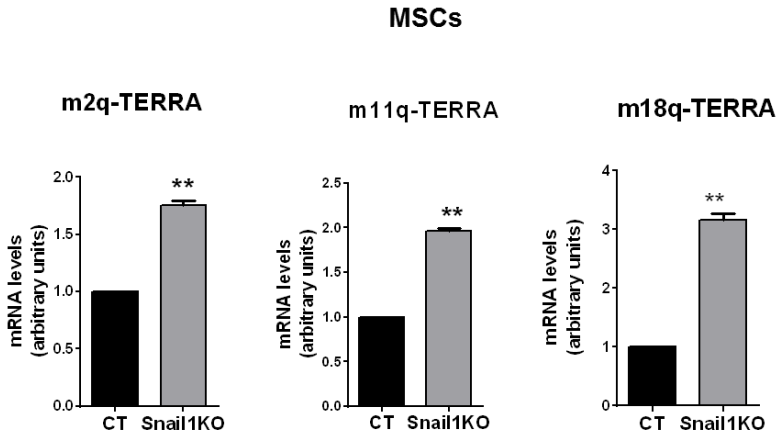


Figure 36. Snail1 regulates telomeric transcription. RT-qPCR shows the changes in the expression of the TERRAs from different chromosomes (m2q, m11q, m18q) in CT and Snail1-KO MSCs. Gene expression was normalized against an endogenous control (*Pumilio*) and presented as RNA levels over those obtained in CT MSCs, which was set as 1. Erro bars show mean ± SEM of at least three independent experiments. *p<0.05. **p<0.01

In the same direction, when Snail1 was re-introduced ectopically to Snail1-KO MSCs, TERRA transcripts were down-regulated (Figure 37).

In order to study whether the regulation of telomeric transcription by Snail1 had a relevant role in a functional tumor scenario, we decided to analyze TERRA transcripts in Cancer-Associated Fibroblasts (CAFs) obtained from murine epithelial tumors [209]. We obtained similar results as in MSCs, where Snail1 depletion up-regulated TERRA transcripts (Figure 38).

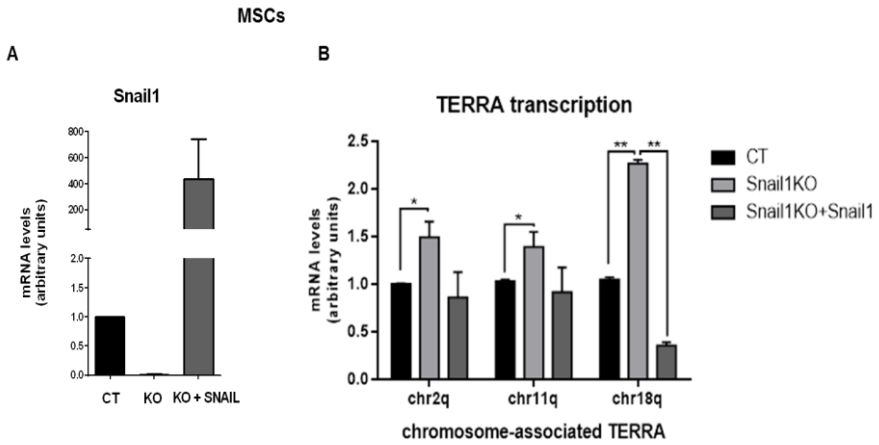


Figure 37. **Telomeric transcription is downregulated after Snail1 rescue A)** RT-qPCR showing Snail1 mRNA levels in CT and Snail1-KO MSCs and upon ectopic Snail1 expression in Snail1-KO cells. **B)** RT-qPCR shows the changes in expression of the TERRAs from different chromosomes (m2q, m11q, m18q) in CT, Snail1-KO and KO+Snail1 MSCs. Gene expression was normalized against an endogenous control (*Pumilio*) and presented as RNA levels over those obtained in CT MSCs, which was set as 1. Error bars show mean ± SEM of at least three independent experiments. *p<0.05. **p<0.01

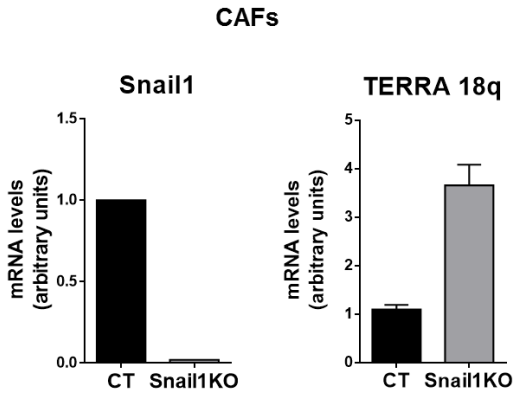


Figure 38. **Snail1 depletion in CAFs induces TERRA transcription.** RT-qPCR shows mRNA levels of Snail1 and TERRA from 18q chromosome of CT and Snail1-KO CAFs. Gene expression was normalized against an endogenous control (*Pumilio*) and presented as RNA levels over those obtained in CT CAFs, which was set as 1. Error bars show mean ± SEM of at least three independent experiments.

All these results confirm that Snail1 is involved in the control of telomeric transcription.

5.2. Snail1 Represses Telomeric Transcription (TERRA) during EMT

We also asked whether TERRA was also regulated during EMT. For that aim, we used again the model of mouse mammary epithelial NMuMG cells that undergo EMT after TGFβ1 treatment. Snail1 was up-regulated upon TGFβ1 treatment and afterwards, as expected, Fibronectin was up-regulated and E-cadherin was down-regulated as previously shown in Figure 27 A and B. We observed that TERRA transcription was significantly decreased during EMT, especially upon 2 hours of TGFβ1 treatment (Figure 39). This down-regulation was transient and after 24h, TERRA levels were the same as in untreated cells.

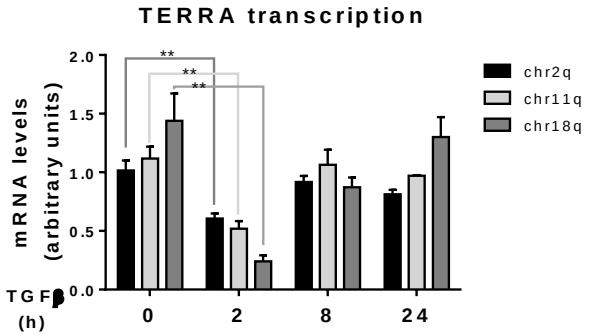


Figure 39. Snail1 represses telomeric transcription during EMT. RT-qPCR shows TERRAs transcripts (m2q, m11q, m18q) levels after normailitzation versus an endogenous control in NMuMG cells treated with TGFβ1 at 0, 2, 8, 24 h time points. Telomeric transcription is downregulated after Snail1 induction. Error bars show mean ± SEM of at least three experiments. *p<0.05, **p<0.01

In order to study whether the down-regulation of TERRA during EMT was functionally relevant, we induced NMuMG cells to produce high levels of TERRA. We used the same strategy as Lieberman and co-workers that constists in transducing a mutant form of the telomere-binding factor TRF1 (TRF1ΔN) alone or fused to the VP16 transcriptional activation domain (VP16-TRF1ΔN) [210]. We used this strategy because TERRA cannot be overexpressed using a plasmid since it does not localize to telomeres and therefore, TERRA overexpression is not functional. The stable expression of these proteins was confirmed by WB (Figure 40 A) and the up-regulation of TERRA by VP16-TRF1ΔN was validated by RNA dot blot (Figure 40 B).

