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Telomere Maintenance Mechanisms in Soft Tissue Sarcomas

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1. Introduction

1.1 Telomeres and tumorigenesis

1.1.1 Telomere structure

Human chromosomes, which are composed of linear double-stranded DNA, are capped at their ends by nucleoprotein complexes called telomeres (reviewed in (Martinez & Blasco, 2011)). This telomeric cap prevents end-to-end fusion events between chromosomes, and protects chromosome ends from being recognized as double-stranded breaks by the DNA damage machinery in the cell. Telomeric DNA is composed of tandem 5′TTAGGG repeats at each end of the chromosome, extending up to tens of kilobases. Poorly understood end-processing reactions after replication result in a 3′ overhang on the G-rich strand, which invades the upstream double-stranded telomeric DNA to form a displacement (D)-loop such that the end of the chromosome is buried thereby preventing detection by the cellular DNA damage response (Greider, 1999). Telomeric DNA is in complex with a number of proteins that act to stabilize the structure and mediate telomeric functions of capping and length regulation (Figure 1). Double-stranded (ds) telomeric repeats are bound directly by TRF1 (Zhong et al., 1992) and TRF2 (Bilaud et al., 1997; Broccoli et al., 1997), while the POT1-TPP1 heterodimer binds to the single-stranded (ss) telomeric DNA (de Lange, 2005; Lei et al., 2002). The ds- and ss-telomeric DNA complexes are linked through their interaction with TIN2 (Abreu et al., 2010; Kim et al., 1999; O'Connor et al., 2006), together forming the telomeric shelterin complex (de Lange, 2005). Additional proteins necessary for telomeric function are recruited by interactions with components of the shelterin complex (reviewed in (Martinez & Blasco, 2011)).

Mammalian telomeres have been shown to contain characteristics of heterochromatin, including the presence of homologues of the heterochromatin binding protein HP1 (Koering et al., 2002; Sharma et al., 2003), enriched tri-methylation of histone H3 lysine 9 (H3K9) and histone H4 lysine 20 (H4K20) (Garcia-Cao et al., 2004), and methylation of CpG dinucleotides in subtelomeric DNA repeats (Gonzalo et al., 2006). Studies in telomerase knockout mice demonstrated that as telomeres become shorter the heterochromatic marks are lost and replaced by marks characteristic of open chromatin, such as increased acetylation of histone tails (Benetti et al., 2007), suggesting that a minimum telomere length is necessary to maintain the appropriate chromatin structure at chromosome ends. Alterations in the level of epigenetic modifications, such as tri-methylation of H3K9 and H4K20 via knockout of the relevant modifying enzymes, led to increased telomere length

without effect on telomere end-capping function (Garcia-Cao et al., 2004). Intriguingly, evidence of increased telomeric recombination is also a result of altering telomeric chromatin structure (Gonzalo et al., 2006).

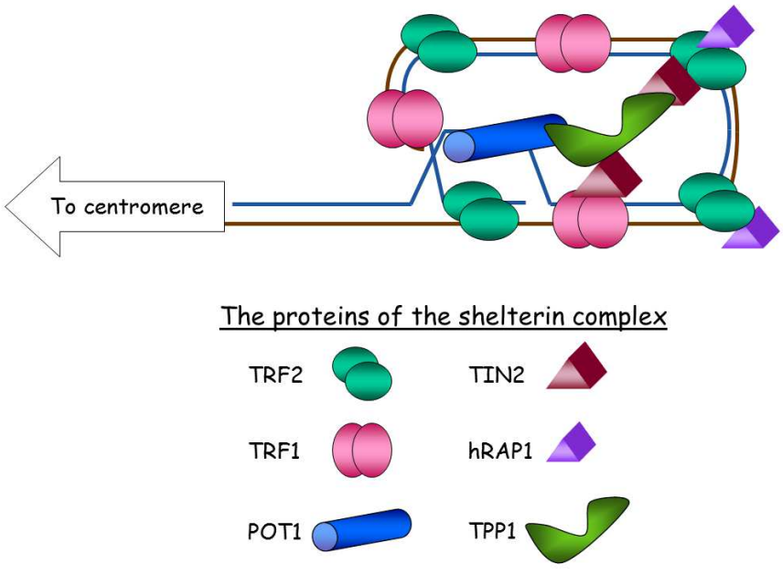


Fig. 1. Proteins of the shelterin complex. TRF1 and TRF2 bind directly to double-stranded telomeric sequence, with TRF2 recruiting RAP1. The POT1-TPP1 binds to single-stranded regions. Interactions with TIN2 link the single-stranded and double-stranded binding complexes.

Despite having chromatin features characteristic of heterochromatin, telomeres are now known to be transcriptionally active, giving rise to a species of long non-coding RNA (lncRNA) termed TERRA (Luke & Lingner, 2009). Long non-coding RNAs are involved in establishing and maintaining chromatin structure (Whitehead et al., 2009). TERRA has been suggested to play a role in telomere heterochromatin formation (Deng et al., 2009). TERRA associates with telomeres and may be involved in maintaining or remodeling telomere structure during development and differentiation (Luke & Lingner, 2009).

