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TELOMERES AND TELOMERASE IN COLORECTAL CANCER STEM CELLS: A CYTOGENETIC CHARACTERIZATION

TELOMERI E TELOMERASI IN CELLULE STAMINALI TUMORALI DI COLON RETTO: UNA CARATTERIZZAZIONE CITOGENETICA

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SOMMARIO

Diverse prove sperimentali e cliniche hanno dimostrato come i tumori solidi siano sistemi complessi e dinamici composti da popolazioni eterogenee di cellule, le quali (1) interagiscono dinamicamente con il microambiente e (2) sono organizzate gerarchicamente, con una sottoclasse di cellule note come staminali tumorali (CSCs) situate all'apice. Le CSCs, sono cellule immature e tumorigeniche e condividono con le normali cellule staminali, diverse caratteristiche tra cui la capacità di auto-rinnovarsi e contemporaneamente generare una progenie differenziata. Le CSCs sono state isolate da diversi tumori, incluso il cancro del colon retto. Le CSCs sono le cellule responsabili dell'inizio, della diffusione e della disseminazione dei tumori e la loro presenza è legata alla resistenza alle terapie convenzionali e alle ricadute. Numerose evidenze sembrano indicare l'esistenza di un certo livello di plasticità nei tumori, inclusa la possibilità che alcune cellule non staminali tumorali siano in grado di riacquisire capacità staminali, sotto l'influenza di determinati stimoli. Inoltre, CSCs fenotipicamente, funzionalmente e geneticamente distinte tra loro possono coesistere all'interno dello stesso tumore. Infine, le CSCs possono evolvere nello spazio e nel tempo, acquisendo e accumulando mutazioni geniche. Queste osservazioni determinano la fine della lunga dicotomia tra il modello stocastico, secondo il quale la trasformazione tumorale è guidata da mutazioni casuali e dalla selezione clonale delle cellule somatiche, e il modello gerarchico, secondo il quale la trasformazione tumorale è legata solo ad una determinata sottopolazione (le CSCs). Ad oggi appare chiaro come sia il modello gerarchico che quello stocastico, siano uniti e come le CSCs esistano e si evolvano [1]. La scoperta di nuove strategie per debellare le CSCs è fondamentale per lo sviluppo di efficienti terapie anti tumorali. Rispetto a ciò, una delle principali strategie è basata sull'evidenza che le CSCs posseggano un'efficiente risposta di danno al DNA (DDR), e quindi possano dipendere specificamente dalla (e essere sensibili a inibitori della) DDR, e probabilmente da meccanismi legati alla biologia del telomero[2].

I telomeri sono complessi nucleoproteici localizzati alla fine dei cromosomi eucariotici e sono costituiti da (1) DNA telomerico, che consiste di ripetizioni in tandem a doppio filamento di 10-15 kilobasi (kb)

dell'esanucleotide TTAGGG, seguite da un filamento singolo 3' ricco in guanine (G), e (2) il complesso Shelterina, un esamero costituito dalle proteine TRF1, TRF2, POT1, RAP1, TIN2 e TPP1 [3]. Uno dei ruoli principali del telomero è quello di impedire l'attivazione della DDR assicurando che le parti terminali dei cromosomi non vengano riconosciute come rotture a doppio filamento (DSBs). La replicazione delle estremità cromosomiche pone un'importante problema nella proliferazione delle cellule somatiche, poiché mette a rischio l'integrità telomerica nelle cellule umane (il cosiddetto "end-replication problem"). Per questo le cellule somatiche possono andare incontro ad un numero limitato di divisioni, un fenomeno noto come "limite di Hayflick" [4]. Infatti, telomeri molto corti (come quelli che hanno raggiungo il limite di Hayflick) possono portare a fusioni telomeriche e riarrangiamenti cromosomici, e potenzialmente all'instabilità cromosomica (CIN) e a una potenziale tumorigenicità. La telomerasi è una polimerasi a DNA RNA-dipendente che catalizza l'estensione del DNA telomerico aggiungendo sequenze ripetute all'estremità 3' dei cromosomi [5]. La telomerasi è solitamente assente nelle cellule somatiche normali, ma è presente (e attiva) nelle cellule della linea germinale, nelle staminali embrionali, nelle pluripotenti, nelle staminali adulte e in ~85-90% delle cellule tumorali [6]. La telomerasi è il meccanismo di mantenimento telomerico più diffuso, ma non è il solo [7]. Infatti, l'allungamento alternativo telomerico (ALT) è indipendente dalla telomerasi, che si basa sul meccanismo di ricombinazione omologa, ed è presente in ~10-15% dei tumori.

In questo progetto, abbiamo caratterizzato a livello citogenetico, un numero consistente di cellule tumorali primarie di colon retto (CRC) arricchite di CSCs (CRC-SCs) isolate da più di 20 pazienti affetti da cancro del colon retto. In particolare, è stata valutata la ploidia, la biologia del telomero e l'attività telomerasica. In netto contrasto con le attuali ipotesi che affermano che le CSCs posseggano un set cromosomico stabile, un'elevata capacità di mantenere una stabilità genomica (e cromosomica) e un'elevata attività telomerasica, abbiamo dimostrato che, almeno nel cancro del colon retto, le CSCs posseggono un'elevata eterogeneità nel numero cromosomico e nei parametri relativi allo status telomerico.

Nel dettaglio, abbiamo osservato che più della metà delle CRC-SCs possiede un cariotipo iperdiploide, suggerendo la loro potenziale

derivazione ed evoluzione da un intermedio tetraploide metastabile, che ha subito uno o più cicli di mal-segregazione cromosomica. Inoltre, abbiamo trovato che le CRC-SCs mostrano valori di lunghezza eterogenei, e nella maggior parte dei casi (11), un valore di lunghezza telomerica più elevato di quello misurato nelle CRCs, con alcune (5) con un valore telomerico simile o più elevato di quello misurato per le cellule di fibroblasti primari umani, il che è in contrasto con le precedenti scoperte [8]. Poiché, l'accorciamento telomerico induce instabilità genomica/cromosomica, abbiamo valutato la correlazione tra il numero di cromosomi e le lunghezze telomeriche, senza trovare una correlazione significativa tra i due parametri. Al contrario, abbiamo dimostrato una correlazione positiva tra la lunghezza telomerica media e la variabilità intra-cellulare delle lunghezze. Questi dati preliminari suggeriscono un ruolo potenziale e rilevante del meccanismo ALT in una parte di CRC-SCs, dal momento che un'alta variabilità intra-cellulare è stata associata al metabolismo dell'ALT [9].

In linea con queste ipotesi, abbiamo osservato un'alta frequenza di telomeri corti e perdita telomerica, nella maggior parte (ma non in tutte) le CRC-SC, in relazione alle cellule tumorali. Inoltre, abbiamo individuate una relazione negativa tra i valori lunghezza media del telomero e la frazione di telomeri corti. Abbiamo inoltre dimostrato che alcune CRC-SCs con telomeri lunghi (ma alta variabilità telomerica inter-cellulare) mostrano una parte significativa di cromosomi senza telomero, confermando la nostra ipotesi sulla possibile attivazione del meccanismo ALT in una parte di CRC-SCs. Abbiamo inoltre dimostrato la presenza di una elevata frequenza di doublets per cromosoma nella maggioranza delle CRC-SCs, indicando un'incompleta o difettosa replicazione telomerica o uno stallo della forca replicativa in queste cellule. Sebbene nessuna relazione sia stata trovata tra la frequenza di doublets e la lunghezza telomerica, in linea con le precedenti osservazioni [10], abbiamo dimostrato che più CRC-SCs con lunghi telomeri presentano anche un elevato numero di doublets. Abbiamo inoltre scoperto che queste cellule sono le stesse che mostrano una risposta di stress replicativa [11]. Alla fine, abbiamo osservato un'elevata variabilità nell'attività telomerasica tra le diverse CRC-SCs, di cui 5 con attività telomerasica assente. Questo dato è in contrasto con i precedenti riportati, nei quali è dimostrato come le CSCs siano telomerasi positive, in maniera

simile alle cellule adulte staminali [12,13] e inoltre suggerisce un possibile ruolo dell'ALT nel mantenimento telomerico delle CSCs.

I risultati ottenuti in questo progetto, fanno luce sullo (e aprono nuovi orizzonti per lo studio dello) stato cromosomico e sulla biologia del telomero nelle CSCs, le quali sono le radici e i semi chemoresistenti del cancro. Questi risultati sono una novità nella ricerca di base sul telomero e siamo certi che avranno importanti implicazioni terapeutiche che saranno fondamentali per lo sviluppo di nuove strategie di annientamento delle CSCs.

SUMMARY

A variety of experimental and clinical evidence demonstrates that solid tumors are complex and dynamic systems composed of heterogeneous populations of cells, which (1) interact dynamically with the tumor microenvironment and (2) are organized hierarchically with a subset of cells, known as cancer stem cells (CSCs) at the apex. CSCs are immature, tumorigenic cells that share with normal stem cells properties, including the ability to self-renew while generating differentiated cells. CSCs have been prospectively isolated from most neoplasms, including colorectal cancer. CSCs are responsible for tumor initiation, propagation and spreading, and their presence drives tumor resistance to conventional/targeted therapy and tumor recurrence. Mounting evidence indicates the existence of a certain degree of plasticity of the tumor system, even including the possibility for non-CSC to re-acquire stemness potential under peculiar stimuli. Moreover, multiple phenotypically, functionally and genetically distinct CSCs can co-exist in an individual neoplasm. Finally, CSCs can evolve in space and time and even acquire and accumulate genetic mutations. These observations wipe off the long-lasting dichotomy between the stochastic model, which assumes that tumor transformation is driven by random mutation(s) and clonal selection of somatic cells, and the hierarchical model, which postulates that tumor is initiated by a fixed subpopulation of CSCs. The current view, indeed, is that the hierarchical and stochastic models are unified and that CSCs exist and evolve [1]. The identification of novel strategies to eradicate CSC is mandatory for efficient anti-cancer therapy. In this context, one main strategy is based on the evidence that CSCs have an

efficient DNA damage response (DDR), and thus can depend specifically on (and be vulnerable by the inhibition of) the DDR and possibly on mechanisms linked to telomere biology [2].

Telomeres are nucleoprotein complexes localized at the end of all eukaryotic chromosomes, composed of (1) telomeric DNA, which consists 10–15 kilobases (kb) of double-stranded DNA tandem repeats of the hexanucleotide TTAGGG and followed by a terminal 3′ G-rich single-stranded overhangs, and (2) the Shelterin complex, which is a hexamer structure consisting of TRF1, TRF2, POT1, RAP1, TIN2 and TPP1 [3]. One main role of telomeres is to prevent the activation of the DDR by ensuring that chromosome ends are not sensed by cells as bona fide DSBs. The replication of chromosome ends poses a serious problem to proliferating somatic cells as it undermines telomere integrity in human cells (so called "end-replication problem"). Therefore, divisions of somatic cells are limited to a finite number of times, a phenomenon known as "Hayflick limit" [4]. Indeed, critically short telomeres (i.e., those reaching the Hayflick limit) can face fusions and rearrangements, potentially leading to chromosome instability (CIN) and conferring a pro-tumorigenic potential. Telomerase is an RNA-dependent DNA polymerase that catalyze the extension of telomeric DNA by adding telomeric repeats to the chromosome 3' end [5]. Telomerase is usually absent in normal somatic cells, but is present (and active) in germline cells, embryonic stem cells, pluripotent cells, adult stem cells as well as in ~85-90% of tumor cells [6]. Telomerase activity is the major, but not the exclusive, mechanism for telomere maintenance [7]. Indeed, alternative lengthening of telomeres (ALT) is a telomerase-independent, homologous recombination-dependent telomere maintenance mechanism occurring in ~10-15% of tumors.

In this project, we have characterized a vast panel of primary CRC cells enriched for CSCs (CRC-SCs) isolated from > 20 colorectal cancer patients at cytogenetic level. In particular, we have evaluated ploidy status, telomere biology and telomerase activity. In sharp contrast with the current hypothesis, which suggests that CSCs possess a stable chromosomal set, high efficient processes for maintaining genomic (and chromosome) stability and elevated telomerase activity, we demonstrated that, at least in colorectal cancer, CSCs present an elevated heterogeneity in chromosome number and telomere-associated parameters.

In more detail, we observed that more than half of CRC-SCs bear a hyperdiploid karyotype suggesting their potential derivation and evolution

from a metastable tetraploid intermediate undergoing one or several rounds of chromosome mis-segregation event(s). In addition, we reported that CRC-SCs display a heterogeneous telomere length, with most of them (11) showing higher telomere length mean value than colorectal cancer cell line, and some (5) higher than primary human fibroblasts, which is in contrast with previous findings [8]. Since telomere shortening is reported to induce genomic/chromosomal instability, we evaluated the correlation between number of chromosomes and telomeres, but we could not find any significant correlation between these parameters. On the contrary, we demonstrated a positive correlation between telomere length mean values and intra-cellular telomeres length variability. This preliminary results suggest a potential relevance of the ALT in a subset of CRC-SCs, as the presence of a high intra-cell variability in telomere length has been associated with an ALT metabolism [9].

In line with this hypothesis, we observed the presence of short telomeres and telomere loss at high frequency in most (but not all) CRC-SCs as compared to tumor cells. Moreover, we reported a negative correlation between telomere length mean values and the appearance of short telomeres. Intriguingly, we also showed that some CRC-SCs with long telomeres (but high intra-cellular telomere length variability) display a significant fraction of chromosome with telomere loss, thereby confirming our hypothesis on the activation of the ALT in a subset of CRC-SCs. Of relevance, we provided evidence of a high frequency of doublets per chromosome in most of CRC-SCs, which indicates incomplete/defective telomere replication or fork replication stalling in these cells. Although we did not find a positive correlation between telomere length values and doublet frequency, which is in line with what previously reported [10], we showed that most long-telomere CRC-SCs present also a high number of doublets. Importantly, these cells are those displaying ongoing replication stress response [11]. Finally, we observed a high variability in telomerase activity within our panel of CRC-SCs, with 5 displaying undetectable telomerase activity. This result is in contrast with previous studies suggesting that, similar to adult stem cells, CSCs are telomerase-positive [12,13] and again suggests a potential relevance of the ALT for telomere biology in CSCs.