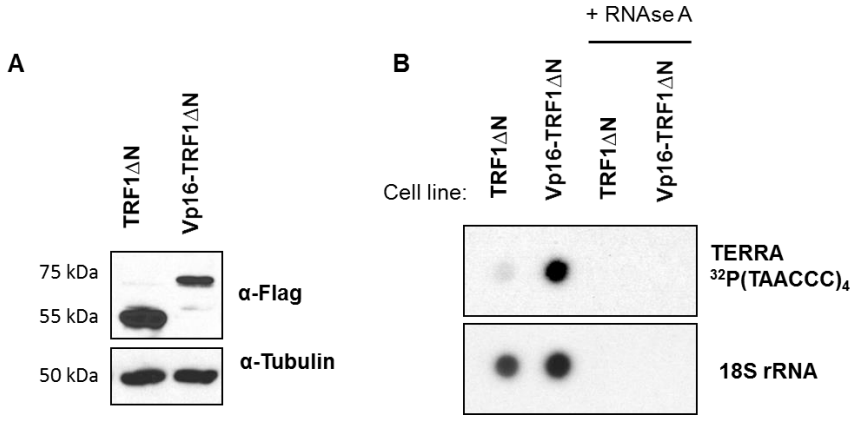


Figure 40. **TERRA overexpression in NMuMG cells. A)** Western Blot from NMuMG cells of TERRA overexpression using Flag-TRF1ΔN alone or fused with the transcriptional activator VP16. Immunoblot with anti-Flag antibody shows TRF1 (ΔN) and VP16-TRF1 (ΔN) fusion protein. **(B)** RNA dot blot from NMuMG cells transduced with vector, TRF1(ΔN), or VP16-TRF1(ΔN) to detect TERRA using a ^{32}p-dCTP-labelled probe; hybridization of 18S rRNA was included as a loading control.

Although TERRA up-regulation did not modify morphology of NMuMG cells after TGFβ1-mediated EMT (Figure 41), we observed

changes in the expression of genes classically associated with EMT, and validated several of these genes by RT-qPCR. As it can be observed in Figure 42, Fibronectin, FGF receptor (FGFR) and Loxl1 presented a lower up-regulation by TGFβ1 in TERRA-expressing cells compared to control cells. Other relevant genes such as E-cadherin, Vimentin or Snail1 were not significantly altered by TERRA overexpression.

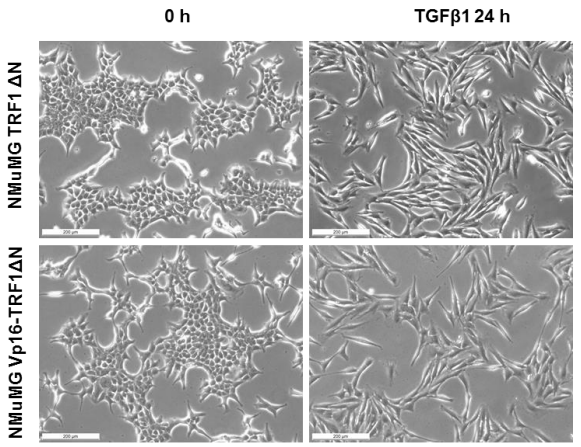


Figure 41. **TERRA overexpression in NMuMG cells does not change NMuMG morphology.** Representative images of phase contrast microscopy of TRF1 ΔN and VP16-TRF1 ΔN NMuMG cells after 24h with or without TGFβ1 treatment

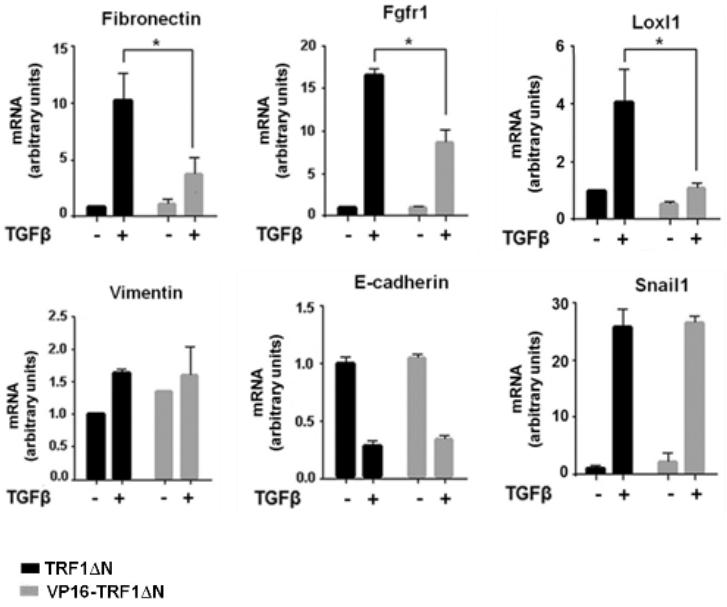


Figure 42. **TERRA overexpression in NMuMG cells leads to regulation of some mesenchymal genes**. RT-qPCR of FN1, Fgfr1, Loxl1, E-cadherin, Vimentin and Snail1 mRNA levels upon TERRA induction. Error bars show mean ± SEM of at least three independent experiments. *p<0.05.

Therefore, these results suggest that a full EMT requires a transient down-regulation of TERRA transcription.

DISCUSSION

Snail1 is a transcriptional factor that activates invasion and metastasis by orchestrating EMT [135,136]. Besides its role in promoting invasion and metastasis, Snail1 is involved in other important hallmarks of cancer: it protects against cell death [211], avoids immune destruction [176] and promotes metabolic cancer cell reprogramming [183-186].

Another important characteristic of cancer cells is replicative immortality, mostly achieved by the reactivation of telomerase [86]. In this thesis, I present results indicating a novel role of Snail1 in the regulation of telomere integrity and transcription as well as telomerase expression.

1. SNAIL1 REGULATES TELOMERE INTEGRITY AND LENGTH

1.1 Snail1 Depletion Leads to Abnormal and Shorter Telomeres

Telomeres consist of a TTAGGG DNA tandem repeats with the associated shelterin [25,26,41]. A large body of evidence suggests that cells respond to dysfunctional telomeres by undergoing senescence, cell death or genomic instability [56,212,213].Telomere integrity depends on the ability to maintain telomere length and / or the ability to mask telomeres from being recognized as damaged DNA. Uncapped telomeres, either by loss of function of telomere-binding proteins or by loss of telomeric repeats, directly associate

with many DNA damage response proteins and induce a response similar to that observed with DNA breaks [214-216]. Several proteins known to play a role in the response to DNA damage (Ku, Mre11, Rad50, etc.) are also integral telomere-associated components playing a dual role in the protection of chromosome ends and the ability to signal cell-cycle arrest in response to dysfunctional telomeres [217-219]. When cells continue to divide, they reach a state of crisis and dysfunctional telomeres cause genomic rearrangements [75] as evidence of teomeric end-capping defects, i.e chromatid associations (SA) and telomere signal-free end (SFE).

Our results show that Snail1 depletion in MSCs promotes SFE and SA, which are genomic rearrangements (Figures 11 and 12). Moreover, these telomere alterations have an impact on the normal function of telomeres (Figure 13). These results suggest that Snail1 is essential for telomere maintenance and integrity. In doing so, Snail1 promotes the acquisition of a new trait for tumorigenesis. Moreover, terminal restriction fragment analysis (TRF) performed in HTC75 cell line show that cells overexpressing Snail1 have an increase in telomere length compared to CT cells or cells overexpressing the mutant Snail1-P2A (Figure 15), confirming that Snail1 transcriptional activity is necessary for maintaining telomere length.

1.2 Snail1 Has no Effect on Shelterin Protein Expression

We decided to study whether Snail1 had a role in the regulation of shelterin protein expression or other telomeric-associated proteins since these proteins are essential for chromosome capping and telomere integrity, stability and length [27,28,39]. Shelterin maintains telomere length and preserves genome integrity by regulating the

access of telomerase to chromosome ends, by controlling end-resection at newly replicated telomeres, and by masking telomeres from the DDR [43,50]. Moreover, several shelterin subunits are negative regulators of telomere length. Although the shelterin complex can exist as a unit, individual subunits have distinct functions and sometimes, unique mechanisms to regulate their stability on telomeres. TRF1 has been described to be the main regulator of telomere length among the shelterin members by inhibiting access of telomerase to chromosome ends [220-221]. TIN2 not only modulates the binding of TRF1 to telomeres, but it is also an essential mediator of TRF1 function [222].

As shown in Figure 16, our results regarding the regulation of some shelterin proteins indicate that Snail1 has no effect in the mRNA expression of TIN2 and TRF1 nor in the mRNA and protein expression of the transient telomeric associated protein Rad51, suggesting that Snail1 regulates telomere integrity and length by other mechanisms. However, other members of shelterin complex and other telomeric associated proteins such as tankyrase, Rad50 and Mre11 have not been explored in this thesis, thus, remaining inconclusive whether Snail1 might have a role in their regulation.

2. SNAIL1 REGULATES TERT EXPRESSION

Since Snail1 is necessary for telomere integrity and length, we explored whether Snail1 regulated telomerase since it is the main mechanism used by cells to elongate and maintain telomeres [70,71]. Telomerase is mainly composed by the reverse

transcriptase (TERT) and telomerase RNA component (TERC). Results from our group indicate that TERC is not regulated by Snai1; thus, in this thesis, we have focused on TERT.