1.1.2 Telomere dynamics

Linear DNA molecules use RNA to prime replication by DNA polymerase. At the completion of synthesis, these primers are degraded and gaps are filled, but the regions at the 5′ ends of the newly synthesized strands cannot be filled in. Thus, with each replication cycle, telomeric sequences shorten at their 5′ ends (Figure 2). End processing events subsequent to replication (Sfeir et al., 2005) may also contribute to sequence loss. Excessive shortening of telomeres disrupts the shelterin complex through loss of binding sites for shelterin complex proteins, which exposes chromosome ends to DNA damage machinery. In the presence of an active DNA damage response (DDR), telomere uncapping activates DNA damage checkpoints, leading to cell cycle arrest or apoptosis (Martinez & Blasco, 2011). Thus, telomere length acts as a molecular clock, limiting the total number of divisions any given cell may undergo. In the absence of a robust DDR, such as often occurs on the road to transformation, exposed telomere ends result in increased genomic instability through

breakage-fusion-bridge cycles induced by joining of chromosome ends. This 'telomere crisis' can only be resolved by restoring telomeres to a length sufficient for functional shelterin complex assembly. Therefore, to circumvent the effects of telomere attrition and attain unlimited replicative potential, cancer cells must solve the so-called 'end replication problem.'

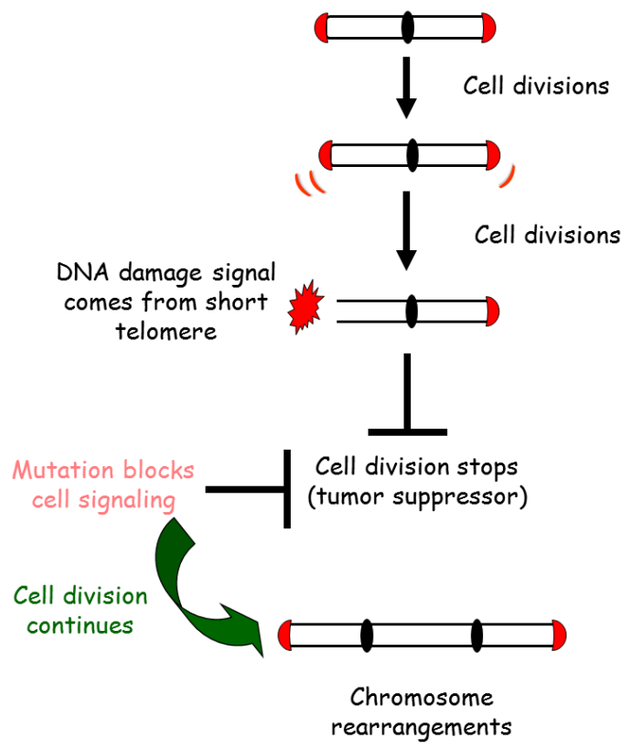


Fig. 2. Telomere attrition from subsequent cell divisions continues until critically short telomeres result in a DNA damage response and the cessation of cell division. In cancer cells, mutations in key regulatory proteins block DNA damage signaling, allowing cell division to continue, potentially leading to chromosome rearrangements from chromosome end-to-end fusions.

Telomere extension occurs by activation of a telomere maintenance mechanism (TMM), via either telomerase or a recombination-based mechanism called alternative lengthening of telomeres (ALT). Activation of either mechanism is sufficient to recover from telomere crisis, thus enabling continued growth of the cancer cells. Resolution of telomere crisis is not a prerequisite to tumor formation, however, as some adult cells contain adequate telomeric reserves to form tumors requiring clinical intervention before loss of telomeric DNA becomes sufficient to induce crisis (Reddel, 2000). Nevertheless, telomere attrition will ultimately limit tumor growth. TMM activation, because it increases replicative potential, is often associated with higher grade tumors and poorer patient prognosis (Costa et al., 2006; Matsuo et al., 2009; Ulaner et al., 2003). Indeed, progress through telomere crisis with its associated genome instability has been suggested to contribute to tumorigenicity by generating a hypermutability environment (Chin et al., 2004), analogous to the tumorigenic potential of cells with microsatellite instability.