The findings obtained in this project shed light upon (and open a new horizon in the study of) chromosome and telomere biology of CSCs, which are the roots and chemo-resistant seeds of cancer. We feel that our results

are a novelty in the basic research dealing with telomere and we are confident that they will have important therapeutic implication providing fundamental insights for the development of novel telomere-based strategies aimed at depleting CSCs.

INTRODUCTION

THE CANCER STEM CELL MODEL

A large body of experimental evidence accumulating over the past years has demonstrated that solid tumors are complex and dynamic systems consisting of heterogeneous populations of cells in close contact with (and highly influenced by) components of the tumor microenvironment [14–16]. Supporting this evidence, high level of intratumor heterogeneity has been detected, both *in vitro* and *in vivo*, in most type of solid cancers and hematological malignancies, and this at multiple levels, including the phenotype, genotype, proliferation rates, epigenotype, karyotype, stemness potential, and therapeutic response [11,17–23].

Two different models have been proposed to elucidate the development of tumors and the acquisition of intra-tumor heterogeneity in established malignancies. The stochastic model postulates that all somatic cells have the potential to initiate neoplasms and maintain tumor growth/survival via mechanisms involving the random acquisition of gene mutations [24–26]. In this model, tumor heterogeneity would result from (and be fueled by) genetic changes occurring during the process of clonal tumor expansion [27,28] and whose positive selection (when they confer an advantage) drive malignant transformation and evolution [29–31]. Alternatively, the hierarchical model postulates that neoplasms are organized in a hierarchical fashion, with a subset of immature, tumorigenic cancer stem cells (CSCs) at the apex [1,32–34]. According to this model, CSCs would be the exclusive cell subpopulation within the tumor mass able to initiate and propagate tumors. The origin of CSCs is still a matter of intense debate. One intriguing hypothesis, which still need to be formally demonstrated, indicates that CSCs could derive directly from the malignant transformation of normal stem cells (the cell-of-origin) experiencing deregulation in networks controlling proliferation, survival or stemness potential [35,36]. However, recent evidence ascribes a high dynamicity to the tumor system, with even differentiated cells displaying some degree of plasticity enabling them to de-differentiate into CSCs in response to certain stimuli [37–39]. These findings indicate that CSC not necessarily originate from normal stem cells. Irrespective to this issue, CSCs share with stem cells peculiar properties, including the ability to self-renew while generating differentiated cells [40,41]. This occurs as CSCs (similar to SCs) can divide either symmetrically,

generating two daughter CSCs, or asymmetrically, generating one CSC and one cell with a certain degree of differentiation, which is known as transient cell [33,42,43]. Of note, in the hierarchical model, transient cells and their differentiated progeny (constituting collectively the pool of non-CSCs) are non-tumorigenic, meaning that only CSCs preserve and propagate the transforming potential within the bulk of tumors [44–46].

A large body of experimental and clinical evidence supports the validity of both models [47,39,48–56]. In addition, the current view is that the hierarchical and stochastic models are not mutually exclusive [1,33,43,50,57–61] (**Figure 1**).

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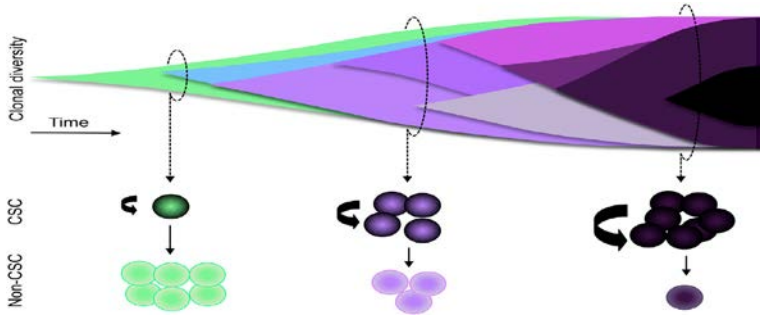


Figure 1. Unified Model of Clonal Evolution and Cancer Stem Cells [1]

According to the unified plasticity model, the tumor is a dynamic system in which non-tumorigenic (non-CSC) cells can re-acquire stemness potential via a process known as plasticity (see also above), which can be driven (among others) by a variety microenvironmental stimuli[59], the most relevant of which are secreted growth[62], immune-mediated[63] and proangiogenic factors [64] . Moreover, there is mounting evidence proving the co-existence, within a neoplasm, of multiple CSCs with phenotypic, functional and genetic heterogeneity, and variable sensitivity to therapeutics[17,65,66]. Thus, CSCs are not a fixed population but a pool that can evolve in space and time, acquire (and accumulate) genetic mutations and thus give rise to distinct subclones on which the evolutionary pressure acts[1,28] (**Figure 2**)

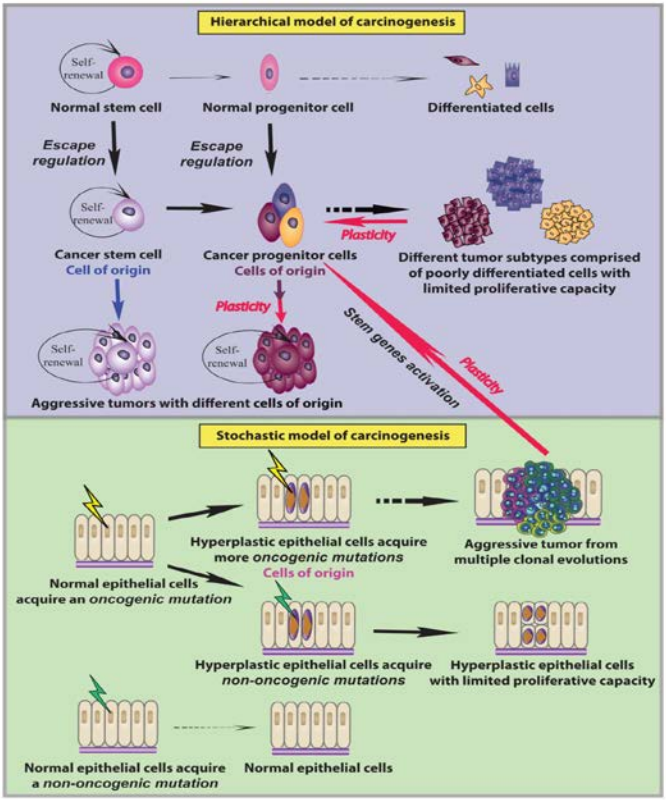


Figure 2. Plasticty model [59]

To add a further layer of complexity, CSCs may exist in different proliferative state depending on the tissue of origin, ranging from quiescence to intense proliferation [33]. Moreover, there exist a dynamic crosstalk between CSCs and their niche, which is fundamental for promoting tumor growth and spreading, and can be behind therapeutic failure and tumor recurrence [67].

At histological level, the colon is organized into four distinct layers: the mucosa, submucosa, muscular layer and serosa (intraperitoneal tissue)/ adventitia (retroperitoneal tissue). The mucosa is composed of three distinct sublayers: the epithelium, lamina propria and muscularis mucosae. Of them, the epithelial layer, which is located at the luminal surface, consists of a single sheet of columnar epithelial cells folded into finger-like invaginations that are supported by the lamina propria to form the functional unit of the intestine called crypts of Lieberkühn (**Figure 3**) [68–70] (see also https://embryology.med.unsw.edu.au/embryology/index.php/Gastrointestinal_Tract_-_Colon_Histology)

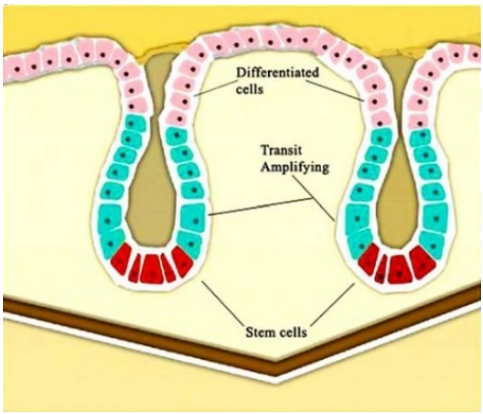


Figure 3. Colonic crypt organization [68]

In the colon, the principal pool of multipotent stem cells is located at the bottom of the crypt. These self-renewing cells are usually proliferative and able to divide asymmetrically giving rise to a transit amplifying cell population that, upon migration towards the crypt, proliferates and differentiates into various epithelial cells of the intestinal wall [71]. However, recent evidence indicates a certain degree of heterogeneity in colorectal stem cells. In particular, it has been clearly demonstrated the existence of distinct pools of stem cells with variable proliferation potential, including a

specific subpopulation of quiescent stem cells, as well as a certain degree of plasticity of non-stem cells [71–73]. The precise description of colon histology and stem cell function/features is beyond the scope of this thesis and can be found in [69].

Colorectal cancer has been diagnosed to 135,430 individuals only in the United States in this year, with an incidence of death approximately of 50% (i.e., 50,260 individuals) [74], thereby constituting a major public health problem. Historically, this tumor type has been an excellent model for investigating malignant development leading to the postulation of the genome instability theory of tumor transformation [75–77]. According to this theory, cancer progression would result from the sequential mutations in oncogenes and tumor suppressor genes. In this context, in 1990, Fearon and Volgestein demonstrated that, in adenocarcinoma, oncogenesis is driven by mutations in APC, WNT signaling pathway regulate (*APC*) and catenin beta 1 (*CTNNB1*; best known as β-catenin), while tumor progression relies on sequential mutations of other genes, such as KRAS proto-oncogene, GTPase (*KRAS*) and tumor protein p53 (*TP53*; best known as p53) coupled to the consequent increase in genomic instability [78]. This model has been highly refined during the last years thanks to the in-depth characterization of driver versus passenger mutations involved in colorectal tumorigenesis, the elucidation of the genetic, epigenetic and cytogenetic evolution of colorectal cancer in space and time, the understanding of the impact of the microenvironment and the integration of the revised CSC model. We briefly describe some of the most relevant discoveries with a major focus on those related to tumor heterogeneity, genomic instability and CSCs. Over the last decades, a variety of inactivating mutations of *APC* have been identified and linked to colorectal transformation and metastasis [79–81]. Moreover, several experimental findings have demonstrated the pleiotropic cellular/molecular consequences of *APC* loss of function, including perturbation of the Wnt pathway, cell adhesion, cell cycle control, chromosome stability, cell survival and cell migration [82–86]. Along with this, gain of function mutations of *KRAS*, have been casually correlated to oncogenesis as well as to cancer cell stemness. In particular, these mutations appear to promote the acquisition of stemness properties in *APC*-mutated colorectal cancer as demonstrated by the upregulation in the expression of specific CSC surface markers (e.g., CD44, CD133, CD166; also refer to next session), nuclear accumulation of WNT/β-catenin complex and activation of MYC proto-oncogene, bHLH transcription factor (*c-MYC*) and cyclin D (*CCND*) [87,88].

Beyond the Wnt/B-catenin axis, other stemness-related pathways, such as those dependent on Hedgehog, Notch [89–91] and Axin 2 [92], have been involved in colorectal cancer stemness. Of these, Lgr5 appears have a key role in CSC generation and maintenance by modulating the expression of WNT as well as in colorectal metastasis [93]. Finally, there is evidence that mutations and karyotypic aberration can occur and accumulate in pools of colorectal stem cells, and this has been causally involved in the generation of CSCs and oncogenesis [68]. In this context, it appears of interest (and possibly consistent) recent discoveries of a high level of aneuploidy, replication stress and genomic/chromosome instability in colorectal CSCs [11,94]

Colorectal CSCs Markers

Compelling evidence supports the relevance of CSCs in several solid tumors, including colorectal cancers [95], but the precise identification (and thus isolation) of CSC fraction is still controversial. This contentious is mainly ascribed to the lack of specific, universal accepted markers of colorectal CSCs [96]. The existence of a subpopulation of CSCs in colorectal cancer was first demonstrated by employing specific plasmatic membrane-surface markers, such as CD133 [97,68], or the combination of CD44 and CD166 [98]. Following experiments confirmed the specificity of these cell-surface markers for colorectal CSCs (e.g., CD166) [98,99] also leading to the identification of novel markers, including (but not limited to) CD29 [117] CD24 [100], MSI-1 [69], CD326 [101] Lgr5 [102,103] and EPCAM [101]. The function of some of these cell surface markers in CSC generation, maintenance and evolution is not always established. CD133 (known as Prominin-1 or AC133) is a transmembrane protein whose biological role is object of intense research [104,105] and whose reliability as CSC marker and contribution to metastasis is a matter of contentious [97,106–110]. P-glycoprotein 1 or CD44 is a transmembrane protein involved in cell to cell and cell-matrix interaction [111], which can exist in diverse isoforms (CD44v) most of which have been involved in colorectal cancer metastasis [112,113]. In particular, CD44v6 is reported to confer migration capability and invasiveness properties to CRC-SCs, and its expression is increased by microenvironmental stimuli, such as cytokines hepatocyte growth factor (HGF), osteopontin (OPN), and stromal-derived factor 1α (SDF-1), in a fashion dependent on Wnt/β-catenin pathway [114]. Finally, Lgr5 is an

established marker of proliferating intestinal stem cells [115,116], which is believed to promote tumor growth by modulating the Wnt pathway [93,117–119]. The specificity of these surface markers for colorectal cancer stem cells (and thus the validity of the cancer stem model in colorectal cancer) has been formally validated by a variety experimental approaches, the most relevant of which are (1) the evaluation of the capability of cells expressing specific cell-surface markers to form tumors resembling (at histological level) the primary tumor from which they are derived once prospectively isolated and serially transplanted into immunodeficient (ideally NSG) mice [120] (2) in vivo lineage tracing experiments in intact tumors to assess the fate of cells expressing specific cell-surface markers [72,121] and (3) analysis of cancer growth upon selective cell ablation [122,123]. As for functional markers, the enzymatic activity of aldehyde dehydrogenase 1 (ALDH1) is still considered one potential marker of colorectal CSCs [14]. Thus, the ALDH1-positive subpopulation is reported to drive cancer progression, while that negative to ALDH1 lacks tumorigenic potential when injected into immunodeficient mice [124–126]. Other known stemness markers in colorectal cancer include the (re)expression of pro-pluripotency genes, such as Oct-4, Sox-2, Nanog, Lin-28, Klf-4, and c-myc [127,128]. Of note, the (re)expression of these genes has been associated with poor prognosis, tumor recurrence and resistance to conventional chemo-/radiotherapy [129–132].