Although we observed that Snail1-KO MSCs presented shorter and abnormal telomeres compared to CT MSCs (Figures 11, 12, 13), these cells showed an increase in mRNA TERT expression compared to CT cells (Figure 17), suggesting that Snail1 is involved in the regulation of TERT expression and activity. Thus, in MSCs, Snail1 depletion leads to an increase in TERT expression although telomeres are shorter and abnormal, suggesting that other mechanisms rather than TERT are involved in telomere length regulation. To further confirm that Snail1 regulates TERT, we overexpressed Snail1 in Snail1-KO MSCs and in two epithelial cell lines that do not normally express Snail1: NMuMG and RWP1 cell lines. In all cases, overexpression of Snail1 produces TERT down-regulation (Figures 18, 20, 22) indicating that Snail1 is a transcriptional repressor of TERT.

It has been widely described that in general, mRNA TERT expression correlates to enzymatic activity. In our model in MSCs, mRNA TERT expression do correlate with enzymatic TERT activity in MSCs as it has been assessed by TRAP assays shown in Figure 22.

2.1. Snail1 Is a Transcriptional Repressor of TERT

Since TERT is the major factor involved in telomerase activity, its regulation is tightly controlled. Regulation of TERT is achieved at many levels: gene expression, alternative splicing, protein folding, post-traslational modification, interacting partners and epigenetic

mechanisms [223]. In fact, transcriptional repression of the TERT gene is the main mechanism by which telomerase is suppressed in normal differentiated cells [224]. Until now, there have been described a variety of transcription factor binding sites within the TERT promoter. Among these, TERT promoter contains binding sites for c-Myc (that binds to a specific E-box motif CACGTG) [225], Smad3, Sp1 [226], Ap2 and ETS [227]. All of these enhance TERT expression.

In this thesis we describe a new transcriptional regulation of TERT, since we show that not only mTERT promoter contains Snail1-binding sites (E-boxes) (Figure 23), but also Snail1 directly binds to TERT promoter in MSCs (Figure 26) repressing its activity (Figure 24). As shown in Figure 25, the repression of mTERT promoter activity by Snail1 has also been demonstrated in RWP1 and 293T cell lines. It has been described that Zeb1 and TERT interacts forming a complex that binds to E-cadherin promoter repressing its expression and consequently, contributing to the occurrence of EMT [228]. Since Snai1 favors Zeb1 binding to E-cadherin promoter to repress its expression [229] we postulate that Snail1 might have the same role in favoring Zeb1 binding to mTERT promoter.

Our results together with what has been published before, reflect the complexity of TERT gene regulation. All of these transcription factors are the effectors of a wide range of cellular signaling pathways, enabling TERT and telomerase activity to be regulated in response to different stimuli.

2.2. TERT Is Downregulated During EMT

Since Snail1 is a transcriptional factor that plays a key role in orchestrating EMT [135,136], and given an important role of EMT in cancer invasion and metastasis, we sought to explore a link of mTERT with EMT. For that aim, we used the well-stablished model of NMuMG cells treated with TGFβ. In Figure 27 we can observe that mTERT transcription decreases upon 2h of TGFβ1 treatment, concomitantly with Snail1 up-regulation. However, although TERT is reduced at the onset of EMT we have not found a role for this down-regulation yet, since mTERT overexpression has neither effect on cell morphology nor in mRNA expression of the main genes related to EMT in our model..

2.3. TERT Has no Effect on Glucose Consumption

Snail1 is involved in promoting metabolic reprogramming by reducing the expression of enzymes necessary to convert glucose in fatty acids in cancer cell lines [183-185]. In fact, the role of Snail1 in regulating metabolic reprogramming has been widely studied in the onset of EMT, where cancer cells undergoing EMT have increased respiration accompanied by elevated oxygen and glucose consumption [183]. In this scenario, and as expected, using the model of NMuMG cell line treated with TGFβ, we observed an increase of glucose consumption upon Snail1 induction, but ectopic TERT overexpression did not affect glucose consumption, suggesting that TERT is not involved in that metabolic reprogramming and that the down-regulation of TERT during EMT is not important for that aim.

2.4. TERT Is Involved in Inflammation

Telomerase plays a major role in protecting telomeres from erosion that results from DNA replication and oxidative damage. In addition to this canonical function, there is an accumulating evidence that indicates that TERT possesses extratelomeric cellular functions called non-canonical functions [230]. These non-canonical activities include stimulation of cell proliferation, protection against oxidative damage and apoptosis, modulation of global gene expression, activation of stem cells and tumor promotion [231,232]. Interestingly, some of these non-canonical functions of TERT are involved in signaling cascades that are important for cancer development and progression, including the NF-κB and Wnt/β-catenin pathways [87]. Moreover, it has been described that telomerase is activated during immune response and wound healing [233,234].

In order to ascertain the biologic relevance of TERT repression by Snail1 we compared the global transcriptome of Snail1-CT and Snail1-KO MSCs upon TERT overexpression. Global transcriptome analysis indicate that 594 genes were differentially regulated by ectopic expression of TERT, most of them involved in pathways associated to cell signaling, inflammatory response and immunological signal. In fact, it has been published that TERT binds to NF-κB p65 subunit that leads to enhanced transcription of a subset of NF-κB target genes such as IL-6 and TNF-α [93], and the roles of IL-6 in tumorigenesis and maintenance of chemo and radio-resistant niches involved in metastasis of cancer cells is well described [235]. We confirmed that a subset of genes were differently regulated upon TERT overexpression and between CT

and Snail1-KO cells. We observed that Snail1 depletion and TERT overexpression produced an increase in the expression of Casp1, Casp4 and Casp12. This group of caspases are involved in inflammation and immune response rather than in apoptosis [205-207], suggesting a role of TERT in the regulation of inflammation as it has been published before [93,95]. These caspases process or induce the secretion of inflammatory cytokines (such as IL-18 and IL-1β) in cells of the innate immune system, as for example macrophages. In our model of MSCs, Snail1 and TERT, through the modulation of these caspases, might be involved in monocyte recruitment: Snail1 disminishes it and TERT increases the recruitment, suggesting that there is a different secreted cytokine pattern expression induced by Snail1 or TERT. The increasing monocyte recruitment by TERT is in accordance of what has been published before in cancer cells; thus, reactivated telomerase /TERT forms a loop with NF-κB in which telomerase cooperates with p65 on a subset of target gene promoters. This enhances NF-κB transcription that drives cellular proliferation, resistance to apoptosis and creates a chronic inflammatory state, which causes infiltration of immune cells such as macrophages creating a favourable microenvironment for tumor growth.

It is also important to consider that macrophages derived from monocyte precursors undergo specific differentiation depending on the local tissue environment. There has been described two main phenotypes: the M1 macrophage phenotype, involved in the classical innate immune response, and the M2 macrophage phenotype, involved in tumor promotion and immune regulation [236]. It remains to be established whether Snail1 and TERT

participate also in the polarization of monocytes towards a M2 phenotype.

Although TERT overexpression induces resistance to apoptosis [87-89], we have not explored that issue in this thesis. This data does not fit with the negative repression of this gene by Snail1, since the action of Snail1 preventing apoptosis have been very extensively documented [176]. In any case, it would be interesting to determine whether TERT repression is also relevant for the Snail1 induced resistance to several proapoptotic stimuli.

3.THE ROLE OF SNAIL1 IN TELOMERE TRANSCRIPTION

3.1 Snail1 regulates telomeric transcription

The results of this thesis show that although Snail1-deficient cells presented shorter telomeres, Snail1 did repress telomerase transcription. It suggests that Snail1 is regulating telomere maintenance by controlling other mechanisms than telomerase and shelterin protein expression. In fact, it is known that telomere maintenance also depends on the proper assembly and regulation of telomeric chromatin [96,237]. Traditionally, telomeres have been considered heterochromatic structures associated with condensed chromatin and transcriptional silencing [237-239]. However, more recent studies have revealed that many eukaryotic telomeres are transcribed, indicating that telomeric silencing is incomplete and that telomere chromatin is dynamic [100,104,110,114]. In addition, the chromatin structure of telomeres is complex due to the specific

variations in the subtelomeric DNA structures, suggesting that telomeric heterochromatin structure and regulation may vary among different chromosomes [240-242].