2. Telomere maintenance mechanisms

2.1 Telomerase

Telomerase is a ribonucleoprotein complex that adds telomeric DNA *de novo* onto chromosome ends (Greider & Blackburn, 1987). The catalytic component of the holoenzyme, TERT, is a reverse transcriptase (RNA-dependent DNA polymerase) that uses the RNA component, TR (aka TERC), as a template for telomere extension (Figure 3). Telomerase is commonly active in germline and some stem cell populations (Artandi & DePinho, 2010), ensuring species-specific telomere length and sufficient reserves to complete developmental programs, respectively. In addition, telomerase may be transiently active during certain differentiation programs (Hodes et al., 2002), leading to regulated telomere extension prior to expansion. Telomerase activity is not enough to entirely prevent telomere attrition over an individual's lifetime (Martinez & Blasco, 2011). Furthermore, stressing stem cell compartments, for example as occurs following bone marrow transplantation, may contribute to premature aging of differentiated cells arising from those stem cell compartments (Allsopp et al., 2001; Lewis et al., 2004). Most differentiated cells do not express hTERT and therefore lack telomerase activity. Expression of telomerase, while conferring immortality on cells, is not directly tumorigenic with transformation requiring additional genetic changes (Bodnar et al., 1998; Hahn et al., 1999; Vaziri & Benchimol, 1998). However, as discussed above, acquisition of telomere length stabilization removes a critical tumor suppressor mechanism. Activation of telomerase is the primary TMM in most carcinomas as well as translocation-associated sarcomas.

Experimentally, telomerase activity is determined through an assay called the telomere repeat amplification protocol (TRAP) (Kim et al., 1994). In this assay, protein extracts are incubated with a short oligonucleotide that can be extended by telomerase, if it is present. After PCR amplification and acrylamide gel electrophoresis of products, extracts containing active telomerase will show a ladder of products with increasing numbers of telomeric TTAGGG repeats. The presence of active telomerase in the extract does not necessitate cellular activity, as *in vitro* activity can be achieved without the additional factors required *in vivo* to support extension of chromosome ends. Indeed, mutants that are catalytically active in vitro but unable to maintain telomeres in vivo, have been described (Counter et al., 1998) and highlight the multiple levels of regulation associated with telomere maintenance by telomerase (Osterhage & Friedman, 2009). The absence of activity by TRAP is also not definitive, as telomerase activity can be easily destroyed by technical challenges during extract isolation and soluble inhibitors of either the initial extension reaction or the PCR amplification step may be present. A definitive lack of activity can be attained by demonstrating the absence of the TERT mRNA by quantitative PCR, as activity cannot occur in the absence of the catalytic component of telomerase.

2.2 ALT

Alternative lengthening of telomeres is an umbrella term for all non-telomerase mechanisms for telomere maintenance (Cesare & Reddel, 2010; Henson & Reddel, 2010). Telomeres in ALT cells are often heterogeneous in length, ranging from very short (<6 kb) to very long (>20 kb), which are easily visualized either by southern blot or quantitative FISH. Despite the broad range of sizes, the average telomere length, as measured by quantitating southern blots, is longer than that observed in normal adult or telomerase-positive cancers (Bryan et al., 1995). ALT cells also frequently show mini-satellite instability (Jeyapalan et al., 2008;

Jeyapalan et al., 2005) and increased telomere sister chromatid exchange (T-SCE) (Londono-Vallejo et al., 2004), which presumably arises from increased recombination at telomeres. Both assays are of limited use in tumor tissue as the mini-satellite instability assay requires the ability to PCR long (~4KB) DNA fragments that may have significant degradation in archival tumor samples and the T-SCE assay relies on the ability to label cells in culture. Also found in ALT cells are double-stranded circular DNA molecules called t-circles that are generated from telomeric DNA and may function in telomere elongation via recombination-independent rolling circle replication (Tomaska et al., 2004). Single-stranded circles, called either c-circles or g-circles based on sequence, are also present in these cells, with c-circles being much more abundant (Henson et al., 2009). The ability to detect these circles with high specificity in archival tumor tissue has not been demonstrated. Perhaps the most frequently assayed characteristic of ALT cells is the appearance of ALT associated PML bodies (APBs), in which telomeric DNA co-localizes with the PML nuclear body (Yeager et al., 1999).

The role of APBs in ALT remains controversial. Conflicting reports have suggested that APBs are a marker of ALT cells that are irreversibly arrested or, conversely, are required for telomere maintenance by ALT. Early studies of ALT-positive cell lines immortalized *in vitro* suggested that perturbation of the p53 pathway might be a common element (Opitz et al., 2001; Rogan et al., 1995). We have previously demonstrated that forced expression of a transactivation-dead p53 suppresses growth of ALT-positive, but not telomerase positive, cells (Razak et al., 2004). We reported that this caused an increase in APB frequency in the absence of downstream effectors of p53, such as p21. We concluded that abrogation of p53 suppression of recombination function was required for ALT activation. In contrast, the Reddel group has reported that over-expression of transactivation competent p53 leads to an increase in APBs, and that this increase in APBs requires p21 (Jiang et al., 2009). These authors conclude that APBs arise in arrested cells rather than cells undergoing telomere elongation by recombination. Furthermore, although ATM is constitutively active in p53-positive ALT-positive cell lines, activation of p53 and downstream effectors only occurs when telomeres are uncapped via perturbation of the shelterin complex (Stagno D'Alcontres et al., 2007). PML is essential for p21 induced cellular senescence in this context, although it is not required for p53 to associate with telomeres.