CSC niche in Colorectal Cancer

Stem-cell niche refers to a complex anatomic structure composed by (1) supportive stromal cells, including (depending on the tissue/organ considered) pericryptal myofibroblasts, fibroblasts, endothelial cells, pericytes and immune cells, which interacts with each other through cell surface receptors, gap junctions and soluble factors, and (2) the extracellular matrix (ECM), which acts as scaffold to maintain the three-dimensional tissue or organ architecture [132,133,134]. Stem cell niche constitutes the specialized microenvironment where stem cells reside. In addition, niche components interact continuously and dynamically with stem cells regulating their function and fate, and ensuing the maintenance of the stem cell compartment [133,134]. There is evidence that CSCs of different origin, including colorectal CSCs, also reside and interact with a specialized but less organized niche, which actively releases (often non-canonical) signals

driving CSC generation, survival, maintenance, plasticity and evolution [20]. Below we will describe some major interactions between the principal niche components and CSCs with a major focus on those involved in plasticity and, thus, resistance to therapy. The precise description of niche composition and functions in CSC biology goes beyond the scope of this thesis project and can be found in these recent and excellent reviews [33, 59].

Cancer-associated fibroblasts (CAFs) are one major component of CSC niche [135]. CAFs are reported to modulate CSC compartment by releasing HGF, which in turn activates b-catenin-dependent transcription and promotes self-renewal in colorectal CSCs [52]. Intriguingly, in this same article, HGF was shown to promote the de-differentiation of non-CSCs, providing the first proof of the existence of plasticity in the tumor system. In line with this evidence, recent findings demonstrate that CAFs promote reprogramming of colorectal cancer progenitors into CSCs through the release of a set of molecules, including HGF, OPN, and SDF1 [114]. Along similar lines, endothelial cells were described to favor the appearance of a stemness phenotype in colorectal cancer by mediating the release of the Notch ligand DLL1 and thus triggering Notch signaling [136]. In addition, immune cells, such as $CD4^+$ T cells, appears to influence colorectal CSCs self-renewal through secretion of IL-22 and activation of the DOT1L methyltranferase, which in turn ignites the transcription of stem-cell-associated genes [137]. Other important components of the CSC niche are transformed myofibroblasts, recruited myeloid cells, other cell types, and extracellular components, all of which have been reported promote oncogenesis and de-differentiation [52,138]. In this context, HGF released from myofibroblast is believed to stimulate Wnt activity [52]. Altogether, these observations suggest that CSCs is rather a dynamic structure, whose components secrete a variety of signals according to the environmental conditions, including the presence of therapeutics. To give an example, CAFs have been shown to secrete specific cytokines and chemokines in response to chemotherapy, including IL-17A, which increased colorectal CSC self-renewal and invasion [139].

CSCs, Epithelial to Mesenchymal Transition and Metastasis

There is mounting evidence indicating that CSCs are able not only to initiate and propagate tumors, but also constitute the roots for distant metastasis [93,114,140]. One crucial step in metastasis is the epithelial to mesenchymal transition (EMT) process [138]. In particular, through the EMT cells acquire a migratory and invasiveness phenotype due to loss of epithelial cell polarization [141]. The early step of the EMT involves the dissolution of epithelial cell–cell junctions that maintain epithelial integrity. This occurs via the decrease of occluding and claudin [142] and the cleavage of epithelial cadherin (E-caderin) [143], and results in loss of apical polarity [141]. Moreover, during the EMT, cells face an extensive reorganization of the cortical actin cytoskeleton and increased contractility [144]. The mechanisms underlying EMT are complex and not fully elucidated. The main transcriptional factors involved in ETM are the transcription factor family Snail, Zeb1 and Twist, which act as transcriptional repressors of epithelial genes, as E-cadherin, and transcriptional activators of mesenchymal genes, including N-cadherin [145,146]. TGFβ constitutes the main regulators of EMT, modulating key downstream targets such as components of the PI3K-AKT-mammalian TOR (MTOR) [147], and MAPK pathways [146]. In addition, the EMT appears regulated by tyrosine kinase receptor, WNT, Notch, and Hedgehog networks [146,148,149], and possibly relies on the cooperating activity of multiple signaling cascades [150]. Of note, TGFβ, WNT, Notch and growth factors are reported to upregulate the expression of SNAIL1 [151]. Confirming a link between EMT and metastasis, metastatic cells are usually characterized by a profound reorganization and deregulation in mechanisms controlling cytoskeleton and cell polarity[152]. In particular, multiple integrins are down- or over-expressed in metastatic cells, and this has been associated with E-cadherin disruption and β-catenin nuclear translocation[153,154]. Moreover, TGFβ plays a relevant role in metastasis and invasion by modulating SMAD receptors[155] pickup or via SMAD-independent mechanisms involving RHO-like GTPases, PI3K and MAPK pathways [156,157] or RHO, RAC and CDC42 GTPases [158].

In the context of CSCs, the EMT has been strongly associated with the acquisition of stemness potential. Thus, migrating cancer stem cells (MCSCs) constitutes a cell subpopulation displaying stemness properties and invasion capabilities, which contributes to cancer metastasis [159,160]. Moreover, the EMT has been associated to cell plasticity by favoring the de-differentiation of non-CSCs in CSCs [47]. Nonetheless, some

experimental findings cast some doubts about role of the EMT in CSCs (reviewed in [33]). One reason explains this controversy may ascribed to the evidence that the EMT in cancer cells may be a transient state and that EMT transition could result in a plastic CSC phenotype [161]. In line with this notion, the EMT inducer ZEB1 has been reported to induce stemness properties in non-CSCs in the presence of EMT-inducing signals from the microenvironment [48]. Moreover, it has been shown that transient expression of TWIST1 induces the acquisition of CSC-like state that persists until the cells have returned to the epithelial phenotype [162].

Therapeutic Target in CRC-SCs

It is well demonstrated that CSCs are endowed with an exquisite resistance to most standard and targeted therapies, and that this specific feature of CSCs is causally linked to therapeutic failure and tumor recurrence after (initially) successful therapy. CSC resistance is often due to the overexpression of ATP-binding cassette transporters (ABC transporters), which promote the efflux of multiple chemotherapeutics, including DNA damaging agents [163–165]. Along with extruding chemotherapeutics, a variety of other intrinsic and extrinsic (i.e., microenvironmental) factors actively promotes the therapeutic resistance of CSCs, all of which de facto constitute potential targets for effective treatment strategies.

Compelling evidence indicates that, similar to normal stem cells, CSCs possess a very efficient DNA damage response (DDR), which, on the one hand, confers them a peculiar resistance to DNA damaging agents and, on the other hand, can constitute a potential vulnerability to be exploited therapeutically [2,166]. In line with this hypothesis, CSC has been shown to depend on CHK1, ATR or RAD51 for survival and proliferation, possibly for these DDR players help CSCs tolerating high levels of replication stress at baseline [11,167]. Along similar lines, inhibition of the ATM or ATR axes reportedly sensitizes CSCs to DNA damaging therapy [168–171]. Other processes whose targeting revert CSC resistance to DNA damage include (1) growth factor beta 1 (TFGB1) signaling, whose blockade radiosensitizes glioma stem cells by abrogating the DDR [172] ; (2) Notch signaling, whose inactivation increases the sensitivity of ovarian CSCs to platinum therapy

by enhancing DDR-driven cell death [173]; and (3) mechanistic target of rapamycin (MTOR) signaling, whose inhibition augments the radiosensitivity of glioma stem cells by preventing DNA repair [174].

Besides the DDR, other CSC-related pathways are being exploited as targets for cancer therapy. First, given its involvement in CSC generation and plasticity (see above), the EMT constitutes an effective target for depleting CSCs. Therapeutic approaches for targeting directly or indirectly the EMT include: (1) the inhibition of the TGF-β pathways by administering agents such as trabedersen, which prevents TGFβ synthesis, and the anti-TGFβ-R II monoclonal antibody IMCTR1 [175,176]; (2) the abrogation of transcription factors involved in EMT such as STAT3[177–179], or (3) the inactivation of the PI3K–AKT–mTOR network [180,181]. The effectiveness of these strategies against CSCs is still object of study. Second, given their peculiar epigenetic status [182], CSCs are selectively eradicated by epigenetic drugs, including DNA methyltransferase 1 (DNMT1) alone[169] or in combination with HDAC inhibitors [183], as well as inhibitors of BMI1 [184]. Third, given their specificity for CSCs, cell-surface markers are being employed as the base of anti-CSC regimens. This applies to CD44 and CD47, whose targeting with specific monoclonal antibodies is effective in colorectal cancer [185,186]. Importantly, an antibody recognizing EpCAM has been shown to selectively deplete colorectal CSCs bearing mutations in KRAS proto-oncogene, GTPase (*KRAS*), phosphatidylinositol-4,5-bisphosphate 3-kinase (*PI3 kinase*) and B-Raf proto-oncogene, serine/threonine kinase (*BRAF*) [187]. Moreover, specific isoforms of ALDH may constitutes promising targets for killing CSCs as they reportedly drive their resistance to DNA damaging agents [188,189]. Finally, some combinatorial strategies seem to be particularly effective in killing CSCs, including (but not limited to) anti-EGFR therapy together with the abrogation of the MAPK pathway, or the inhibition of PIK3 and mTOR [190], double blockade of $BRAFV^{600E}$ and EGFR in combination with PIK3 inactivation [191], and co-administration of VEGF (bevacizumab) and c-MET inhibitors [192].

TELOMERES: STRUCTURE AND FUNCTIONS

Telomeres are nucleoprotein complexes localized at the end of all eukaryotic chromosomes [193,194]. The DNA component of human telomeres (i.e., the telomeric DNA) consist of 10–15 kilobases (kb) of double-stranded DNA tandem repeats of the hexanucleotide TTAGGG and followed by a terminal 3′ G-rich single-stranded overhangs [193,195]. At structural level, telomeric DNA is organized in a double stranded locked t-loop structure, which is generated through a mechanism involving strand invasion of the duplex telomeric repeat by the 3' G-rich single-stranded overhang and the consequent displacement of the TTAGGG repeat strand (d-loop_displacement loop) at the loop-tail junction [196,197] (**fig. 4**).

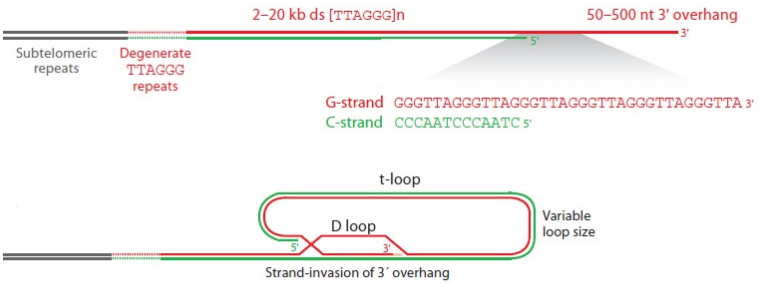


Figure 4. Telomeres structure in Human [198]

Telomeric DNA is associated with the Shelterin complex, a hexamer composed of telomere repeat binding factor 1 (TRF1), TRF2, protection of telomeres 1 (POT1), repressor activator protein 1 (RAP1), TRF1-interacting protein 2 (TIN2) and Tripeptidyl peptidase 1 (TPP1) [3,194]. As for the role and interactions of Shelterin proteins in telomere: (1) TRF1 and TRF2 bind directly the double-stranded DNA tandem repeats as homodimer [199,200]; (2) POT1 has a strong specificity for the single-stranded DNA fraction [199,200] (3) TIN2 acts a linker protein stabilizing the Shelterin complex through a

direct association with TRF1, TRF2 and TPP1 [201]; (4) TPP1 associates with POT1 and TIN2 promoting the physical connection between the G-overhang and the duplex telomeric repeats [202]; and RAP1 interacts with TRF2 [203]. Once assembled to telomeric ends, Shelterin complex contributes to the preservation of telomere integrity and functionality by stabilizing the t-loop structure [194,204] (**fig. 5**). Moreover, the presence of the Shelterin complex ensures that telomeric ends are not sensed as DNA double strand breaks (DSB) and thus do not activate the DNA damage response (DDR)[205].