Telomeres are transcribed into telomeric repeat-containing RNAs (TERRA), a large non-coding RNAs that form an integral part of telomeric heterochromatin. Although the function of TERRA is not completely understood, it seems that TERRA acts as a molecular scaffold for proteins required for correct telomere function. There is increasing evidence that shows that telomeric transcription controls telomere structure and function [106]. In that direction, TERRA interacts directly with TRF2 and ORC1 to form a stable complex, which plays a central role in telomere structure maintenance and heterochromatin formation [106].

The results in this thesis indicating that Snail1 regulates telomere integrity and length and represses telomeric transcription are consistent with previous observations in cells from the human pathology ICF (immunodeficiency, centromeric region instability, facial abnormalities), a disease caused by hypomethylation of subtelomeric regions [243]. In ICF patient cells, TERRA levels are abnormally elevated and telomeres shortened, with associated telomere aberrations such as SFE but with unaltered levels of telomerase [243]. In fact, it has been previously suggested that up-regulation of TERRA transcription leads to several telomeric aberrations including SFE and SA, heterochromatin reorganization and telomere shortening [100]. Other results have also shown that TERRA is required for proper telomere organization and maintenance [103,105,106,108, 109,114,115,244]. It is likely that the

levels of TERRA are tightly controlled and increases or decreases of TERRA transcripts might cause telomeric defects.

Moreover, few transcription factors have been involved in the control of telomeric structure and function, such as CTCF and cohesin subunits [245]. A recent report has shown that Rb increases 15q TERRA transcription through direct binding to its promoter [246]. In that direction, our results show that Snail1 is involved in the 2q, 11q and 18q TERRA repression since its depletion leads to a significant increase in TERRA transcripts, and when Snail1 is reintroduced in Snail1-KO MSCs, TERRA transcripts are down-regulated. Furthermore, we analyzed TERRA expression in CAFs obtained in our laboratory from murine epithelial tumors [209]. These fibroblasts are activated and show elevated Snail1 levels, necessary for their role in stimulating epithelial tumor cell invasion [209]. The results regarding TERRA expression were in the same direction. Thus, we describe another level of regulation of telomeric transcription by Snail1. Further work is necessary to elucidate the mechanism by which Snail1 represses of 2q, 11q and 18q TERRAs. The repression might be consequence of the interaction of Snail1 with the proximal promoters; alternatively or additionally, it might be related to the Snail1-induced expression of transcriptional repressors such as Zeb1 or Zeb2 that potently repress epithelial genes [247].

3.2. Snail1 represses telomeric transcription during EMT

We also investigated the role of TERRA during EMT in the widely used model of NMuMG cells treated with TGFβ1. We observed that telomeric transcription is transiently downregulated upon 2 h of exposure of this cytokine, correlating with the up-regulation of Snail1

and previously to the expression of most mesenchymal genes (Figure 39). This results confirm and extend previous work indicating that Snail1 regulates pericentromeric heterochromatin transcription [173]. Snail1 repression of pericentrometric Major Satellite RNA also takes place early during EMT and is required for the completion of the process. Compared with Major Satellite, ectopic TERRA up-regulation shows a lower impact on EMT since I did not detect changes in cell morphology (Figure 41) and preliminary results in our lab indicate that there are also no changes in cell migration or invasion with respect to control cells. It is probably that the global contribution of TERRA is lower than that of Major Satellite in the cellular organization of heterochromatin; therefore, its role in the chromosome repositioning during EMT [173] should be lower. Accordingly, we have not detected alterations in essential transcriptional factors such as Zeb1 and Zeb2, contrarily to what is observed when Major Satellites are overexpressed [173]. In any case, we observed that ectopic expression of TERRA prevented the transcription of some EMT-related genes: FN, Fgfr1 and Loxl1 (Figure 42). However, it remains to be established what are the functions of these genes and if their lower expression prevent some other actions of mesenchymal genes such as their communication with tumoral cells or with other roles from stroma.

CONCLUSIONS

1. Depletion of Snail1 in MSCs leads to abnormal and short telomeres.

2. The expression of some members of shelterin protein complex and telomeric associated proteins is not affected by Snail1.

3. The transcriptional repressive activity of Snail1 is necessary for effective telomere elongation in HTC75 human cells.

4. Although Snail1-deficient cells present shorter telomeres, Snail1 represses telomerase transcription and binds to mTERT promoter.

5. mTERT expression is down-regulated during EMT associated to Snail1 up-regulation.

6. Global transcriptome analysis indicate that 594 genes were differentially regulated by ectopic expression of mTERT, most of them involved in pathways associated to cell signaling, inflammatory response and immunological signal.

7. Snail1 depletion and mTERT overexpression produced an increase in the expression of Casp1, Casp4 and Casp12 mRNA levels in MSCs, suggesting a differential role of mTERT and Snail1 in the regulation of inflammation.

8. In MSCs, Snail1 and mTERT are involved in monocyte recruitment: Snail1 diminishes it and mTERT increases the recruitment, suggesting that there is a different secreted cytokine pattern expression induced by Snail1 or mTERT.

9. Snail1 regulates transcription of TERRA telomeric transcript. Snail1 is involved in the 2q, 11q and 18q TERRA repression.

10. Telomeric transcription is transiently down-regulated correlating with the up-regulation of Snail1 during EMT.

11. Ectopic expression of TERRA prevented the transcription of some EMT-related genes: FN, Fgrf1 and LOXL1.

MATERIALS AND METHODS

1. CELL CULTURE

1.1. Stable Cell Lines

Cell lines were obtained from the Cancer Cell Line Repository Facility (IMIM). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), except for THP-1 cells that were grown in Roswell Park Memorial Institute medium (RPMI, Invitrogen). Medium was supplemented with 4.5 g/L glucose (Life Technologies), 2 mM glutamine, 100 U/mL penicillin, 56 µg/L streptomycin, and 10% fetal bovine serum (FBS; all from GIBCO). Cells were maintained at 37ºC with humid atmosphere containing 5% CO_2 and 95% air.

Cell lines used in this thesis:

- **RWP-1**: Human pancreatic cell line. Epithelial morphology growing in colonies. Stable RWP-1 cells expressing pcDNA3-Snail1-HA and Snail1-P2A-HA were generated in our laboratory [204] and maintained through the addition of G418 antibiotic (GIBCO) to the medium at 500 µg/mL in the cell culture.
- **NMuMG**: Mouse mammary cell line. Epithelial morphology with high levels of E-cadherin expression.They grow forming colonies and undergo EMT when treated with TGFβ1 (5 ng/mL). NMuMG cells were also supplemented with insulin (10 µg/mL). Cells were collected after 0, 2, 8 and 24h hours.
- **HEK293T**: Human embryonic kidney cell line derived from the HEK 293 cell line. Epithelial morphology. They are very easy to grow and transfect due to the expression of SV40 large T antigen. Transfected plasmid DNAs that carry the SV40 origin

of replication can replicate in 293T and will transiently maintain a high copy number of expressed protein.

- **MEFs**: Mouse embryonic fibroblasts obtained from the Snail1-conditional KO mice in our laboratory [173]. Mesenchymal morphology.
- **HTC75**: Human fibrosarcoma cell line. HT1080-derived clonal cell line that expresses high levels of telomerase (TERT) and maintains its telomeres at a constant length. Mesenchymal morphology. HCT75 cells were a gift from Susan Smith (Skirball Institute of Biomolecular Medicine, New York).
- **CAFs**: Cancer Associated Fibroblasts obtained from tumors in our laboratory [209].
- **THP-1**: human monocytic cell line derived from an acute monocytic leukemia patient. They exhibit a large, round, single-cell morphology and grow in suspension.