In contrast to experiments suggesting that APBs only occur in arrested cells, we and others have shown that DNA replication occurs in APB-positive cells, suggesting that these structures are present in actively cycling cells (Grobelny et al., 2000). Others have found that disruption of APBs prevents telomere maintenance by ALT and leads to loss of culture viability, arguing for an active role of APBs in telomere maintenance by ALT (Jiang et al., 2005). The spatio-temporal dynamics of telomeric DNA association with PML bodies have been recently described, and the authors of this study conclude that telomere recombination takes place in these structures (Draskovic et al., 2009). Furthermore, new PML bodies form at telomeric DNA regardless of which TMM is active (Brouwer et al., 2009) and APBs form transiently in human diploid fibroblasts following high LET radiation (Berardinelli et al., 2010), suggesting that the association of telomeric DNA with PML bodies may be a component of a DNA damage response. Indeed, telomeres are transiently recognized as DNA damage during normal DNA replication (Verdun & Karlseder, 2006). The consistent theme within these data is that uncapped/short telomeres are localized to the PML body.

TERRA expression is increased in ALT cell lines and is accompanied by a less dense, albeit variable, pattern of sub-telomeric CpG methylation relative to telomerase positive and

normal cells (Ng et al., 2009). It is not clear if the increase in TERRA occurs subsequent to altered sub-telomeric chromatin (i.e., as a consequence) or contributes to generating changes in sub-telomeric chromatin (i.e., as a cause). Recent work implicates TERRA as being critical for telomere structure in the ALT-positive U2OS cell line and telomerase positive HCT116 cells (Deng et al., 2009). TERRA was shown to interact with the end-capping protein TRF2, and decreasing TERRA expression levels led to loss of heterochromatin marks and telomere instability. On the surface, then, one might expect the increased TERRA in ALT cells to increase heterochromatin-associated chromatin condensation making telomeres less accessible to recombination. Conversely, we have shown that TERRA levels increase as telomeres shorten (associated with gaining histone marks consistent with an open chromatin structure) and in response to telomere uncapping (Caslini et al., 2009). The increase in TERRA, in this context, requires activity of the histone H3K4 methyltransferase MLL and the p53 DNA damage response. How might these observations be reconciled? It is likely based on published results that a minimum level of TERRA expression is necessary to support functional end-capping. However, TERRA may play additional roles in ALT-positive cells. Increased TERRA may be necessary to propagate and maintain a more open chromatin structure, which in turn promotes telomeric recombination, by titrating essential factors and modulating the formation of telomeric heterochromatin. Alternatively, increased TERRA in ALT cells may simply reflect the presence of ultra-long telomeres present in these cells. Future studies will elucidate the role of TERRA in telomere stability and telomere maintenance mechanisms.

Many questions remain about the nature of the ALT mechanism. A single ALT cell may show only some of the features associated with ALT, and may show a different subset of features than other ALT cells (Fasching et al., 2005; Slatter et al., 2010). This may result from the existence of multiple mechanisms currently described as ALT. It is unclear to what extent these different mechanisms rely on similar or overlapping pathways for telomere maintenance, and thus whether and to what extent they share genetic requirements for activation and regulation. For example, if the appearance of APBs is related to DNA damage and repair, does the lack of APBs in some ALT cells indicate lower levels of DNA damage and thus lower levels of genome instability? Or are APBs associated with a specific recombination-based pathway for telomere maintenance? An essential step on the pathway to understanding ALT regulation, and the pathways leading to telomere stabilization through recombination, would be the development of an ALT cell line lacking APBs. It remains essential as further work is done to characterize ALT to be cognizant of the potential impact the features used to define cells as ALT-positive have on any conclusions about the characteristics of ALT in general.

ALT seems to be more common in tumors of mesenchymal origin and with complex karyotypes when compared to those of epithelial origin and translocation-driven, respectively (Johnson & Broccoli, 2007; Montgomery et al., 2004; Ulaner et al., 2004), which may provide some insight into the genetic origins of ALT. Both ALT and telomerase can be active in a single tumor (Costa et al., 2006; Hakin-Smith et al., 2003; Johnson et al., 2005; Ulaner et al., 2003), suggesting that lack of functional telomerase is not a prerequisite for ALT activation, although it is not known whether both mechanisms can be active in a single cell. Studies investigating TMM in tumors rely upon a variety of assays to identify telomerase-positive and ALT-positive tumors, respectively (Costa et al., 2006; Montgomery et al., 2004; Ulaner et al., 2003). At least some of the tumors without characteristics of either mechanism are also ALT-positive based upon increased mini-satellite instability (Jeyapalan

et al., 2008; Jeyapalan et al., 2005). However, a substantial fraction of mesenchymal tumors defy TMM characterization using the currently available techniques. In part this is due to many assays not being consistent with the quality of DNA isolated from tumor tissue, particularly archival tissues that are only available as formalin-fixed, paraffin-embedded samples. An alternative possibility is that only a rare subset of tumor cells, e.g. tumor stem cells, actively maintain telomeres while the bulk of the cells comprising the tumor do not have active telomere maintenance.