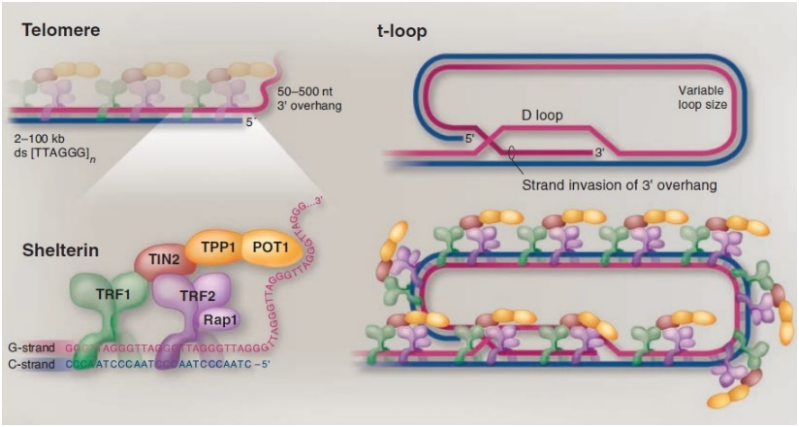


Figure 5. Telomere loops and Shelterin complex [206]

Telomere is involved in several biological processes, including (but not limited to): (1) protection of chromosome ends from nuclease degradation, recombination, end-to-end fusion, and recognition by the DDR as lesioned DNA; (2) correct DNA replication and chromatin spatial/functional organization; and (3) regulation of gene expression [195]. Moreover, as we will discuss in the next session, telomeres constitute the cellular molecular clock, controlling the replicative and proliferation potential of human cells [207,208].

Telomere Maintenance Mechanisms (TMMs): Telomerase and ALT

The replication of chromosome ends poses a serious problem to proliferating somatic cells undermining telomere integrity at each round of replication. During DNA replication, DNA polymerase catalyzes the addition of nucleotides only at the 3'overhang (5'-3' direction). Consequently, one of the two strands (the lagging strand) is replicated discontinuously in short segments (known as Okazaki fragments) starting from short 5′ RNA primers, which are then replaced by DNA synthesis prior to ligation [209–211]. However, at the end of the chromosome, the removal of the terminal RNA primer is not followed by gap filling for the absence of a 3'-OH end, which results in the loss of small portion of telomeric DNA (50-100 nucleotides at each round of cell division) thereby causing the so-called *end replication problem* [212,213]. In addition, telomere shortening can also arise from the activity of specific nucleases, which degrade nucleotides at the 5' end of the lagging strand and generate the G-strand [214]. One major consequence of the end replication problem is that human cells can only divide a finite number of times in culture [213]. This phenomenon, which is known as "Hayflick limit", was proposed to act as a mitotic clock within the cells [4]. Following studies demonstrated that the Hayflick limit is determined by the initial length of telomeres and the rate of telomere shortening [215,216] and confirmed that telomere shortening operates as a molecular clock in the cells [207,217]. According to the current accepted model, in the presence of critically short telomeres, cells enter in a phase of permanent growth arrest known as replicative senescence or mortality stage 1 (M1) [218,219]. One major mechanism promoting the escape from replicative senescence in the presence of short telomere, and thus reinstating cell cycle progression and cell division, is the inactivation of cell cycle checkpoint due for instance to defects in the p53 network [220,221]. Deregulated cell proliferation in turn provokes a further shortening of telomeres, which results in telomere dysfunction or telomere loss and triggers the activation of massive cell death during a phase known crisis or mortality stage 2 (M2) [222,223]. Of note, some rare cells can also survive telomere-dysfunction- or telomere loss-mediated crisis by (re)instating mechanisms for telomere maintenance (i.e., the process of counteracting the natural telomere attrition), which in most cases relies on the (re)activation of a ribonucleoprotein enzymes known as telomerase (see below), thereby acquiring an unlimited proliferative

capacity (i.e., cellular immortalization) and potential pro-oncogenic properties [223,224].

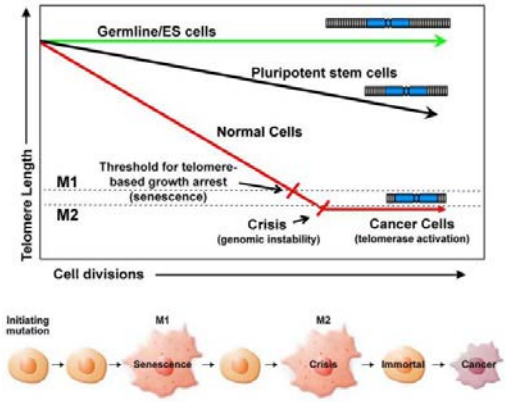


Figure 6. Telomere shortening during tumor transformation [224]

Telomerase is an RNA-dependent DNA polymerase that catalyzes the extension of telomeric DNA by adding telomeric repeats to the chromosome 3' end [225]. Telomerase activity thus constitutes the major, but not the exclusive, mechanism for telomere maintenance [7]. Telomerase exerts its functions as it is assembled as a complex composed of a catalytic subunit (hTERT), which acts as a reverse transcriptase, and a RNA component (hTERC), which constitutes the template for telomeric DNA synthesis [226,227]. Telomerase was first discovered in *Tetrahymena thermophila* [228], and then identified and characterized in all eukaryotes [227]. Human cells display heterogeneous telomerase activity. Thus, while telomerase is usually absent in normal somatic cells, this ribonucloprotein enzyme is present (and active) in germline cells, embryonic stem cells, pluripotent cells, adult stem cells as well as in most (approximately 85-90%) of tumor cells [6,229,230]. At experimental level, the gold standard to detect telomerase activity is the Telomerase Repeated Amplification Protocol (TRAP) assay [231–233].

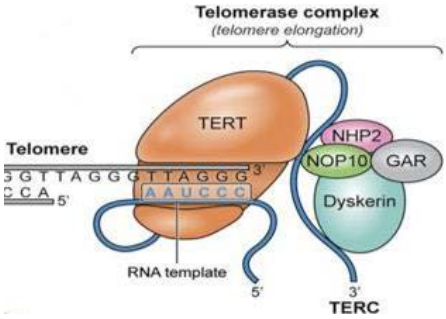


Figure 7. Telomerase complex (source Web)

Alternative Lengthening of Telomeres (ALT) is a telomerase-independent telomere maintenance mechanism discovered in the nineties and possibly acting in the minority of tumor cells lacking telomerase activity [234,235]. Despite the precise molecular mechanisms of ALT is still unclear [205], a large panel of experimental evidence indicates that ALT relies on homologous recombination (HR) [7,205]. Of note ALT mechanism has been detected in a significant fraction (approximately 10-15%) of tumors [236]. The presence of ALT activity can be estimated by distinct observations, including the presence of (1) high heterogeneity in telomere length [237], (2) undetectable telomerase activity [235], (3) linear [238] or circular [239] telomeric repeats, (4) telomere-sister chromatid exchanges (T-SCE) [240] and (5) complexes called ALT-associated promyelocytic leukemia nuclear bodies (PML-NBs) or APBs [241]. Mounting evidence indicate the potential coexistence of telomerase activity and ALT in tumor cells. This has been proven either *in vitro* in glioblastoma cells [242] and transformed lung fibroblasts [243] or *in vivo* in glioblastoma multiforme [244]; astrocytomas [237], osteosarcomas [245,246], gastric carcinomas [247]. Finally, two distinct cell population presenting telomerase or ATL activity were detected in individual neuroblastoma [248], while no TMM was observed in melanoma metastases [249].

Telomeres, Telomerase and Chromosomal Instability (CIN)

As discussed above, a main role of telomeres is to prevent the activation of the DDR by ensuring that chromosome ends are not sensed by cells as bona fide DSBs. In line with this function, critically short telomeres once being uncapped and dysfunctional activate DSB repair pathways [250,251]. In particular, telomere uncapping and dysfunction is reported to triggers the ATM, ATR and p53 axes [252], often leading to the activation of cell senescence or cell death, and thereby acting as oncosuppresive mechanism [253]. However, telomere shortening and uncapping generates sticky ends, which are particularly prone to telomeric fusions [250,251]. These aberrations are reported to increase the level of genomic instability via a mechanism known as breakage-fusion-bridges (BFBs) cycle [254,255]. In more detail, during mitosis, telomere fusions result in the generation of anaphase bridges, which are often (but not always) broken once sister chromatid segregates [256,257]. These breakages result in the acquisition of karyotypic aberrations and the generation of novel sticky ends, which, upon fusion and anaphase bridge generation, enter a novel round of BFBs. As a result, karyotypic aberrations accumulates at high rate during each cell division [258], a condition known as chromosome instability (CIN) often found in tumors [259–262]. Along with being frequent in cancer, CIN (at least when occurring at high but tolerable level) may also drive oncogenesis, tumor evolution and therapeutic resistance [263,264]. In this context, telomere shortening has been also reported to foster CIN during oncogenesis, as it promotes endoreplication and consequent generation of tetraploid cells [265–267], which reportedly bear an intrinsic high level of chromosomal instability [268]. Of note, besides dysfunctional telomeres, other CIN-inducing mechanisms include both pre-mitotic defects (e.g., replication stress or deregulated centrosome cycle) and mitotic defects (e.g., weakened/impaired spindle assembly checkpoint, defects in sister-chromatid cohesion, cytokinesis failure), whose detailed description can be found in this recent review [269].

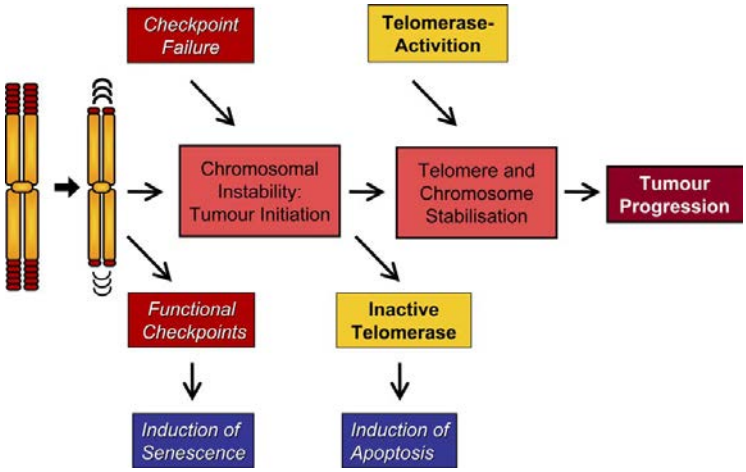


Figure 8. Telomere shortening and telomerase activation in tumor progression [270]

Telomeres and TMM in CSCs

It is well established that normal stem cells have a detectable telomerase activity, which ensures the preservation of stemness properties and perpetuation of the stem-cell pool [271]. However, although embryonic stem cells preserve long telomeres, telomeres of adult stem cells shorten during aging despite a detectable telomerase activity in these cells [272]. Telomere shortening is often accompanied by stem cell dysfunction, differentiation and demise, thereby resulting in the potential exhaustion of the stem cell pool [223]. Moreover, telomere shortening may increase the risk of stem cell immortalization and transformation [6]. Contrarily to normal stem cells, the status of telomeres and telomerase in CSCs is poorly investigated, and remains matter of contentious. On the one hand, in some studies CSCs of different origin are described to have very short telomeres as compared to the bulk of tumor, which, in the case of neural CSCs, is coupled to high expression levels of TERT and detectable telomerase activity [273–275]. On the other hand, breast and pancreatic cancer stem cells were shown to display similar telomere length and telomerase activity in comparison to the bulk of

the tumor [8,276], while glioblastoma stem cells were reported to present long and heterogeneous telomeres [277]. Of note, in this latter study, long telomeres were maintained by ALT mechanisms while telomerase activity was undetectable in these cells. These observations seem to indicate a heterogeneous level of telomere length and variable telomerase activity in CSCs. Nonetheless, further studies are required to elucidate the telomere biology in CSCs.

Some intriguing findings, which need further experimental confirmation, suggests a role for telomerase subunits in CSC generation and function, which is not always dependent on their role in telomere biology. First, overexpression of TERT in epidermal stem cells promotes the clonogenic potential and increases the susceptibility to develop skin tumors [278,279]. Second, TERT overexpression increases the fraction of CSCs in glioma possibly by inducing EGF expression [280]. Third, ß-catenine is reported to modulate TERT expression [281], and this has been surmised to promote CSCs proliferation [282]. Finally, TERT seems to promote the EMT and stemness potential of gastric cancer cells [283].

Telomere and TMM, as Therapeutic Target in Cancer and CSCs

Over the last years, telomerase is being investigated as a specific target for anti-cancer therapy, mainly based on the evidence that most tumors reactivate this nucleoprotein enzyme. The main telomerase targeting strategy consists in the use of specific pharmacological inhibitors, such as MST312, BIBR1532 and GRN163L (best known as Imetelstat). MST312 is a chemically modified derivative from tea catechin, epigallocatechin gallate and its activity has been validated in breast cancer cells, showing decreased telomerase activity and induced telomere dysfunction and growth arrest [284] and similary results have been observed in glioblastoma cells [285]. BIBR1532 is a synthetic, non-nucleosidic compound that acts in a non-competitive manner by hampering the addiction of TTAGGG hexanucleotide, and thus promoting telomere erosion and cell death [286–289]. Imetelstat is a compound acting via a mechanism involving its direct binding to hTERC, which in turn results in telomerase inhibition, telomere

shortening/uncapping, and ultimately cell cycle arrest and/or death [290,291]. Imetelstat is an oligonucleotide complementary to the RNA template hTERC, which has demonstrated elevated *in vitro* and/or *in vivo* anti-neoplastic activity either alone [292], or in combination with chemotherapeutics, such as the BCL-2_inhibitor venetoclax [293], antimitotic paclitaxel plus VEGF inhibitor bevacizumab[294] and HSP90 inhibitor alvespimycin [295], or together with radiotherapy [296,297]. Moreover, as opposed to BIBR1532, Imetelstat has entered into clinical trials and is still being investigated in some ongoing clinical studies [291,298,299] (https://clinicaltrials.gov/). Of note, imetelstat is not recognized and thus not extruded by the ATP-binding cassette transporters [300]. Other strategies to inactivate telomerase include (1) nucleosid analogs, such as 3'-azido-2',3'-dideoxythymidine (AZT) that is reported to interact (and inhibit) with TERC at high affinity [301], (2) immunotherapeutic compounds targeting telomerase [290,302], and (3) TERT subunit depletion by transfecting specific small interfering RNA (siRNA) or small-hairpin RNA (shRNA) [303–305]. In this context, a particular efficacy in killing cancer cells has been proven by combining siRNA directed against TERT subunits and radiotherapy[306]. Finally, alternative telomere-perturbing anticancer regimens include G-quadruplex ligands, such as BRACO19 and RHPS4, which affect telomeric DNA replication and telomere functions [307,308] and inhibitors of HSP90, which has been involved in telomerase assembly [289].