1.2. Primary Cell Isolation and Culture

1.2.1. Murine MSC:

Mouse mesenchymal stem cells were obtained from bone marrow as indicated [248]. Briefly, 6-8 weeks old mice were sacrificed by cervical dislocation and their femurs and tibiae were carefully cleaned from the adherent soft tissue. Proximal and distal epiphysis were cut with a scalpel and the bone marrow was harvested by insertion of a syringe needle (31G) into the bone and flushing with cold DMEM (Invitrogen) 10% FBS (GIBCO). Bone cavities were washed at least three times using the syringe until the bones became pale. Collected medium from the washes was filtered by 100 µm filter. For each mouse, collected medium was split in two and each one plated in a gelatin-coated p60 cell culture dish and cultured at

37 ºC in 5 % CO_2. After 6h, cells were washed three times with PBS and maintained with DMEM (Invitrogen) supplemented with 100 U/mL penicillin 100 µg/mL streptomycin, 2 mM glutamine, and 10 % FBS (GIBCO). This procedure was carried out every 12 h the following three days post extraction and then once a day until colonies of MSCs appeared. When the colonies were big enough (about 50 % of confluence), cells were trypsinized for 2 min at 37ºC and plated again. All the attached cells after the 2 min of trypsinization were discarded. The medium was changed every three days. MSCs were isolated from a conditional knockout (KO) mouse (Snail1$^{Flox/Flox}$) in order to obtain MSCs Snail1-KO (see retrovirus infection).

2. TRANSFECTION AND INFECTION PROCEDURES

2.1. Retrovirus Infection

2.1.1. Depletion of Snail1 in MSCs

In order to obtain MSCs Snail1-KO cells, Snail1 deletion was induced by transfection of MSC (Snail1$^{Flox/Flox}$) with pcDNA3-Cre or pcDNA-GFP as a control. We used Plat-E Retroviral Packaging Cell Line [249] for retrovirus production since they stably produce retroviral structure protein. Plat-E were seeded on a 10 cm plate and when they reached 70-80% confluence were transfected. Cells were transfected (day 0) using Lipofectamine PLUS reagent (Invitrogen) either with pcDNA3-Cre or pcDNA3-GFP according to manufacturer's instructions. 24 h (day 1) and 48 h (day 2) post

transfection, the transfection medium was collected and filtered through 0.45 µm filter (Millipore), mixed 3:1 with Retro-X Concentrator (Clontech) and incubated 24h at 4ºC. Afterwards, conditioned medium was centrifuged at 1000 g for 45 min; pellets were resuspended in 1 mL DMEM, 10% FBS, aliquoted in 100 µL and kept at -20ºC until their use to infect MSCs.

The host cells (MSCs) were seeded on a 10 cm culture plate in complete growth medium. At 40% confluence, 100 µL of concentrate virus was added with 0.8 µg/mL polybrene, and 24h later, the medium was replaced for fresh medium with 2 µg/mL puromycin (Sigma) and cells were selected for 48h.

2.1.2. Overexpression of mTERT in NMuMG and MSCs

NMuMG cell lines overexpressing mTERT were generated by stable infection with retroviruses with pBABE-mTERT (addgene #36413) or pBABE-empty vector as a control. The same protocol described above was used. Stable transfectants were obtained after selection with 1 µg/mL of puromycin.

2.1.3. Generation of HTC75 sublines

HTC75 sublines expessing either wild-type (WT) Snail1 (pBABE-Snail1-HA) or the transcriptional inactive P2A mutant (pBABE-Snail1-P2A-HA) [135] were generated by retroviral infection using the same protocol as above (2.1.1). pBABE-empty was used as a control.

Two important concepts should be differentiated:

The passage number simply refers to the number of times the cells in the culture have been sub-cultured, often without consideration of the inoculation densities or recoveries involved.

Population Doubling (PD) refers to the total number of times the cells in the population have doubled since their primary isolation in vitro.

One important mark when studying telomeres is the PD. In our case, after the infection of Snail1 and Snail1 P2A and selection with puromycin, the PD was set to PD0. PD indicates the “age” of the cell population, and differences in telomere length can be studied between early and late population doubling. For this aim, once cells were infected and selected with 2 µg/mL of puromycin, they were grown until confluence and cells were always harvested at the same time. Every time cells were trypsinized, two cell pellets were collected to study telomere length (TRF assays) and to perform protein and RNA analysis.

In summary, on day 1, 10 cm dishes containing $2x10^6$ cells were infected. On day 2, infected cells were selected with 2 µg/mL puromycin. On day 3, cells were sub-cultured 1:2 and upon confluence (day 4 for HTC75) were designed PD 0. Cells were grown in DMEM supplemented with 10% calf serum and were continuously selected in puromycin until PD 72 (approximately 2 months).

2.1.4 TERRA Overexpression

To overexpress TERRA, we took the same approach as Wang Z et al. in [210]. NMuMG cells were infected with retroviruses (see above) using pLU-Flag-Vp16-TRF1ΔN or pLU-Flag-TRF1ΔN vectors (a gift of Paul Lieberman, The Wistar Institute, Philadelphia, USA) and selected with puromycin (1 µg/mL). Cells were trasduced with the ectopic expression of TRF1ΔN (44-439), a truncated form of TRF1 shelterin component (localize to telomeres and displace the full

length endogenous TRF1 protein from telomeres) or TRF1ΔN fused to the transcription activation domain of Vp16, Vp16- TRF1ΔN able to activate TERRA transcription [210].

2.2 Transfection

NMuMG cells and Snail1-KO MSCs were transiently transfected with pcDNA3-Snail1-HA. For that procedure, cells were seeded in 10 cm plates, and when 60% of confluence was reached, they were transfected with 200 ng of pcDNA3-Snail1-HA or pcDNA3-empty using Lipofectamine-Plus reagen (Invitrogen) for 6 hours according to the manufacturer's instructions. After 24h of transfection, cells were harvested and RNA or protein levels were analyzed.

3. CLONING PROCEDURES

Generation of pBABE-mSnail1-HA and pBABE-mSnail1-p2A-HA was previously described [142]. pcDNA3-Snail1-HA vector was generated as previously specified [135].

To obtain the pGL3-TERT Prom reporter vector, a fragment of mTERT gene (5kb-mTert-EGFP) corresponding to -599 to +53 with respect to the transcriptional start site was cloned by PCR with primers containing KpnI and HindIII restriction sites into the KpnI / HindIII sites of the pGL3 plasmid (Promega) (Table 1).

Primer	Sequence
Tert-P- 1F	5'-ATGGTACCTTGCTATGGGTGCGTGAGTT-3'
Tert-P-1R	5'-ATCAAGCTTGGGAACCAAGATGCAAGGG-3'

Table 1. mTERT promoter cloning primers. Primers used to obtain the pGL3-mTERT plasmid.

pEGFP-N1 vector [250] containing 5 kb-mTERT was a gift from Alfonso Gutierrez-Adan, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid. The pEGFP-m5KbTERT cDNA was amplified by PCR with primers Tert-prom 1F and TERT-prom 1R.

4. RNA ANALYSIS

4.1. Phenol-chloroform RNA Extraction

Cells were washed twice with cold PBS and lysed in 800 µLTrizol® reagent (Invitrogen). The lysate was vortexed, 200 µL chloroform added, mixed and incubated at room temperature for 2 min. The solution was centrifuged at maximum speed (15,000 rpm) at 4ºC for 15 min and the transparent supernatant was transferred into a new tube and mixed with 500 µL isopropanol. Incubation for 20 min at -20ºC precipitated the RNA, which was pellet at 15,000 rpm at 4ºC for 15 min. The pellet was washed with 1 mL 75% ethanol and centrifuged at 15,000 rpm at 4ºC for 5 min. After evaporation of all ethanol in a bath at 55ºC, the RNA pellet was resuspended in RNase-free water and dissolved for 10 min at 55ºC prior to quantification. Quantifications were performed in a NanoDrop TM

1000 spectrophotometer (Thermo Scientific) after treating samples with DNAse Turbo (Ambion) to eliminate DNA contamination.

For microarray approach, RNA was extracted using GenElute™ Mammalian Total RNA miniprep Kit (Sigma-Aldrich) according to manufacturer's instructions.

4.2. Microarray Gene Expression Analysis

Gene expression levels of MSCs Snail1-CT and Snail1-KO transfected with or without mTERT was assessed by microarray approach at the IMIM core facility SAM (Microarray Analysis Service). For this aim, microarrays analysis, amplification, labeling and hybridizations were performed according to protocols from Ambion WT Expression Kit (Ambion). 100 ng total RNA samples were labeled using the WT Terminal Labeling Kit (Affymetrix), and then hybridized to GeneChip Gene 2.0 ST Array System for Mouse (Affymetrix) in a GeneChip Hybridization Oven 640. Washing and scanning were performed using the Hybridization Wash and Stain Kit and the GeneChip System of Affymetrix (GeneChip Fluidics Station 450 and GeneChip Scanner 3000 7G). After quality control of raw data, they were background corrected, quantile-normalized and summarized to a gene-level using the robust multichip average (RMA). Genes with an absolute fold change (FC) value above 0.5 were selected as significant.