3. Telomere maintenance mechanisms in tumorigenesis

Although it is used in a considerable proportion of cell lines (~35%), until recently ALT had only rarely been documented in human tumors (Bryan et al., 1997). Accordingly, comparative studies have relied largely upon cell culture systems or investigation of tumors arising in late generation telomerase-deficient mouse models, which are thus forced to use ALT. Although each system has inherent limitations with respect to extrapolation to human cancer, several key insights have come from these studies. Most importantly, the two mechanisms of telomere maintenance are not equivalent with respect to their contribution to the tumorigenic phenotype. Exogenous expression of hTERT, the catalytic subunit of telomerase, in combination with activated Ras and the SV40 early region is sufficient to transform human cells and render them tumorigenic in nude mice (Hahn et al., 1999). In contrast, human cells which rely on ALT instead of telomerase for telomere maintenance, while immortal, are unable to form tumors in nude mice when injected subcutaneously, although they are competent to form tumors when injected under the renal capsule (Sun et al., 2005). If hTERT is introduced into these cells, tumorigenicity is restored in the subcutaneous setting even though telomerase is not required for telomere maintenance *per se*. Likewise, immortalized mouse embryo fibroblasts (MEFs) that use telomerase are readily able to colonize lungs and proliferate when injected into tail veins, while MEFs that use ALT are not (Chang et al., 2003). Thus, while telomere maintenance by either TMM is sufficient to ensure replicative immortality, these studies suggest that telomerase may provide additional growth advantages during *in vivo* tumorigenesis.

Both ALT and telomerase can be active in a single tumor, suggesting that lack of functional telomerase is not a prerequisite for ALT activation, although it is not known whether both mechanisms can be active in a single cell. Given tumor heterogeneity it is possible that distinct regions of a tumor utilize telomerase and ALT respectively. In cell based studies in which telomerase expression was forced in an ALT background, telomerase specifically elongated the shortest telomeres in the population (Grobelny et al., 2001). Despite extensive culturing, characteristics of ALT such as APBs were not altered in the presence of telomerase. This suggests that reconstitution of telomerase, and associated telomere stabilization, is not sufficient to suppress the ALT mechanism once it has been activated.

4. Telomere maintenance mechanisms in liposarcomas

Soft tissue sarcomas (STS) are rare malignancies of mesenchymal origin, with approximately 10 500 cases in 2010 (Jemal et al., 2010). Given the rarity of mesenchymal tumors, studies assessing TMM activation have either used multiple histological types or focused on the more common STS types. The most common adult STS, accounting for ~20% of cases, is liposarcoma, named for its morphological resemblance to adipose tissue. Liposarcomas are

divided into several subtypes based on histological features: well-differentiated (WDLS), de-differentiated (DDLS), pleomorphic (PLS), myxoid (MYXLS), and round cell (RCLS). Based upon expression profiling, it has been suggested that these tumors can be further classified along a developmental pathway from the mesenchymal stem cell to mature adipocytes based on their state of differentiation (Matushansky et al 2008), with de-differentiated liposarcomas most closely resembling mesenchymal stem cells, and well-differentiated liposarcomas most closely resembling mature adipocytes. The remaining histotypes fall between these, with pleomorphic liposarcomas appearing less differentiated than myxoid/round cell tumors. Due to their relatively high frequency among adult STS, several studies of TMM have focused on liposarcomas. Intriguingly, the frequency of specific TMM utilization appears to vary with histological subtype within this category of STS (Figure 3).

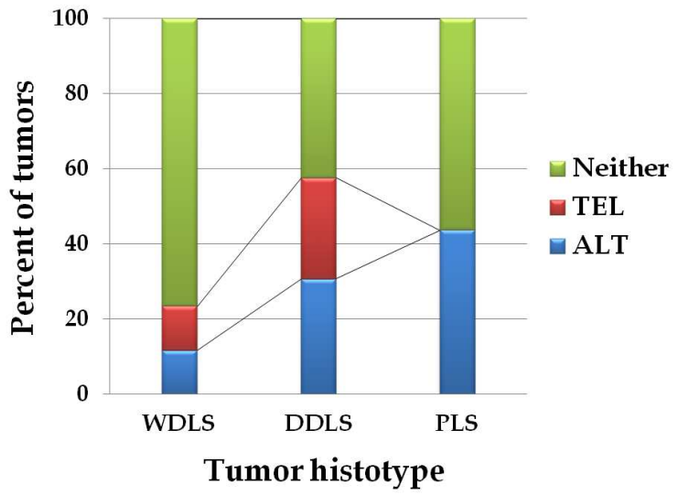


Fig. 3. TMM utilization varies among histological subtypes of liposarcoma.