Few studies investigated the effect of telomerase inhibitors on CSC survival. Thus, the telomerase inhibitors MST312, have shown a decrease in ALDH-positive CSC population and promote telomeres shortening in lung CSCs in vivo [309]. Along similar lines, imetelstat was shown to impair self-renewal and clonogenic potential in multiple myeloma CSCs [8], and to target specifically the CSC fraction either in breast and/or pancreatic CSCs, when administered together with trastuzumab [8,310], or in glioblastoma stem cells, when combined with ionizing radiation and temozolomide [274]. As described in the previous section, the presence, relevance and activity of telomerase in CSCs is debated thereby casting doubts on the use of telomerase inhibitors in cancer therapy. A better comprehension of telomere biology of CSCs is thus urgent so to elucidate the anti-neoplastic effectiveness of telomerase inhibitors, identify the markers of the response to these compounds and

investigate the true therapeutic potential of alternative regimens based on compounds such as G-quadruplex ligands and ALT inhibitors.

AIM OF THE PROJECT

Colorectal cancer is the third leading cause of cancer death worldwide causing approximately 700.000 deaths/year worldwide. Despite diagnostic and therapeutic advances (with surgery constituting a common clinical approach in early tumor stages), the 5-year relative survival rate of CRC is 66% for colon cancer and 62% for rectum cancer, according to the recent publication of the Italian Association of Cancer Registries (AIRTUM). Moreover, in most cases diagnosis occurs when tumor is in an advanced stage and distant metastases had appeared. Tumor recurrence and metastasis, which are the most critical survival-influencing factors of CRC, have both been associated with the presence of subpopulations of immature and self-renewing cells within the tumor mass, known as cancer stem cells (CSCs). The identification of novel strategies to eradicate CSC is thus mandatory for efficient anti-cancer therapy.
Our and other groups have previously shown that CSCs share a variety of properties with normal stem cells, including the ability to self-renew while generating differentiated cells and a significant rewiring of the DNA damage response. Whereas embryonic stem cells are characterized by long telomeres and high telomerase activity, in adult stem cells telomeres shorten during age even in the presence of a detectable telomerase activity. Cancer cells usually possess short and dysfunctional telomeres, which are maintained by the enzyme telomerase and contribute to increase of chromosome instability. To date, few studies have analyzed the ploidy and biology of telomere in CSCs, and their findings are rather contrasting.

In this project, we investigated ploidy status, and telomere length and functionality in a vast panel of primary CRC cells enriched for CSCs (CRC-SCs) isolated from > 20 patients.

Our major objectives were:

1) to characterize the ploidy status of CRC-SCs;
2) to determine telomere length in CSCs, and how it varied in our panel of CRC-SCs (inter-cellular variability) and within each CRC-SC (intra-cellular variability);

3) to analyze the frequency of short telomeres and telomere loss as well the presence of telomere abnormalities (i.e., telomere doublets) in CSCs;
4) to analyze the mechanisms for CSC telomere maintenance, with a major focus on telomerase activity.

To the best of our knowledge, this is the first time that telomere biology is investigated so in-depth and in such a large number of patient-derived

CSCs. This study can thus provide fundamental tools to the scientific community (and our group) for the design of novel telomere-based strategies able to eradicate CSCs and identification of potential biomarkers of the response to these regimens.

RESULTS

CHARACTERIZATION OF CHROMOSOMAL NUMBER AND PLOIDY STATUS IN CRC-SC

We took advantage of a panel (total number 21) of CSCs derived from human colorectal cancer patient samples (hereinafter referred to as CRC-SCs), which were previously generated by our collaborators at the Biobank of CSCs (*Istituto Superiore di Sanità*). All CRC-SCs used in this study have been validated for their stemness and tumorigenic potential *in vitro* and *in vivo* through (1) morphological analyses and determination of the expression of stem cell markers by immunohistochemistry and cytofluorimetry, and (2) evaluation of the capability to recapitulate tumor patient's heterogeneity and hierarchy when subcutaneously injected in immunodeficient NSG mice [311]. Moreover, our group previously characterized these CRCs at genetic level by short tandem repeat (STR) analysis and whole exome sequencing, and at (phospho) proteomic level by reverse-phase protein array (RPPA) [11,312].

To pursue the characterization of our panel of CRC-SCs we decided to launch an extensive cytogenetic analysis. In particular, we counted the number of chromosome by metaphase spreads upon treatment of CRC-SCs with the microtubular poison colchicine followed by fixation with a *Carnoy*'s solution and DAPI counterstaining. By this analysis, we observed a heterogeneous modal chromosome number with values ranging from 44 to 90. We were able to classify CRC-SCs in two categories according to their ploidy status: 1) near-to-diploid CRC-SCs (modal chromosome number < 50) and 2) hyperdiploid CRC-SCs (modal chromosome number > 50). As shown in **Figure 9**, 11 out of 21 (i.e., approximately 52%) of CRC-SCs display a chromosome number exceeding the diploid set, while the remaining 10 (i.e., approximately 48%) show a near-to-diploid modal chromosome number, which is consistent with what reported in the Mitelman database of chromosome aberrations and gene fusions in cancer (https://cgap.nci.gov/Chromosomes/Mitelman). Moreover, the intracellular heterogeneity in CRC-SC ploidy has been confirmed by cytofluorimetric-mediated analysis of the cell cycle upon staining with a DNA dye (data not shown)[11].

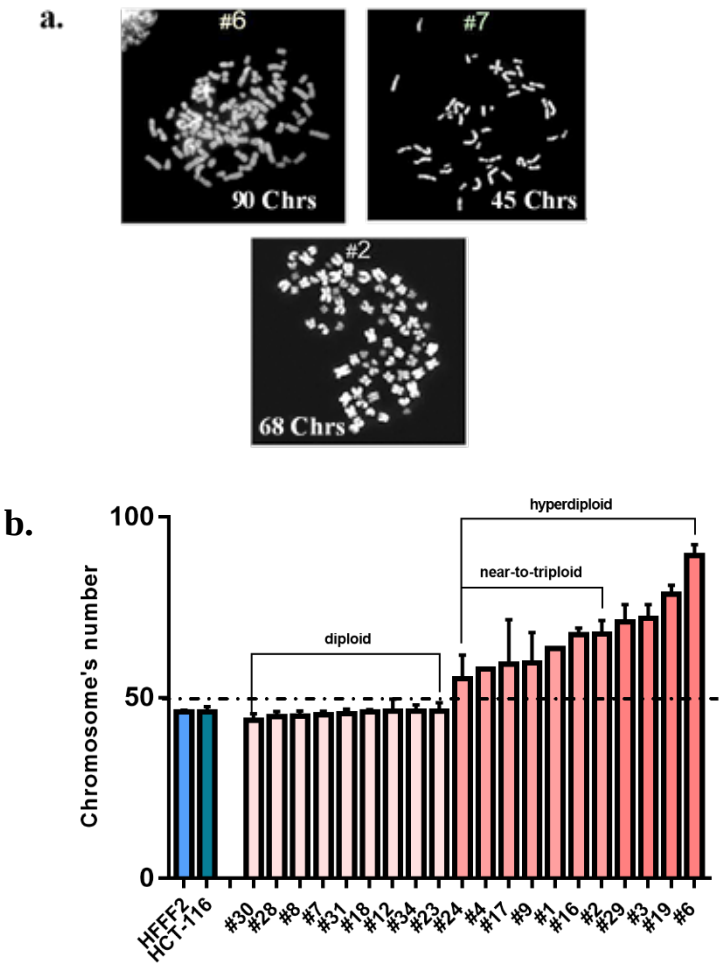


Figure 9. Ploidy status in CRC-SCs. a) Metaphase spread analysis of CRC-SCs treated with colchicine, fixed in Carnoy's solution and then counterstained with DAPI. One representative near-to-diploid (#7), near-to-triploid (#2) and hyperdiploid (#6) CRC-SCs are shown. **b)** Histogram representing chromosome number mean values of our panel of CRC-SCs. The dotted line refers to the threshold value of 50 that discriminates near-to-diploid from hyperploid chromosome set. One hundred metaphases per cell lines were analyzed and experiments repeated three times in independent experimentations. Chromosomal numbers of #32 and #35 is missing due to technical difficulties, and novel experiments are ongoing. Parallel cell cycle analyses by FACS indicate that CRC-SCs are both near-to-diploid.

CHARACTERIZATION OF TELOMERE LENGTH IN CRC-SCs

In parallel analyses, we also investigated the status of telomeres in our panel of 21 CRC-SCs by performing Quantitative-Fluorescence *in situ* Hybridization (Q-FISH) studies. To this aim, CRC-SCs were subjected to metaphase spreads followed by co-staining with the whole telomeric peptide nucleic acid (PNA) probe and a chromosome 2 centromeric specific PNA probe. Finally, CRC-SCs were counterstained with DAPI and metaphases analyzed in fluorescence microscopy upon image capturing. Telomeric length (TL) was calculated using a dedicated analysis software (Isis Fluorescence Imaging System) as the ratio between total telomeres fluorescence and fluorescence of the centromere of chromosomes 2 (T/C), with this latter constituting the internal reference in each metaphase analyzed (**Fig. 10**).

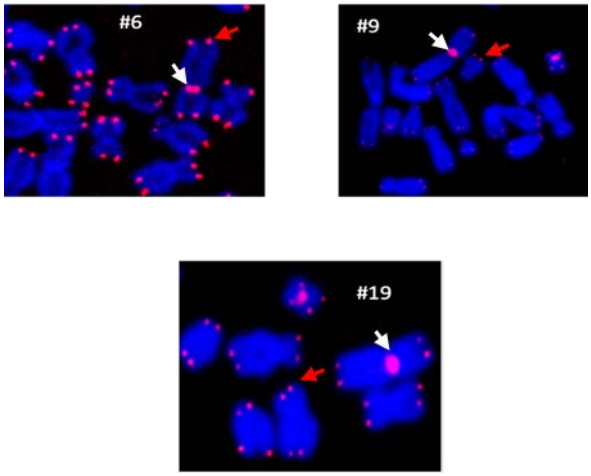


Figure 10. Quantitative-Fluorescence in situ Hybridization analysis (Q-FISH) in CRC-SCs. CRC-SCs were co-stained with telomeric (red arrows) and chromosomes 2 (white arrows) probes to measure telomere lengths. Images of representative metaphases of CRC-SCs with very long (#6), short (#19) and very short telomeres (#9) are illustrated.

Telomere length values of all CRC-SCs were assessed in comparison with those of human fetal foreskin fibroblasts (HFFF2), which present long telomeres (T/C% value of 20), and of the human colorectal cancer cell line HCT116, which show short telomeres (T/C% value of 8) [313]. Similar to chromosome number (**Fig. 9**), we observed a high variability in telomere lengths among CRC-SCs (**Fig. 11**). Importantly, approximately 50% of CR-CSCs presented a telomere length mean value equal or lower than that of HCT116, while approximately 80% of CRC-SCs displayed a telomere length mean value equal or lower than that of HFFF2. Strikingly, two CRC-SCs (#4 and #6) showed very high telomere length mean (T/C% value of 30.4 and 43.2, respectively) as compared to HFFF2

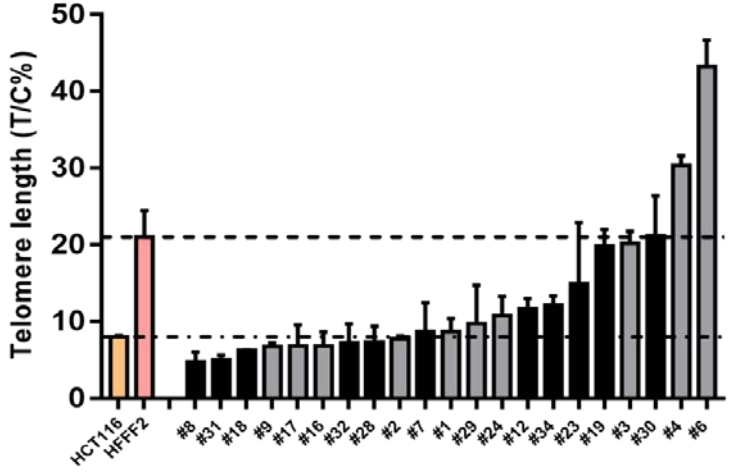


Figure 11. Telomere length mean values of CR-CSCs. Histogram depicting telomere length mean values of the illustrated CRC-SCs determined by Q-FISH analyses and evaluated in comparison to that of human fetal foreskin fibroblasts HFFF2 and human colorectal cancer cell lines HCT 116. At least 10 metaphases per CRC-SCs were analyzed (n=3). Diploid and hyperdiploid CRC-SCs are depicted in black and gray, respectively.

Taken together, these findings demonstrate the presence of a high inter-cellular variability in telomere lengths in our panel of CRC-SCs, which is in contrast with previously results showing the prevalence of short telomeres in CRC-SCs [273–275].

As expected, we could find no correlation between telomere length and ploidy either when we considered all (near-to-diploid + hyperdiploid) CRC-SCs (linear coefficient $R^2=0.279$, $P=0.016$; Spearman correlation $R=0.342$, $P=0.14$) or exclusively hyperdiploid CRC-SCs (linear coefficient $R^2=0.375$, $P=0.04$), although in this case there was a trend for an exponential correlation ($R^2_{exp}=0.53$) (**Fig. 12a and 12b**).