4.3. Reverse Transcription and Real Time qPCR

RNA was retrotranscribed using oligo dT and the Transcriptor First Strand cDNA Syntesis Kit (Roche) at 65ºC for 10 min following manufacturer's instructions. Analyses were carried out in triplicates

with 50-100 ng of cDNA with SYBR Green I Master Reagent using a LightCycler 480 Real Time PCR System (Roche) with the following conditions: 40 cycles amplification, 94ºC for 30 sec, 60ºC for 15 sec, 72ºC for 10 sec. The primers used for the quantitative RT-PCR are indicated in Table 2. Relative quantification for the studied genes was calculated using Light Cycler 480 Software 1.5.0 by the ΔCt method. All values obtained were normalized by housekeeping gene Pumilio.

Primer	Sequence 5'-3'		Ann T[a]
mSnail1	Fw	GCGCCCGTCGTCCTTCTCGTC	60ºC
	Rw	CTTCCGCGACTGGGGGTCCT	
mTert	Fw	ACCAGGCACAATGAGCGCCG	62ºC
	Rw	AGCTCCCGTAGCCGCACTCT	
mchr2q-TERRA	Fw	TTTCCAGTGATGGCCGACTAG	60ºC
	Rw	CCCCGGAGCTCTTGACTCT	
mchr11q-TERRA	Fw	TGCCATTGGAACACAGCAA	60ºC
	Rw	CGTCTGCTGAGGTCCACAGA	
mchr18q-TERRA	Fw	CAGGCCAAAGAAGGGACAGA	60ºC
	Rw	GCTTCCTCACTGATCCACAGTACA	
mE-cadherin	Fw	TTCAACCCAAGCACGTATCA	60ºC
	Rw	ACGGTGTACACAGCTTTCCA	
mFibronectin	Fw	AGCAAGCCTGAGCCTGAAGAG	60ºC
	Rw	GCGATTTGCAATGGTACAGCT	
mVimentin	Fw	GGCTGCGAGAGAAATTGC	60ºC
	Rw	TCTCTTCATCGTGCAGTTTCTTC	
mFgfr1	Fw	GGAGTTAATACCACCGACAAGG	60ºC
	Rw	TTGGTGCCGCTCTTCATCTT	
mZeb1	Fw	TCAGCTGCTCCCTGTGCAGT	60ºC
	Rw	AAGGCCTTCCCGCATTCAGT	
mLoxl1	Fw	ATGGTCGCGGCCTCCCTGACTTA	60ºC
	Rw	TCCGCTGTGCCTTGGTTTTTCACT	
mCaspase 1	Fw	TGGCAGGAATTCTGGAGCTT	60ºC
	Rw	CTTGAGGGTCCCAGTCAGTC	
mCaspase 4	Fw	TCTCACTGAGGTATGGGGCT	60ºC
	Rw	GCTCCTCTTTCACCACCACA	

mCaspase 12	Fw	GCACCGAAACAAAAAGCCAGA	60ºC
	Rw	AGCTCAACACACGTTCCTCA	
mIrf9	Fw	GTCTGGAAGACTCGCCTACG	60ºC
	Rw	TGGTTCCGTGGTTGGTTAGG	
mMmp9	Fw	GTCCAGACCAAGGGTACAGC	64ºC
	Rw	ATACAGCGGGTACATGAGCG	
hTert	Fw	CGGAAGAGTGTCTGGAGCAA	60ºC
	Rw	GGATGAAGCGGAGTCTGGA	
mTIN2	Fw	TCCCGTGGCTCCGCGAAATG	62ºC
	Rw	TTCCCGGACGCTCCCGTAGG	
mTRF1	Fw	CTGCGGGCTGGATGCTCGAC	60ºC
	Rw	TAGCCTCGGCGCTGTCACGA	
mRad51	Fw	TGATGAGTTTGGTGTCGCAGTG	60ºC
	Rw	CGAACATGGCTGCTCCATCTAC	
Pumilio	Fw	CAGGTAATTAACGAGATGGTGCG	60ºC
	Rw	ACGGGTGCGTAGACAAAGC	

Table 2. Primers used for qPCR

4.4. Dot Blot Analysis

TERRA RNA expression was determined by dot blot analysis. 5 µg RNA were resuspended in 1mM EDTA pH 8.0 to a final volume of 50 µL, mixed with denaturing solution, incubated at 65ºC for 30 min and then immediately cooled down on ice. The RNA was spotted on a Hybond-N membrane (Amersham) using a dot blot apparatus. The membrane was then UV cross-linked at 125 mJ in UV Stratalinker 2400 (Stratagene) and pre-hybridized in Church buffer at 55ºC for 2 h. The 32P-labeled $(TAACCC)_4$ probe was added and incubated at 55ºC overnight. The blot was washed twice in wash buffer 1 at room temperature, and once in wash buffer 2 at 50°C. Radioactive signals were collected with a phosphorimaging screen, and the signal was measured using a Typhoon 9410 Imager (GE Healthcare). A 18s rRNA probe (5'-CCATCCAATCGGTAGTAGCG-3') was 5′ end-

labeled with ^{32}P using a T4 Polynucleotide Kinase (New England Biolabs) and used as internal control.

5. PROTEIN ANALYSIS

5.1. Total Cell Extracts

For protein analysis, the whole cell extract was used. Total cell extracts were obtained using 2% SDS (sodium dodecyl sulfate) lysis buffer. Cells were washed three times with cold PBS and scraped in the plate with 100 µL of 2% SDS lysis buffer. Cell extracts were kept at room temperature to avoid precipitation of the SDS, syringed five times, centrifuged at 13,200 rpm for 10 min and boiled at 95ºC for 3 min.

5.2. Protein Quantification

Protein extracts were quantified in triplicate using the *DC* Protein Assay kit (Lowry method; Bio-Rad).

5.3. Western Blot

Protein was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) by loading 1-40 µg of sample previously mixed with 5X Loading Buffer and boiled at 95ºC for 5 min. Gels had a 7.5-15% polyacrylamide concentration. The Mini-Protean System (Bio-Rad) was used to run gels in TGS buffer that were then transferred to Protran nitrocellulose membranes (Whatman) at 4ºC, during 75 min or overnight, depending on the molecular weight of the protein.

Transfer buffer was used for this procedure. Prior to blocking, Ponceau S staining was performed to each membrane in order to be sure that protein was correctly transferred onto the membrane. Membranes were placed directly from the Transfer buffer to Ponceau S staining and rocked for 5 min. The solution was removed and various washes with distilled water were performed to remove excess stain. Membranes were then blocked in 5% skimmed milk in TBS-T for 1 hour and incubated in the desired antibody for another hour at room temperature or overnight at 4ºC. In table 3 are summarized all the antibodies used. After three 10 min washes with TBS-T, Horseradish peroxidase (HPR)-combined secondary antibody (Dako) was diluted in 5% skimmed milk and the membrane was incubated for 1h at room temperature. Three more 10 min washes with TBS-T were performed. Afterwards, membranes were developed using Luminata Western HRP Substrates (Millipore) and exposed on Agfa-Curix or Hyperfilms ECL (Amersham) for proteins that were more difficult to detect.

Antibody	Specie	Dilution
E-cadherin (610182, Transduction labs)	Mouse	1:2000
Fibronectin (A0245, Dako)	Rabbit	1:5000
Flag (F7425, Sigma)	Rabbit	1:1000
HA (H6908, Sigma)	Rabbit	1:200
Rad51 (sc-8349, Santa Cruz Biotechnology)	Rabbit	1:200
Snail1 [147]	Mouse	1:5
Tubulin (T9026, Sigma)	Mouse	1:50000

Table 3. Antibodies used for Western Blots

6. LUCIFERASE REPORTER ASSAYS

6.1. Reporter Assays in MSCs

For the luciferase reporter assays, mTERT promoter [pGL3-mTERT (-599/+53)] that contains two E-boxes, was transfected to CT and Snail1-KO MSCs. CDH1 promoter [pGL3-E-cad (-178/+92)] was used as a positive control.