WD/DDLS together account for 55% of all LS (40% and 15% respectively) (Conyers et al., 2011). Both WDLS and DDLS may contain a diagnostic Chr12q13-15 amplification (Rieker et al., 2010; Tap et al., 2011), although this genetic change is found more frequently in DDLS. WDLS are not typically included among the complex karyotype malignancies; however, upon dedifferentiation this tumor is included in the complex karyotype subdesignation. Combined, these tumors are called atypical lipotomous tumors, which, like alternative lengthening of telomeres, is commonly abbreviated ALT. WDLS are low grade tumors that are histologically similar to adipose tissue, showing primarily local recurrence with limited metastasis, in contrast to DDLS, which are higher grade tumors with greater propensity for metastasis. The WD/DDLS that have an active TMM use ALT and telomerase with similar frequency, but WDLS have a higher fraction of malignancies with no evidence of telomere maintenance (75% for WDLS, 50% for DDLS) (Costa et al., 2006).

MYX/RC LS, which together account for ~40% of all LS, are characterized by the TLS/CHOP (aka FUS/CHOP) translocation (Conyers et al., 2011). This translocation is transformative when over-expressed in mice (Perez-Losada et al., 2000) and other cell lines (Riggi et al., 2006; Schwarzbach et al., 2004), indicating that FUS/CHOP drives tumorigenesis in these malignancies. Of the two, MYX LS is the lower grade, and RC LS is the higher grade. To our knowledge, no malignancies containing the characteristic

translocation have been shown to use ALT. Thus, if one uses this molecular alteration to classify MYX/RC LS, then these tumors use only telomerase for telomere maintenance. This is consistent with early reports suggesting that, as a whole, translocation-associated sarcomas utilize telomerase, rather than ALT, for telomere maintenance.

The rarest form of liposarcoma, PLS, accounts for only 5% of all LS cases (Conyers et al., 2011). Like WD/DDLS, PLS is a complex karyotype malignancy. Unlike the lower grade tumors, PLS are highly metastatic and do not contain the chromosome 12q13-15 amplification characteristic of DDLS. Thus far, no PLS tested in our hands have shown telomerase activity, using the stringent criteria of not only absence of enzymatic activity using TRAP but also absence of hTERT expression. Using the presence of APBs as a marker for ALT activation, we have found that PLS exclusively use ALT for telomere maintenance or fall in the category of tumors without evidence of either pathway. Given the advanced nature of PLS, their high likelihood to metastasize relative to other types of liposarcoma, and poor prognosis for patients (Guillou & Aurias, 2010), it is likely that these tumors have active telomere maintenance even in the absence of APBs. However, the rarity of PLS has limited the ability to test this hypothesis as most tumors are only available as archival formalin-fixed, paraffin-embedded samples, which will not provide DNA of sufficient size to allow testing for microsatellite instability. Archival samples are also not amenable to analysis for T-SCE, which requires hemi-substitution of the DNA molecule with BrdU and therefore dividing cells. Additional markers of ALT activation/utilization await a better understanding of the various recombination-based pathways utilized for telomere maintenance.

4.1 Genome instability and TMM

Telomeres serve the essential function of providing stability to the ends of linear chromosomes. The cyclical behavior of a single marked telomere in ALT cells (gradual shortening over time in culture, punctuated by rapid increases in length and resumption of telomere attrition) suggests that ALT may act after telomeres reach a critically short length. Thus, it is possible that a steady state level of compromised telomere function might be a feature of cells that utilize ALT for telomere maintenance. Consistent with this possibility, a number of chromosome ends in any given metaphase cell of ALT-positive cell lines do not contain detectable telomeric DNA when analyzed by FISH (Cerone et al., 2001; Perrem et al., 2001). Furthermore, comparison of osteosarcoma-derived cell lines that used either ALT or telomerase for telomere maintenance, using both telomere and multiplex fluorescence in-situ hybridization (FISH), revealed telomere length heterogeneity and increased chromosomal rearrangements in ALT-positive cell lines compared to telomerase-positive cell lines (Scheel et al., 2001). One phenotype exhibited by cells that have lost telomere end-protection function is the end-to-end fusion of chromosomes (Counter et al., 1992; van Steensel et al., 1998). During anaphase, these fusions are manifested as bridges of unresolved DNA between the separating daughter nuclei. Although such bridges can also arise through telomere independent mechanisms, an increase in anaphase bridges is characteristic of the loss of telomere capping function (Blasco et al., 1997; van Steensel et al., 1998). It is noteworthy that ALT-positive sarcomas that were identified in archival sections by virtue of having APBs are reported to have higher levels of genome instability as measured by anaphase bridge index (Montgomery et al., 2004). These tumors also tended to have a complex karyotype. In contrast, tumors with translocations were predominantly non-ALT (i.e., APB negative) and had lower instability.

As discussed above, shortening of telomeres leads to DNA damage and fusion-bridge-break cycles that result in increased genome instability in cancer cells with compromised DNA damage response. We previously reported that tumors using ALT for telomere maintenance have, on average, higher levels of genome instability than those using telomerase, and that tumors with any TMM active have, on average, higher genome instability than those falling in the category without evidence of either telomerase or ALT (Johnson et al., 2007). Genome instability in this study was defined as the percentage of SNPs deviating from diploid copy number. Intriguingly, genome instability was also increased in peritelomeric regions in ALT tumors, i.e., within 200 kb of the most telomeric SNP. These results are consistent with many tumors passing through telomere crisis prior to activation of a TMM.