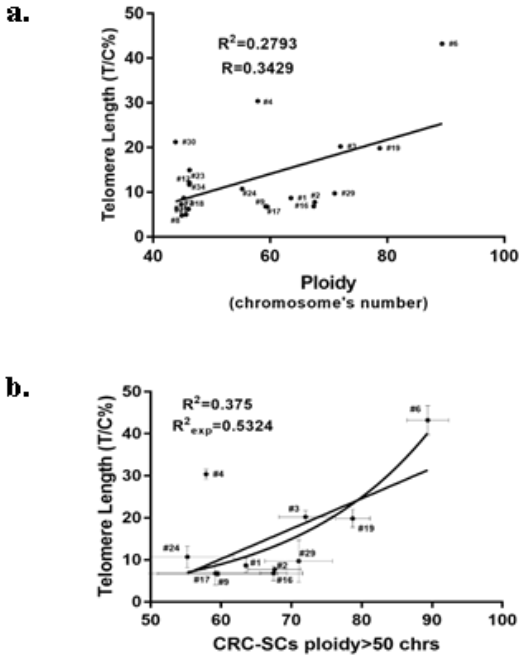
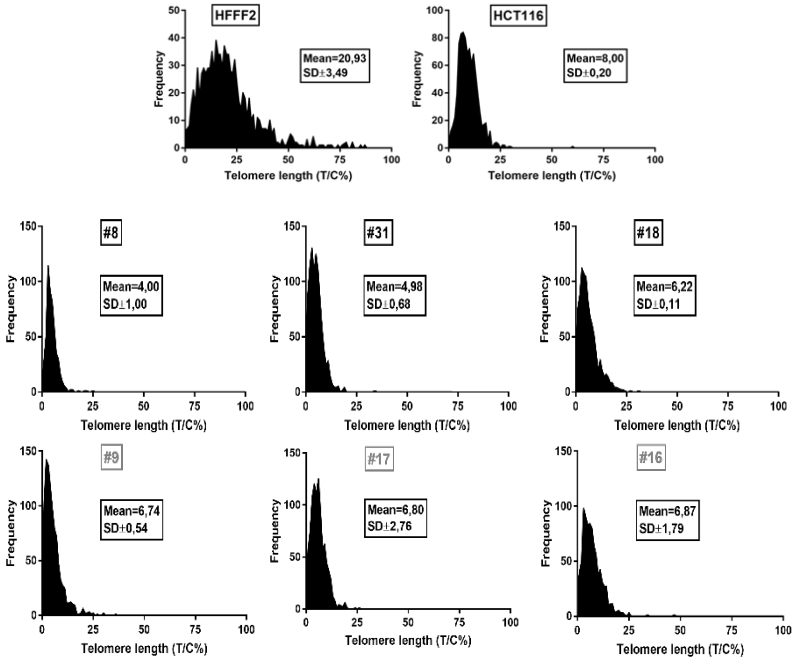


Figure 12. Correlation between ploidy and telomere length in CRC-SCs. Bi-parametric correlation analysis between chromosomal number and telomere length mean values (expressed as T/C%) in all CRC-SCs **(a)** or exclusively in hyperdiploid CRC-SCs **(b).** Linear coefficient R^2 or R^2exp values and Spearman correlation R values are reported. Chromosomal number and telomere length mean values were determined as reported in Figure 1 and 3, respectively.

Analysis of Telomere Length Distributions in CRC-SCs

We then investigated the level of intra-cellular variability in telomere length in CRC-SCs. To this aim, we analyzed telomere length distributions in each CRC-SCs by considering each individual telomere length value for all metaphase analyzed per cell lines (determined by Q-FISH) and then extrapolating its frequency in cell line population. By this approach, we could confirm the presence of a high inter-cell variability in telomere length mean values and provide evidence of a heterogeneous intra-cellular variability in telomere length frequencies in most CRC-SCs (**Fig. 13 and 14**). Interestingly, we observed that intra-cell variability increased with increasing telomere length mean values, as demonstrated by (1) the positive correlation between variance (a parameter indicating the heterogeneity in the distribution) and telomere length (R^2 = *0.9242*, *P<0,0001*; **Figure 6**), and (2) the distribution of frequency peaks reported in **Figure 13**, with a narrow or wide curve in CRC-SCs presenting short telomeres (e.g., #8) or long telomeres (e.g., #6), respectively.



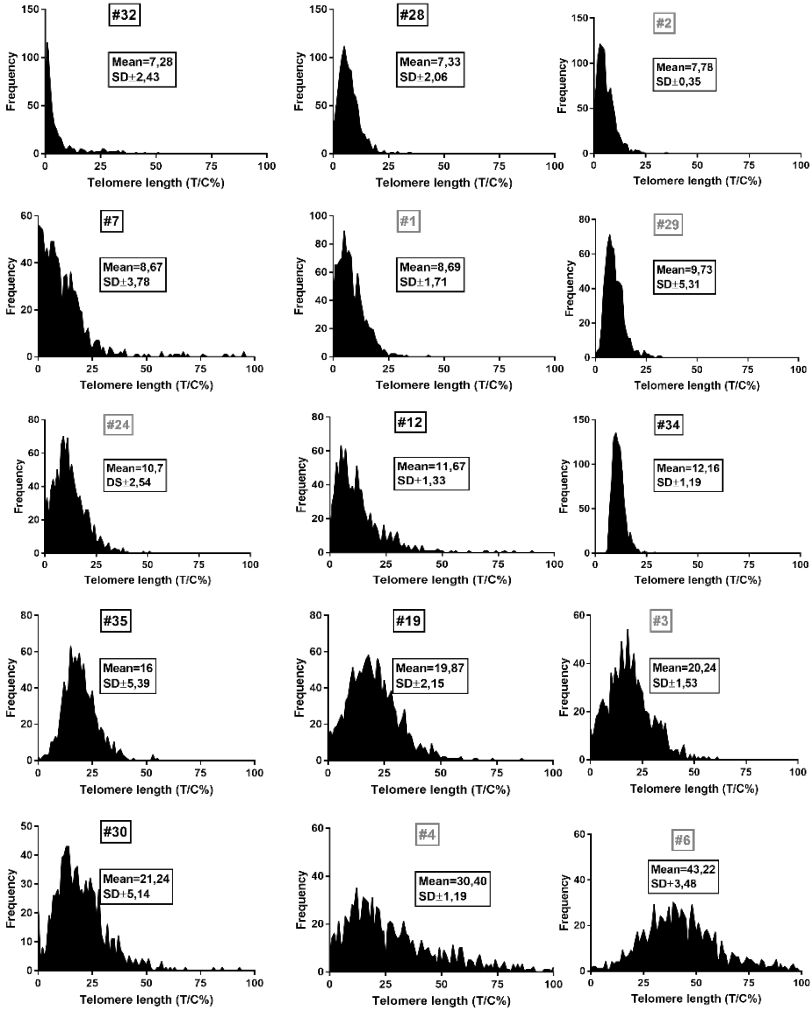


Figure 13. Telomere length distribution in individual CR-CRCs. Distribution curves representing the frequency of individual telomere length value (TL) determined by Q-FISH analyses for individual CRC-SCs. One representative experiment of three yielding similar results is reported. Numbers refer to TL mean value and standard deviation (SD). Cell lines are ordered following the increase in telomere length. Diploid and hyperdiploid CRC-SCs are depicted in black and gray, respectively.

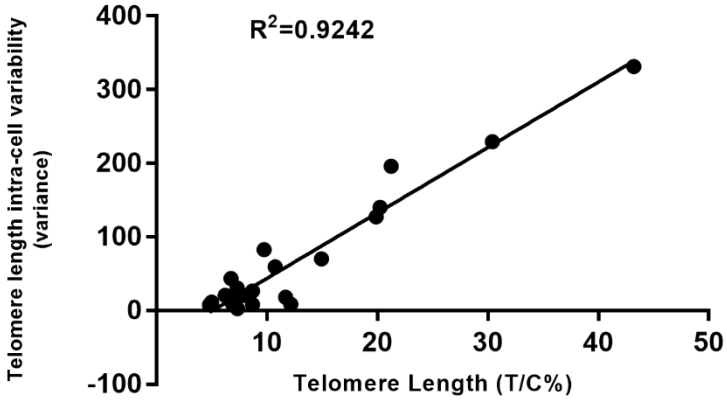


Figure 14. Correlation between intra-cellular variability and telomere length mean values in CRC-SCs. Bi-parametric correlation analysis between the telomere length distribution variance calculated from the distribution curves in Figure 5 and telomere length mean values (expressed as T/C% as in Figure 3) in all CRC-SCs. The linear coefficient R^2 value is reported.

Identification of short telomere side population in CRC-SCs

As reported in the introduction section, telomeres are nucleoprotein complexes involved in several biological functions, including end-chromosome's protection from nuclease degradation, recombination, end-to- end fusion and the DDR, whose loss/dysfunction has been linked to telomeric fusions and chromosomal aberrations. In particular, the presence of critically short telomeres in tumors is reportedly correlated with increased levels of CIN [222,314].

To evaluate the presence and frequency of short telomeres, we clustered telomere length values in each single CRC-SCs (calculated by Q-FISH analyses as reported in Figure 2 and 3) in three categories according to distinct thresholds: (1) telomere length values lower than 6 T/C% (which correspond to short telomeres), (2) telomere length values lower than 3 T/C% (which correspond to very short telomeres) and (3) telomere length values lower than 0.5 T/C% (which correspond to telomere loss).

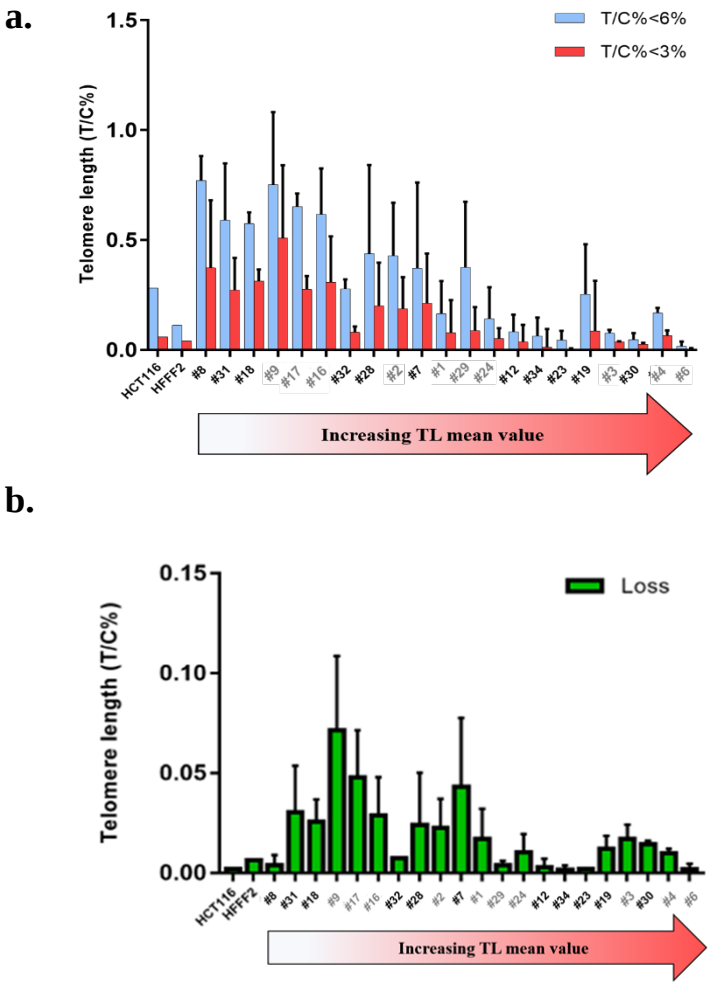
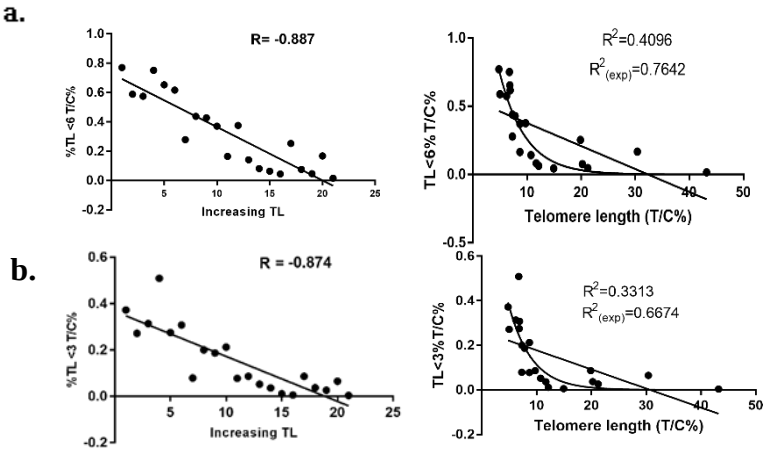


Figure 15. Short telomere and telomere loss frequency in CRC-SCs. Histograms representing the frequency of telomeres with values < 6 T/C% (**a**), < 3 T/C% (**b**), and < 0.5 T/C% (i.e., telomere loss) in our panel of CRC-SCs, which are ordered following the increase in telomere length mean value. Diploid and hyperdiploid CRC-SCs are depicted in black and gray, respectively

Through this approach, we could observe a high variability in the frequency of (very) short telomeres and telomere loss in CRC-SCs (**Fig. 15**). In particular, approximately half of CRC-SCs display short (T/C% values <6) and very short (T/C% values <3) telomeres at higher frequency than HCT 116. Moreover, the vast majority (approximately 81%) of CRC-SCs present an elevated telomere loss frequency as compared to HCT 116, whose telomere loss frequency is of 0.22%.