For this experiments, $3x10^4$-$4x10^4$ cells were seeded in 24 wells-plate and each condition was transfected in triplicate by using Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions (Promega). The amounts for each well were 200 ng of mTERT promoter or CDH1 promoter. 10 ng of Renilla luciferase (pRL-SV40) were co-transfected as a control for transfection efficiency. 24 h after transfection, cells were washed twice with cold PBS and afterwards, the firefly and Renilla luciferase activities were analyzed using the Dual Luciferase Reporter Assay System (Promega) in a FB 12 luminometer (Berthold Detection System) according to the manufacturer's instructions.

6.2. Reporter Assays in HEK-293T Cells

Reporter assays were also carried out in HEK-293T cells. In this case, cells were also co-transfected with 500 ng of pcDNA3-Snail1-HA or pcDNA3-empty by using Lipofectamine Plus (Invitrogen). CDH1 promoter was also used as a positive control. The procedure was similar as above.

6.3. Reporter Assays in RWP-1 Cells

In this case, we took advantage of stable RWP-1 cells expressing pcDNA3-Snail1-HA generated in our laboratory [204]. The procedure was the same as in MSCs, and again, CDH1 promoter was used as a positive control.

7. CHROMATIN IMMUNOPRECIPITATION (ChIP)

Chromatin immunoprecipitation (ChIP) assays were performed using p150 plates of MSCs at 80% of confluence. Cells were crosslinked with 1% formaldehyde in serum-free DMEM at 37ºC for 10 min. Crosslinking was stop using a final concentration of 125 mM glycine for 2 min at room temperature. From this point on, all the processes were at 4ºC. Cells were scraped in 1 mL cold soft lysis buffer, incubated 20 min on ice and centrifuged at 3,000 rpm for 15 min. Nuclei pellets were resuspended in 0.5 mL SDS lysis buffer and extracts were sonicated 10 times for 10 seconds at 40% (Branson) to generate DNA fragments of around 200-500 bp. Incubation of 20 min on ice and 10 min centrifugation at 13,000 rpm was used to verify lack of sedimentation of the chromatin. Supernatants were diluted 1:10 with dilution buffer, and precleared by incubating with 20 µL of protein G magnetic beads (Millipore) and 1 µg irrelevant immunoglobulins G at 4ºC for 3 hours. Immunoprecipitation was done by adding the primary antibody or the irrelevant G immunoglobulins to the samples and incubating on a rotating wheel at 4ºC overnight. The day after, the immunocomplexes were recovered by incubation with protein G/A magnetic beads, previously blocked with 1 mg/mL salmon sperm (Ambion) at 4ºC for 3 hours.

The beads were then sequentially washed with the following buffers (4 times each): low salt washing buffer, high salt washing buffer and LiCl buffer. Chromatin was eluted from the beads by adding 100 µL elution buffer and shaking at 37ºC for 1 hour. Crosslinking was reverted in the eluted samples by the addition of NaCl at 200 mM final concentration and incubate at 65ºC overnight. The day after, the protein complexes were treated with proteinase K Solution and incubated 1 hour at 55ºC. The DNA was purified with Qiagen PCR Purification kit, eluted in water and analyzed by RT-qPCR.

The primers used in ChIP experiments are shown in Table 4.

Primer	Sequence 5'-3'	
mTert promoter fragment 1	Fw	AGCCCGAGAAGCATTCTGTA
	Rw	CACTGAGAGTCCACGACGAA
mTert promoter fragment 2	Fw	ATCTACCGCACTTTGGTTGC
	Rw	ACCAGCTCTTTCAGGGATGA

Table 4. Primers used for ChIP experiments

8. TELOMERE REPEAT AMPLIFICATION PROTOCOL (TRAP)

Quantitative measurement of telomerase activity in vitro was assessed using the TRAPeze® RT Telomerase Detection kit (Millipore), which is a highly sensitive in vitro assay system for detecting telomerase activity. The assay is a one buffer, two enzyme system utilizing the polymerase chain reaction (PCR).

8.1. Extract Preparation

Cell pellet was resuspended in 200 µL of 1X CHAPS Lysis Buffer. A positive control cell pellet provided in the kit, which has telomerase activity, was also resuspended following the manufacturer's instructions. The suspension was incubated on ice for 30 min and then centrifuged at 12,000 g for 20 min at 4ºC . The supernatant was collected and protein concentrations were determined in triplicate by *DC* Protein Assay kit (Lowry method; Bio-Rad).

8.2. Experimental Design

8.2.1 Controls

TRAP is a very sensitive assay, which allows detection of telomerase activity in a very small number of cells. Special laboratory set up and precautions are required to prevent PCR carry-over contamination and RNase contamination. Therefore, some controls are necessary to set a base line.

8.2.1.1 For each sample: Telomerase is a heat-sensitive enzyme. As a negative control, every sample was tested for heat sensitivity. Thus, analysis of each sample consists of two assays: with or without a heat-treatment. For that treatment, 10 µL of each sample was incubated at 85ºC for 10 min to inactivate telomerase.

8.2.1.2 For each TRAP assay:

- *telomerase-positive cell extract* provided in the kit was used as a positive control. It was prepared following the manufacturer's instructions.

- *internal PCR amplification control* was included to monitor PCR inhibition in every lane. Since many cell extracts contain inhibitors of Taq polymerase and can give false-negative results, the kit contains internal control oligonucleotides K1 and TSK1 which together with TS produce a 36 bp band (S-IC) in every lane, and this band serves as a control for amplification efficiency in each reaction and can be used for quantitative analysis of the reaction products.

- *primer-dimer / PCR Contamination Control*: 2µL of 1X CHAPS Lysis Buffer was substituted for the cell extract. No product except for the 36 bp internal control band is expected to be present.

- *telomerase quantitation control template – TSR8*: TSR8 is an oligonucleotide with a sequence identical to the TS primer extended with 8 telomeric repeats $AG(GGTTAG)_7$. This control serves as a standard for estimating the amount of TS primers with telomeric repeats extended by telomerase in a given extract.

8.3. Experimental Procedure

8.3.1 Assay Set-up

First, a Master Mix with the next reagents was prepared: 10X TRAP Reaction Buffer, 50X dNTP Mix, TS Primer, TRAP Primer Mix, Taq Polymerase and dH_2O. 200 ng of total protein extract was used in each PCR reaction.

8.3.2 PCR Amplification

The PCR conditions were the follow: 30ºC/30 min, 34 cycles of 94ºC/30 sec, 59ºC/30 sec, 72ºC/60 sec, and kept at 4ºC

8.3.3 PAGE and Data Analysis

25 µL of PCR products were mixed with a loading dye containing bromophenol blue and xylene cyanol. PCR products were then separated in a 12.5% non-denaturing polyacrylamide gel in 0.5% TBE buffer. Gels were run 1.5 hours at 400 V. After electrophoresis, the gel was stained with SYBR® Green according to the manufacturer's instructions. Afterwards, the images were quantified with ImageJ software.

9. PNA-FISH OF PROMETAPHASE SPREADS AND ANALYSIS

Fluorescence in situ hybridization using peptide nucleic acid probes (PNA-FISH) is a cytogenetic technique to detect and localize the presence or absence of specific DNA sequences on chromosomes, in our case TTAGGG telomeric repeats.

For prometaphase spread analysis, CT and Snail1-KO cells were seeded in 10 cm plates and when 70% of confluency was reached, they were incubated with colcemide (0.5 mg/mL) for 90 min and collected by trypsinization. Pellets were resuspended in 300 µL of RSB buffer for 10 min at 37ºC, centrifugated for 5 min at 1000 rpm and afterwards resuspended in 4 mL freshly prepared methanol : acetic acid (3:1) to fix them. Metaphase spreads were performed by dropping methanol:acetic acid resuspended pellets onto the slides, incubating at room temperature for 15 min, and air dry overnight in fume hood. The day after, slides were washed with PBS, fixed in 3.7% formaldehyide for 2 min, washed in PBS three times for 5 min, and treated treat with pre-warmed freshly prepared pepsin for 10 min

at 37ºC. The samples where then washed in PBS twice for 2 min, fixed in 3.7% formaldehyde for 2 min, washed in PBS three times for 5 min, and dehydrated in ethanol (70%, 95%, 100% ethanol series for 5 min), air dried and incubated in denaturing pre-warmed solution for 10 minutes at 75ºC in hyb oven. Chromosomes were then hybridized in HYBMIX solution that contained 0.5 µg/mL of a Cy3-conjugated $(TTAGGG)_3$ PNA telomere repeat probe (Applied Biosystems) for 1-2 h at room temperature on humid dark chamber. Then, coverslips were washed twice for 15 min in washing 1 solution, washed three times for 5 min in washing 2 solution, dehydrated in ethanol and stained with DAPI (0.2 µg/mL). Images were acquired using a fluorescent microscope (Axioplan 2, Carl Zeiss, Inc) and processed and merged using ImageJ software.