We have re-evaluated our conclusions regarding genome instability as a function of TMM in the light of our recent realization of histological bias in TMM. Of the 32 tumors included in the study, 9 used ALT, 6 used telomerase, 1 used both ALT and telomerase, and 16 did not show characteristics of either mechanism. Within each category, tumors are distributed among multiple histological types, minimizing the effect of histology. Thus, it is likely that the differences observed in genome instability between ALT and TEL occur despite differences in histological bias rather than because of it. The more uniform telomere length and DNA damage protection conferred by telomerase may help control genome instability in telomerase-positive tumors.

4.2 TMM and survival

Previous work has shown a link between ALT activation and decreased survival in liposarcomas (Costa et al., 2006). The authors of this study did consider the potential contribution of histological type to this result, and concluded that the survival decrease associated with ALT was significant even when tumor grade, location, and histotype were taken into account. It is unclear from the methods in the paper how these factors were accounted for in the statistics. What is clear is that approximately two thirds (20/33) of the ALT-positive tumors are DDLS/other (potentially PLS), and greater than two thirds (27/34) of the telomerase-positive tumors are MYX/RC. Thus, this tumor set contains a histological bias. The authors do note that histology still needs to be considered when interpreting their multivariable analysis given the obvious bias. Further, they comment that the reduced prognostic value of telomerase-positivity is due to round cell tumors already having poor prognosis. This study, therefore, highlights the need for single histotype (and possibly even single-grade) data for survival.

In light of the discussion above regarding differences in characteristics of ALT-positive cell lines, it is important to note that Costa *et al.* defined tumors with 0.5% of nuclei containing APBs as ALT-positive. This is a fairly relaxed definition as co-localization of telomeric components with PML nuclear bodies is assayed by immunofluorescence and there is a possibility that signals will overlap by chance. We define ALT-positive as tumors in which at least 35% of the nuclei exhibit co-localization of telomeric components and PML nuclear bodies. This criterion is likely overly stringent and will exclude some ALT-positive tumors. Some of the differences may be resolved if clear criteria could be established to ensure a rigorous and consistent definition for APB-positive (and therefore ALT-positive) cells.

We recently performed a new analysis of the relationship between histotype, TMM, and survival, using 52 tumors from complex karyotype liposarcomas (WDLS, DDLS, PLS). Our analysis did not include any myxoid/round cell tumors, which comprised about a third of

the samples in the earlier work. Consistent with the previous study, tumors that had activated a telomere maintenance mechanism were associated with poorer survival, but the survival difference between ALT and telomerase tumors was not significant (p=0.06). However, as the majority of tumors with a detectable TMM are higher grade, i.e., DDLS and PLS, it is possible that the difference in survival observed between TMM-positive and TMM-negative tumors reflects differences in tumor grade rather than an independent marker of patient prognosis. Within DD-LS, which use both TMMs with similar, high frequency, there was no survival difference associated with TMM (p = 0.64). Thus, it is possible that the apparent poorer prognosis for ALT seen in the larger population is biased by higher grade pleomorphic tumors that contribute only to the ALT survival data.

4.3 Genetic characterization of ALT versus telomerase-positive tumors

It has been reported that the hTERT promoter is repressed in mesenchymal stem cells (Zimmermann et al., 2003). In cell based studies, epigenetic silencing of hTERT expression has also been documented (Serakinci et al., 2006). Thus, it is possible that the increased use of ALT in mesenchymal tumors is a result of tight repression of hTERT expression in this lineage.

The observation of TMM-based differences in survival suggested that fundamental genetic differences might be present between telomerase-positive and ALT-positive tumors. In a genome-wide screen of liposarcomas, we identified deletion of Chr 1q32.4-44 as an ALT associated genetic alteration (Johnson et al., 2007). This deletion differentiates between telomerase positive and ALT-positive DDLS. It is also present in ALT-positive (i.e. APB-positive) PLS. This is a large deletion containing many genes that might be implicated in tumorigenesis. When prioritized on the basis of known biological activity, SMYD3 stands out. SMYD3 is a methyltransferase that modifies histone H3 on lysine 4 (H3K4) (Hamamoto et al., 2004). Methylation of H3K4 is found at actively transcribed regions in the genome (Eissenberg & Shilatifard, 2010). In fact, this chromatin mark is associated with the 5' end of transcribed genes. Increased SMYD3 expression is associated with advanced cancers while reducing SMYD3 levels inhibits cancer cell growth and promotes apoptosis *in vitro* (Chen et al., 2007; Hamamoto et al., 2004; Hamamoto et al., 2006). Comparion of the expression profiles of cells with forced SMYD3 expression to cells with unaltered SMYD3 levels found alterations consistent with tumor progression, including activation of NF-kB pathway genes (Yamamoto et al., 2011). SMYD3 has recently been reported to be important for epigenetic modification of the hTERT promoter resulting in transcription of this locus (Liu et al., 2007). Importantly, over-expression of SMYD3 in both primary human fibroblasts and ALT-positive Saos-2 cells resulted in telomerase expression while siRNA-mediated knockdown led to inhibition of hTERT gene expression. However, hTERT expression does not necessarily lead to enzymatic activity capable of stabilizing telomeric repeats because, as discussed above, both telomerase activity and access to telomeres are regulated at multiple levels. In addition, ectopic SMYD3 expression likely affected multiple sites within the genome. Finally, SMYD3 expression in tumors utilizing different TMMs was not assessed. Thus, the relevance of SMYD3 deletion to ALT utilization in human tumors is not yet established.