These results demonstrate the existence of a side population of chromosomes with short telomeres in CRC-SCs.

When we investigated the correlation between telomere length and the three different categories, we observed a strong inverse correlation between telomere length (expressed either as telomere length mean value or telomere lengths ranking, i.e., the classification of CRC-SCs according to their telomere length mean value) and the frequency of short telomere ($R^2exp = 0.764$; Spearman correlation $R = -0.89$, $P=0.0018$; linear correlation $R^2 = 0.41$) and very short telomere <3 ($R^2exp = 0.667$; Spearman correlation $R = -0.87$, $P=0.0063$; linear correlation $R^2=0.33$) (**Fig. 16a and 16b**). On the contrary, the correlation between telomere length and telomere loss was weaker ($R^2exp=0.206$, Spearman correlation $R = -0.553$, $P=0.0759$; linear correlation $R^2 = 0.15$) and this for the presence of a significant fraction of telomere loss in some CRC-SCs (e.g. #30, #4, #6) with long telomeres (**Fig. 16c**).



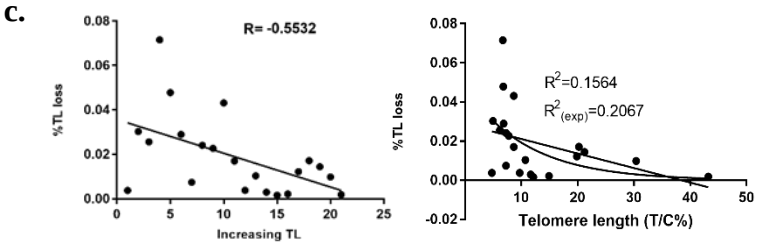


Figure 16. Correlation between telomere length and short telomeres or telomere loss frequency in CRC-SCs. Bi-parametric correlation analysis between telomere length mean values or telomere length ranking and the frequency of telomere length <6 T/C% (**a**), telomere length <3 T/C% (**b**) and telomere loss (**c**) in all CRC-SCs. Linear coefficient R^2 or R^2exp values and Spearman correlation *R* values are reported.

Detection of Fragile Telomeres: Telomeric Doublets

When we performed telomere status analysis by Q-FISH, we often detected the presence of two clear, distinct telomere signals on single chromatids (**Fig. 17**), a telomere aberration known as telomere doublets (or simply doublets). Doublets are reportedly associated with incomplete telomeric replication or fork replication stalling, a condition that leads to the exposure of stretches of single stranded DNA, acquisition of a fragile phenotype and activation of the DDR [315,316]. In this context, Pennarum and colleagues demonstrated that doublets can result from an improper t-loop formation and can occur in either leading or lagging strands [317].

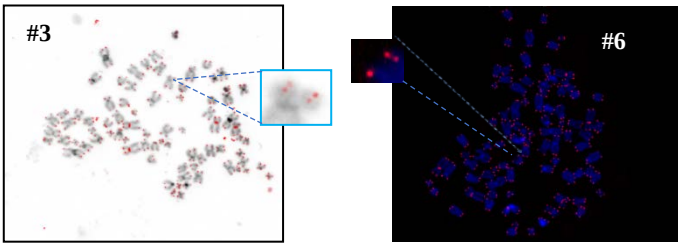


Figure 17. Fragile telomeres in CR-CSCs. Image of metaphases of two representative CRC-SCs (#3 and #6) subjected to Q-FISH analysis as reported in Figure 2. Higher magnifications show the presence of one or two telomere doublets in individual telomeres.

We thus launched an analysis to determine the frequency of telomere doublets in each CRC-SC of our panel by extrapolating the number of doublets per chromatid. As shown in **Figure 18**, most CRC-SCs present high levels of doublets (range between 0.12 and 0.49 per chromosome), with few of them (#3, #30 and #6) displaying a particularly high frequency of doublets (0.49, 0.47 and 0.28 per chromosome, respectively).

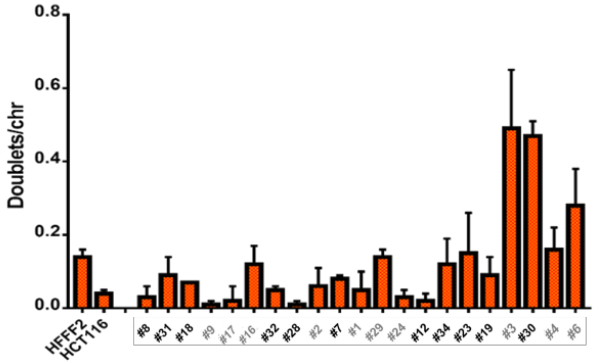


Figure 18. Frequency of telomere doublets in CRC-SCs. Histogram illustrating the frequency of doublets per chromosome in the depicted CRC-SCs, ordered according to the increase in telomere length value. Diploid and hyperdiploid CRC-SCs are illustrated in black and gray, respectively.

In line with what reported in literature [10], there was no significant correlation between the frequency of doublets and telomere length mean value ($R^2 = 0.37$, $P=0,0022$) (**Fig. 19a**). However, there was a positive correlation trend when we considered telomere length's ranking (Spearman correlation $R= 0.68$, $P=0,0004$). (**Fig. 19b**), thereby indicating the need of confirming this result in a larger sample size.

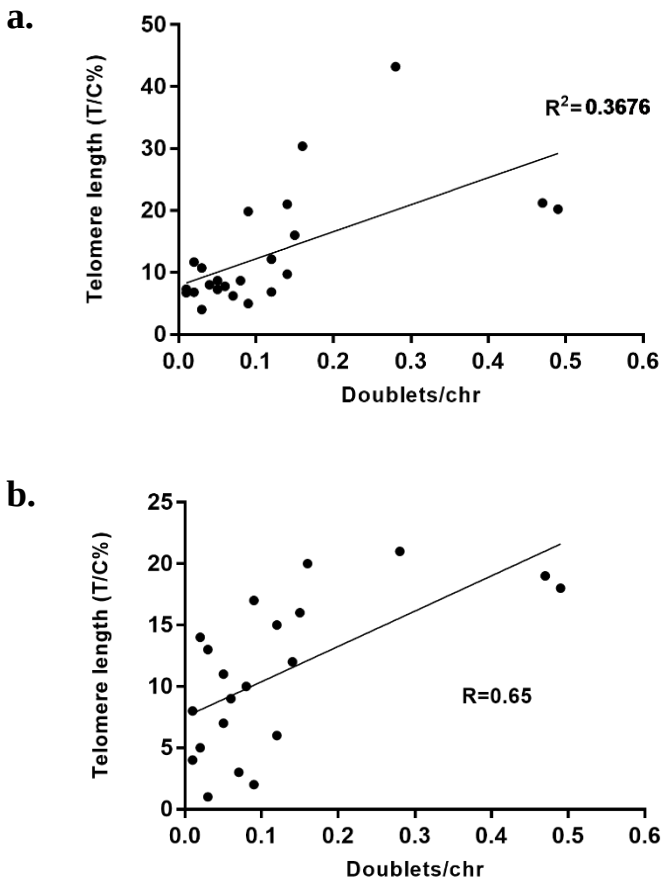


Figure 19. Correlation between telomere and telomere doublets in CRC-SCs. Bi-parametric correlation analysis between telomere length mean values and frequency of doublets per chromosome (**a**) or telomere length ranking and frequency of doublets per chromosome (**b**) in all CRC-SCs Linear coefficient R^2 and Spearman correlation R values are reported.

These findings demonstrate that a significant fraction of CRC-SCs display fragile telomeres.

Telomerase Activity in CRC-SCs

The reactivation of telomerase is a well-known marker of tumor transformation in somatic cells [318–320]. In this context, a range of evidence, which needs further experimental investigation, seems to indicate the presence of telomerase activity also in CSC [13,271,321,322]. We thus decided to analyze telomerase activity in our panel of CRC-SCs. To this aim, CRC-SCs were collected and lysed, and then protein extracts were subjected to the real time-PCR-based Telomerase Repeated Amplification Protocol (TRAP) assay. As shown in **Figure 20**, we observed a high variability in telomerase activity among CRC-SCs. Interestingly, the vast majority (approximately 90%) of CRC-SCs displayed a telomerase activity lower than that of HCT 116.

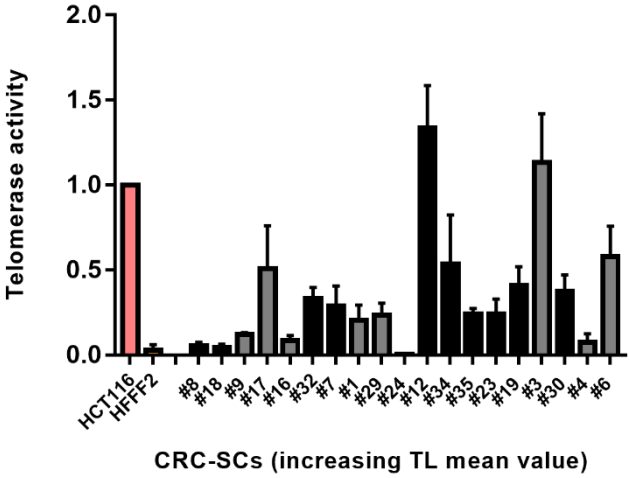


Figure 20. Telomerase activity in CRC-SCs. CRC-SCs were collected and their protein extracts subjected to TRAP assay to evaluate telomerase activity HCT116 and HFFF2 were used as internal reference point. Histogram shows telomerase activity of all CRC-SCs ordered according to the increase in telomere length mean value. Diploid and hyperdiploid CRC-SCs are depicted in black and gray, respectively. The value of telomerase activity for #31, #28 and #2 is missing as the analyses are ongoing.

Although we found no correlation between telomerase activity level and telomere length (R^2= *0,060, P= 0,3247*), we observed that a significant subset of CRC-SCs with very short telomeres (e.g., #8, #18 and #16) presented a very low telomerase activity, which seems at odds with what previously published and what expected. Finally, we did not observe any correlation between chromosomal number and telomerase activity (data not shown) and between fragile telomeres (as indicated by the presence of doublets) and telomerase activity (*R^2=0.12, P=0.16*) (**Fig. 21a and 21b)**.

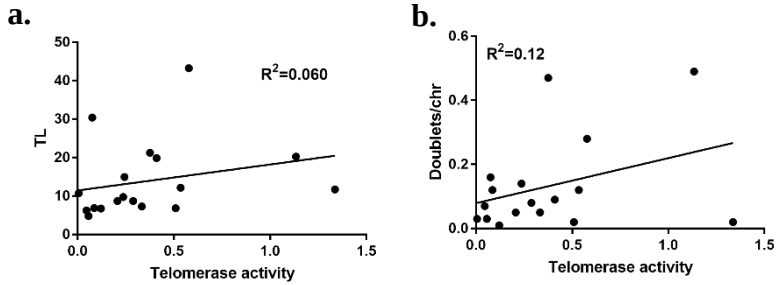


Figure 21. Correlation between telomerase activity and telomere length or doublets. Bi-parametric correlation analysis between telomerase activity (determined by TRAP assays reported in Figure 11) and telomere length mean values (**a**) or telomerase activity and telomere doublets frequency (**b**).in all CRC-SCs. Linear coefficient R^2 values are reported**.**

Taken together, these results suggest that, as opposed to what expected, there is a great variability in telomere activity in CRC-SCs

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DISCUSSION

Despite most solid tumors, including colorectal cancers, are genetically instable, the status of genomic stability in CSCs and the regulatory mechanism for its preservation in this cancer subpopulation have been poorly investigated so far and are matters of contentious. The current hypothesis is that, beyond having stemness properties, CSCs would share with normal stem cells a stable chromosomal set and high efficient processes for maintaining genomic (and chromosome) stability[2], including a robust DDR and possibly a constitutive telomerase activity. In contrast with this hypothesis, here, we provided evidence that - at least in colorectal cancer - CSCs display an elevated heterogeneity in chromosome number and telomere-associated parameters, a finding that can have important implication for cancer therapy. In particular, by performing chromosomal counting, FACS-mediated cell cycle analysis, Q-FISH studies and TRAP assays on a vast panel of patient-derived CRC-SCs, we showed of a high inter-cellular variability of ploidy, telomere length and telomerase activity in CRC-SCs.

When analyzing chromosomal numbers, we observed that more than half of CRC-SCs bear a hyperdiploid karyotype, which suggests their derivation and evolution from a tetraploid intermediate undergoing one or several rounds of chromosome missegregation event(s) [323]. As a matter of fact, hyperdiploid cells display an imbalance in copy number of multiple chromosomes and an elevated number of chromosomes (> 50). These features reportedly boost the level of genomic/chromosomal instability by altering the expression levels of components (and thus the stoichiometry) of the machineries for cell cycle progression, DNA repair and chromosome segregation, and/or by overloading these cellular processes [18]. In this context, we previously demonstrated that cells undergoing a round of whole-genome duplication display an intrinsic inability to correctly execute mitosis, increased level of CIN and proneness to activate mitotic catastrophe [268]. Moreover, in a recent study we discovered that the acquisition of a hyperdiploid karyotype induces a dramatic augmentation of the level of replication stress in CRC-SCs by perturbing the DNA replication process [11]. Telomeres dysfunction is known to increase the level of structural and/or numerical CIN via a mechanism involving the fusion of telomeric ends followed by the breakage-fusion-bridge cycle, [324]. Moreover, telomere shortening is reported to induce genomic instability by promoting

endoreplication and consequently whole-genome duplication [265–267]. In this study, we did not reveal a correlation between increased chromosomal number and either telomere length or telomerase activity in CRC-SCs. This observation, which requires further experimental confirmation, seems to indicate the relevance and (co)existence of other CIN-inducing mechanisms in CRC-SCs, such as defect in centrosome cycle, spindle assembly checkpoint regulation and/or sister-chromatid cohesion (reviewed in [269]). However, to definitely elucidate the precise link between telomere biology and CIN in CSCs, we have started a series of experiments to determine the level of CIN in all CRC-SCs (by clonal FISH studies, SNP microarray analysis and next-generation sequencing studies to evaluate copy-number variation) and then analyze how it correlates with telomere length and telomerase activity.