10. Q-FISH

Telomere length depends on the TTAGGG repetition numbers at telomeres. Q-FISH is a cytogenetic technique based on the traditional FISH methodology. Therefore, it is a good technique to get information about telomere length, and employs Cy3-conjugated $(TTAGGG)_3$ PNA telomere repeat probe labeled with a fluorocrom to stain telomeric repeats on prepared metaphase spreads of cells that have been treated with colcemid, hypotonic shock, and fixation to slides via methanol/acetic acid treatment (see above).

Images were acquired using a fluorescent microscope with oil immersion (Axioplan 2, Carl Zeiss, Inc), and were processed using

TFL-Telo software (kindly provided by P. Lansdorp, Terry Fox Laboratory, Vancouver, Canada), where telomere signals were determined as pixels showing brightest intensities within each selected nuclear region. Data was accumulated by using 40 metaphases for each histogram. The “frequency” or number of telomeres within a given range of telomere DNA intensities was plotted against the telomere DNA signal intensity arbitrary units (0 no telomere DNA signal, and increasing increments of arbitrary telomere fluorescence units up to 161). With these quantitative fluorescence values, telomere length was estimated. Analysis were performed as a blind study as described in [251], in which the presence or absence of Snail1 (CT or KO MSCs) was not known.

11. TERMINAL RESTRICTION FRAGMENT (TRF) ANALYSIS

Telomeric length can be indirectly measured by a technique called Telomere Restriction Fragment analysis (TRF). This technique is a modified Southern Blot, which measures the heterogeneous range of telomere lengths in a cell population using the length distribution of the terminal restriction fragments [116]. This technique is the classical method for measuring telomere length.

Genomic DNA was isolated from HTC75 cells and digested with Hinfl, Rsal, Alul and Mbol, leaving the telomeric and sub-telomeric region uncut and intact. The DNA fragments were then separated by gel electrophoresis on a 0.8% agarose gel for approximately 4 hours at 120 V or until the dye front migrated 15 cm. The separated fragments were then transferred onto a nylon membrane by blotting

overnight using capillary transfer. Telomere restriction fragments were detected by Southern blotting with a ^{32}P-labeled $(CCCTAA)_4$ probe, as previously described in [251]. The telomeric DNA was visible as a smear, and to convert the telomeric specific smear on the Southern Blot image into mean terminal restriction fragment length (TRF), the TIF file was analyzed by densitometry analysis. The mean length of telomere restriction fragments was determined using Telomeric software0 (Fox Chase Cancer Center).

12. GLUCOSE CONSUMPTION

Glucose uptake was assessed in MSCs and NMuMG cells with or without mTERT overexpression by using a glucose assay kit (Abcam, ab65333), in which a Glucose Enzyme Mix specifically oxidizes glucose to generate a product which reacts with a dye to generate color (λ = 570 nm). The generated color is proportionally to the glucose amount. For that aim, $1{\cdot}10^5$ cells were seeded in triplicate in 6 cm plate. In NMuMG cells, 24h later, TGFβ1 was added. 5 days after cells had been seeded, glucose uptake was measured following the manufacturer's instructions.

13. MIGRATION ASSAYS

To measure the effect of TERT expression on cell motility and the effect on the abilities of cell migration, we used special incubation chambers. Only positively migrating cells can pass through the

membrane filters and attach to the underside. Therefore, we can easily stain and count such cells with this experiment.

5 x 10^4 THP-1 cells were seeded on a Matrigel transwell in RPMI FBS 1% in a final volume of 150 µL. After 4h, 5 x 10^4 CT, Snail1-KO or CT overexpressing mTERT MSCs were seeded in DMEM FBS 1% in the lower chamber of the migration system in a final volume of 500 µL. The migration was stopped at 18 h. Cells were washed with PBS and fixed with 4 % p-formaldehyde 20 min. Non-migrating cells were removed from the upper surface of the membrane with a cotton swab. Membrane with the migrating cells (lower surface) were stained with DAPI and mounting for microscopy analysis. Images were obtained and analyzed with ImageJ software by counting nuclei.

14. STATISTICAL ANALYSIS

Values are expressed as the relative mean of at least three independent experiments and error bars represent ± standard error of the mean (SEM). To determine whether two quantitative variables differed significantly, the t-test (when normally distributed) or Mann-Whitney test (non-normally distributed) were applied and statistical significance has been considered * when $p \leq 0.05$ and ** when $p \leq 0.01$. Statistical analyzes were performed with GraphPad Prism 6 software.

15. BUFFERS AND SOLUTIONS

PBS: 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4

Dot Blot:

Denaturing solution (Dot Blot): 30 µL 20X SSC, 20 µL 37% formaldehyde.

Church buffer: 0.5 N Na-phosphate, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA

Wash buffer 1: 0.2 N Na-phosphate, 2% SDS, 1 mM EDTA

Wash buffer 2: 0.1 N Na-phosphate, 2% SDS, 1 mM EDTA

Protein analysis:

2% Total Lysis Buffer: 50mM Tris-HCl pH7.5, SDS 2%, 10% glycerol

5X Loading Buffer: 250mM Tris-HCl pH 6.8, 10% SDS, 0.02% Bromophenol blue, 50% glycerol, 20% β-mercaptoethanol

Running Buffer (TGS): 25 mM Tris, 192mM Glycine, 1% SDS

Transfer Buffer: 50mM Tris-OH, 386mM Glycine, 20% Methanol, 0.1% SDS

Ponceau S Stain: 0.5% Ponceau S, 1% acetic acid

TBS-T: 25 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% Tween-20

ChIP

Cold soft lysis buffer: 50mM Tris, pH 8, 10 mM EDTA, 0.1% NP-40, 10% glycerol

SDS lysis buffer: 1% SDS, 10mM EDTA, 50 mM Tris pH 8.0

Dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7 mM Tris pH 8, 167mM NaCl

Low Salt Washing Buffer: 20 mM Tris HCl pH 8, 5 mM NaCl, 2 mM EDTA 0.1% SDS, 1% Triton X-100

High Salt Washing Buffer: 20 mM Tris HCl pH 8, 150 mM NaCl, 2 mM EDTA 0.1% SDS, 1% Triton X-100

LiCl Buffer: 10 mM Tris HCl pH 8, 1 mM EDTA, 250 mM LiCl, 15 NP-40, 1% sodium deoxycholate

Elution Buffer: 1% SDS, 0.1 M Na_2CO_3

Proteinase K Solution: 10 µl 0.5 M EDTA, 20 µl Tris 1 M pH 6.5, 40 µg proteinase K

TRAP assay

1X CHAPS Lysis Buffer: 10mM Tris-HCl pH 7.5, 1mM $MgCl_2$, 1mM EGTA, 0.1 mM Benzamidine, 5mM β-mercaptoethanol, 0.5% CHAPS, 10% glycerol

10X TRAP Reaction Buffer: 200mM Tris-HCl pH 8.3, 15mM $MgCl_2$, 630mM KCl, 0.5% Tween 20, 10 mM EGTA

50X dNTP Mix: 2.5mM each dATP, dTTP, dGTP and CTP

TRAP Primer Mix: RP primer, K1 primer, TSK1 template

Loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 50% glycerol, 50 mM EDTA

TBE Buffer: 89mM Tris-borate, 2mM EDTA pH 8.3

PNA-FISH

RSB Buffer: 10mM Tris pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$

Freshly prepared pepsin: 1mg/mL pepsin in 10 mM glycine pH 2.8

Denaturing prewarm solution: 70% formamide, 2X SSC

HYBMIX Solution: 10 mM $NaHPO_4$, 10 mM NaCl, 20 mM Tris pH 7.5, 70% formamide, 1X Denhardts, 0.1 µg/mL tRNA, 0.1 µg/mL Herring Sperm DNA, PNA 0.5 µg/mL

Washing 1 Solution: 70% formamide, 10 mM Tris pH 7.5, 0.1% BSA

Washing 2 Solution: 0.1M Tris pH 7.5, 0.15 M NaCl, 0.08% Tween

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