Importantly, this project provides also four novel, relevant insights in CSC telomere biology. First, we reported that most CRC-SCs display higher telomere length mean value than colorectal cancer cell lines, and, of them, 5 CRC-SCs present very long telomeres also in comparison to primary human fibroblasts. This result is in contrast with previous findings showing that CSCs have telomere length either similar [8,276] or shorter [224] than bulk tumor cells. Although some CRC-SCs with very short telomeres has an almost undetectable telomerase activity, we could find no correlation between telomere length and telomerase activity. This evidence seems to rule out the possibility that telomerase activity can contribute by itself to telomere length maintenance or elongation in CSCs. In this context, it appears of interest to evaluate the mutational status of Shelterin genes and *TRF1* as well as the presence of secondary structures as G-quadruplex in CRC.SCs and correlate them to telomerase activity. The presence of long telomere in cancer cells have been ascribed to the activation of the alternative lengthening of telomeres (ALT) mechanism. This telomere maintenance process seems frequent in cancers, with an estimated percentage of 15% in all tumor analyzed [325]. On the contrary, only few studies have investigated ALT mechanisms in CSCs, including one in which ALT activation has been detected in glioma stem cells [277]. The presence of a high intra-cell variability in telomere length is believed to be associated with an ALT metabolism [9] . In this study, we demonstrated a positive correlation between telomere length mean values and intra-cellular telomeres length variability. This result is consistent with previous findings showing that ALT-positive cell lines display a long telomere length mean value and a very wide telomere length distribution [234,235,314,326,327]. At odds with this evidence, a recent study reported a correlation between telomeres length variability and

very short telomeres frequency in blood lymphocytes [133], which casts doubts on high intra-cell variability in telomere length as an ALT marker. In view of this consideration, novel experiments have been launched in order to confirm ALT status and the importance of ALT mechanism in (at least some) CRC-SCs of our panel.
Second, we observed the presence of short telomeres and telomere loss at high frequency in most (but not all) CRC-SCs as compared to tumor cells. Moreover, we reported a negative correlation between telomere length mean values and the appearance of short telomeres. Intriguingly, we also showed that not only CRC-SC with short telomeres but also some CRC-SCs (e.g., #19, #3, #30, #4, #6) with long telomeres (but high intra-cellular telomere length variability) display a significant fraction of chromosome with telomere loss. It will be interesting to analyze whether these cells display ALT activation and/or high levels of CIN, as short telomeres and telomere loss have been proven to boost CIN [328].

Third, we provided evidence of a high frequency of doublets per chromosome in most of CRC-SCs, which indicates incomplete/defective telomere replication or fork replication stalling in these cells. Although we did not find a positive correlation between telomere length values and doublet frequency, which is in line with what previously reported [10], we showed that most long-telomere CRC-SCs present also a high number of doublets. Of relevance, these cells were previously reported as those having an ongoing replication stress response due to high levels of replication stress at baseline, coupled to a high chromosome content [11]. To demonstrate the presence of alteration in telomere replication and thus telomere damage in these CRC-SCs, we have now started immune-FISH experiments aimed at assessing telomere dysfunction induced foci (TIFs) by analyzing the co-localization between telomere (by Q-FISH studies or using antibodies directed against TRFs) and DNA damage markers, such as ɣH2AX or 53BP1.
Finally, we observed a high variability in telomerase activity within our panel of CRC-SCs, which is in line with what reported in cancer, *in vivo* [329,330]. Of note, we can find that 5 CRC-SCs of our panel were telomerase negative. This result is in contrast with previous studies suggesting that, similar to adult stem cells, CSCs are telomerase-positive [12,13,331,332] and suggests a potential relevance of ALT mechanisms for telomere biology in cancer stem cells, as most of anticancer therapies are aimed to target telomerase activity. Therefore, ALT could be an important therapeutic target, contributing to eradicate tumors definitely.

CONCLUSION AND PRESPECTIVE

In this study, we performed an extensive cytogenetic and functional characterization of chromosome number and telomere-associated parameters in a panel of patient-derived CRC-SCs, showing the presence of high levels of inter-cellular heterogeneity in ploidy, telomere length mean value and telomerase activity. We also found elevated intracellular heterogeneity in telomere lengths in most (often long-telomere) CRC-SCs, with a significant fraction of CRC-SCs showing chromosomes with short telomeres, telomere loss and fragile telomere at elevated frequency.

These findings are important for cancer and cancer therapy as they shed light upon (and open a new horizon in the study of) chromosome and telomere biology of CSCs, which are the roots and chemo-resistant seeds of cancer, and thus they can have important implication providing fundamental insights for the development of novel telomere-based strategies for depleting CSCs.

In this context, our main perspectives are

(1) to correlate results of this study with CRC-SC drug-sensitivity data, which have been obtained in the context of other projects of the group using compounds included in specific cancer-related drug libraries, commonly employed in the clinics to treat colorectal cancer patients (5-fluorouracil, oxaliplatin, irinotecan), related to telomere biology (DDR, G-quadruplex or telomerase inhibitors as imetelstat and MST312) or having immunotherapeutic potential;
(2) to analyze the expression levels and mutational status of proteins, including (but not limited to) those forming the Shelterin complex or involved in the DDR;
(3) to evaluate the presence of the ALT pathway in CRC-SCs by analyzing telomere sister chromatid exchange (T-SCE), c-circles, ALT-associated PML-NBs (APBs), telomeric repeat-containing RNA (TERRA)
(4) to correlate telomere status with *in vivo* aggressiveness of CRC-SCs as well as patient's data.

The successful completion of these ongoing experiments will uncover the true potential of this study and could pave the way for its future clinical translation.

SUPPLEMENTARY DATA

MATERIALS AND METHODS

CSC Isolation and Culture

Human colorectal carcinoma HCT 116 were routinely cultured in McCoy's 5A medium supplemented with 10% fetal calf serum (FCS), 10 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer, 100 units/mL penicillin G sodium salt and 100 µg/mL streptomycin sulfate, while Human Fetal Foreskin Fibroblasts (HFFF2) (ECACC, UK) in D-MEM supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. Cells were maintained in a 95% air/5% CO2 atmosphere at 37° C. Colorectal cancer stem cells (CRC-SCs) were isolated from human colorectal cancer patient samples via mechanical and enzymatic dissociation followed by resuspension in CSC isolation medium containing advanced DMEM/F12 medium supplemented with 2 mM L-glutamine, 2% B-27 supplements, 1% N2-supplement, 20 ng/mL human epidermal growth factor (EGF), 10 ng/mL human fibroblast growth factor (FGF2) (basic) (both from PeproTech Inc., London, UK), 10 mM nicotinamide and 1 µM Y-27632 (both from Sigma-Aldrich, St. Louis, MO) as previously reported [312]. Surgical specimens from patients were all obtained in accordance with the standards of the institutional Ethics Committee on human experimentation, authorization no. CE5ISS 09/282. Once isolate, CRC-SCs were grown as spheroids in ultra-low attachment tissue culture flasks and routinely maintained in CSC culture medium composed by DMEM/F12 medium containing 2 mM L-glutamine, 0.6% glucose, 9.6 mg/mL putrescine, 6.3 ng/mL progesterone, 5.2 ng/mL sodium selenite, 4 mg/mL heparin sodium salt, 100 ng/mL hydrocortisone, 0.025 mg/mL insulin, 0.1 mg/mL apotrasferrin (Euroclone, Pero, Italy) and supplemented with 20 ng/mL human EGF, 10 ng/mL human FGF2, 10 mM nicotinamide (Sigma-Aldrich) as described in (Manic et al., 2017). CRC-SCs were passaged once a week at dilution 1:2 by mechanical (micropipette) or enzymatic [<5 min at 37°C with TripLE™ Select and Accumax (Sigma-Aldrich) (1:1 dilution)] dissociation and incubated in standard culture conditions in ultra-low attachment tissue culture flasks.

Collection of Chromosome Spreads

CRC-SC spheroids were dissociated enzymatically and then seeded at a density of $2x10^5$ cells/well onto 6-well (ultra-low attachment) plates. After 24 h, CRC-SCs were treated with with 5 μM colchicine (Sigma-Aldrich) for 5 h to enrich the fraction of mitotic cells. Successively, cells were collected and incubated with 75 mM KCl (J.T.Baker) for 12 min at 37°C prior to fixation in freshly prepared Carnoy solution (3:1 v:v methanol:acetic acid, both from Sigma-Aldrich) and storage at -20°C. Cells were then seeded onto slides and utilized for cytogenetic analysis.

Ploidy Analysis

Ploidy analyses were evaluated by performing chromosome counts and cell cycle profile. To determine modal chromosome number, CRC-SCs treated with colchicine and seeded as reported in the previous section were stained and mounted with a solution containing the DNA dye 4',6-diamidino-2-phenylindole DAPI and Prolong-Gold antifade (both from Molecular Probes-Thermo Fisher Scientific). Metaphases were captured using the Axio-Imager M1 microscope equipped with a coupled charged device (CCD) camera (Zeiss, Oberkochen, Germany) and images (100 metaphases for each sample in at least three independent experiments) analyzed with ImageJ v1.5. Alternatively, to determine the exact cell ploidy. To assess cell cycle profiling, CRC-SC spheroids were dissociated, seeded at a density of 3×10^5 cells in 6-well (ultra-low attachment) plates (3 mL of medium/well) and cultured for 24 h. Thereafter, cells were collected and fixed in ice-cold 80% (v/v) ethanol (Sigma-Aldrich). Samples were incubated at least 24 h at -20 °C, then washed twice with PBS and stained with 50 μg/mL propidium iodide (PI) (Sigma-Aldrich) in 0.1% (w/v) D-glucose in PBS supplemented with 1 μg/mL (w/v) RNAse A (Sigma-Aldrich) for 30 min at 37°C. Cells were then incubated overnight at 4°C upon flow cytometry-mediated analyses. Cytofluorometric acquisitions were performed by means of a MACSQuant® Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) while data were statistically evaluated using the FlowJo software (FlowJo LLC, Ashland, OR). Only the events characterized by normal forward

scatter (FSC) and side scatter (SSC) parameters were gated for inclusion in the statistical analysis. Cell cycle analyses were performed upon exclusion of the sub-G1.

Telomeric Quantitative FISH (Q-FISH)

For the assessment of telomere length, CRC-SCs were treated with colchicine and seeded as reported in "Collection of chromosome spreads" section. After seeding (48h), slides were rinsed with PBS pH 7.5, and fixed in 4% formaldehyde for 2 min. After two rinses in PBS, slides were incubated in acidificated pepsin solution for 10 min, rinsed, and dehydrated through graded alcohols. Slides and probes (Cy3 linked telomeric and chromosome 2 centromeric PNA probe, Panagene and DAKO Cytomatation, respectively) were co-denatured at 80°C for 3 min and hybridized for 2h at RT in a humidified chamber. Slides were the hybridized, washed twice for 15 min with 70% formamide, 10mM Tris pH 7,2 and 0,1% BSA and the thrice for 5 min in a solution containing 0,1 M Tris pH 7,5, 0,15 M NaCl and 0,08 % Tween 20. Slides were then dehydrated with an ethanol series, air dried and finally counterstained with DAPI in Vectashield (Vector Laboratories). Images were acquired at 63x magnification using an Axio Imager Z2 microscope (Carl Zeiss) equipped with a Cool Cube 1 CCD camera (MetaSystems). Telomere size analysis was performed with the ISIS software (MetaSystems). The software calculates telomere lengths as the ratio between the total telomere fluorescence (T) and the fluorescence of the centromeres of the two chromosomes 2 (C), which is used as the internal reference in each metaphase spread analyzed, and expressed as percentage (T/C%). At least 10 metaphases were analyzed for each sample in three independent experiments. Telomere doublets were analyzed on 100 Q-FISH metaphase spreads and counted using the ISIS software (MetaSystems).

Real Time Quantitative–Telomerase Repeat Amplification Protocol Assay (RTQ-TRAP)

To assess telomerase activity, dissociated CRC-SC spheroids were seeded at a density of $1x10^5$ cells/well onto 6-well plates. Twenty-four h later, cells were collected and lysed in TRAPeze® 1X CHAPS Lysis Buffer (MerckMillipore) and telomerase activity was measured by the SYBR green RTQ-TRAP assay, which was conducted as described elsewhere [333] with minor modifications. Briefly, the reaction was performed with protein extracts (1,000 cells), 0.1 µg of telomerase primer TS, and 0.05 µg of anchored return primer ACX, in 25 µl of SYBR Green PCR Master Mix (Biorad). Primer sequences are reported in the following manuscript (Kim and Wu, 1997). The reaction was performed using the Applied Biosystems 7900HT fast Real-Time PCR System, samples were incubated for 20 min at 25°C and amplified in 35 PCR cycles with 30 sec. at 95°C and 90 sec. at 60°C (two step PCR). The threshold cycle values (Ct) were determined from semi-log amplification plots (log increase in fluorescence as a function of cycle number) and compared with standard curves generated from serial dilutions of telomerase-positive (tel+) HCT116 and (tel-) HFFF2 cell extracts. Each sample was analyzed in triplicate in at least three independent experiments. Telomerase activity was expressed relative to the telomerase-positive (tel+) sample.

Statistical Analysis

Statistical analysis was performed with Microsoft Office Excel 2016 (mean, standard deviation) and subsequently, data were graphed with the software GraphPad Prism 7. Linear, exponential and Spearman correlation values were obtained from GraphPad Prism analysis.

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