Levels of H3K4 methylation at mammalian telomeres have not been determined. However, studies in yeast suggest that loss of H3K4 methylation is an intermediate step in assembly of silent chromatin. Telomeres are now known to be sites of active transcription and SMYD3 is

located in a region often deleted in ALT-positive, but not telomerase-positive, liposarcomas. Decreased H3K4 methylation at ALT telomeres would be predicted to increase heterochromatin marks resulting in decreased TERRA expression. However, ALT-positive cells show increased TERRA levels, and increased heterochromatin would be expected to decrease telomeric recombination, the hallmark of telomere maintenance in ALT. Further experimentation will be required to resolve the apparent paradoxes concerning the role of H3K4 methylation in telomere maintenance.

Previous studies of telomere maintenance in liposarcomas have primarily grouped them as a single malignancy rather than separating them into histological subtypes. The histological bias in telomere maintenance mechanism, as described above, had not been fully realized, but inevitably created a strong histotype bias when separating tumors on the basis of TMM alone. The importance of this bias was highlighted by a recent publication reporting a gene expression signature distinguishing between tumors and cell lines using ALT and telomerase (Lafferty-Whyte et al., 2009). In that study, the authors combined expression data from cell lines and tumors to identify 297 genes that were differentially expressed between ALT and telomerase. When we applied this signature to an independent set of tumors, we found that our samples clustered not on the basis of TMM but rather by histological subtype (Doyle et al., 2011). A closer analysis of the samples used to generate the published gene signature revealed that the cell lines used for the telomerase cohort were primarily of epithelial origin and tumors were primarily MYX LS. In contrast, cell lines used for the ALT cohort were primarily of mesenchymal origin and tumors were primarily DDLS. Thus, the reported cell line signature contained a strong epithelial versus mesenchymal component, while the tumor signature was heavily influenced by histological type. Even when we applied this published signature within a single histological subtype, DDLS, it failed to discriminate tumors on the basis of TMM. This suggests that expression differences between ALT-positive and telomerase-positive tumors are subtle, if present at all.

4.4 TMM and drug sensitivity

A number of studies have established that inhibition of telomerase and/or compromising telomere integrity increase the chemosensitivity of cells. Telomerase-deficient mice exhibit an increased sensitivity to DNA damaging agents that is correlated with the level of telomere dysfunction (Lee et al., 2001). Because telomere dysfunction leads to widespread genome instability, it was proposed that the increased chemosensitivity is a consequence of the underlying increase in genome instability. Indeed, reconstitution of telomerase in this system resulted in genome stabilization and an increased resistance to DNA damaging agents. Experiments carried out using human cell lines have also established an increased sensitivity to DNA damaging agents following inhibition of telomerase (Cerone et al., 2006a, b; Saretzki, 2003). Furthermore, when telomere integrity is compromised by expressing a mutant template RNA that results in repeats unable to recruit the shelterin complex, this also results in increased sensitivity to DNA damage (Cerone et al., 2006a). Increased drug sensitivity may occur before detectable telomere shortening (Masutomi et al., 2005), raising the possibility that telomerase may provide a protective function independent of its role in maintaining telomeric DNA arrays (Martinez & Blasco, 2011). For example, telomerase may contribute to telomere capping function thereby enhancing telomere stability. Recent work has implicated telomerase in modulating the cellular response to DNA damage (Masutomi et al., 2005). In this study, reducing telomerase in primary human cells blocked the DNA

damage response, thereby rendering cells more sensitive to damage. Exogenous expression of mutant hTERT alleles with reduced affinity for telomeric DNA rescued the DNA damage response as long as the introduced allele retained catalytic activity. These data suggest that telomerase may contribute to the cellular DNA damage response.

Drugs that target telomerase are increasingly used in cancer therapy with great success (Gilley et al., 2005). By reducing telomerase activity, treatment produces shorter telomeres and results in apoptosis and tumor shrinkage. In addition, telomerase inhibition increases the sensitivity of the cells to other chemotherapeutic agents, either directly through telomerase inhibition, as seen with imatinib (Deville et al., 2011), or indirectly via telomere shortening, as seen with cisplatin (Uziel et al., 2010). Both mechanisms may depend on the intersection of telomere maintenance and DNA repair. Thus ALT-positive tumors may be more sensitive than telomerase-positive tumors to DNA-damaging chemotherapies, as they possess higher levels of genome instability and lack the anti-apoptotic activity and DNA-damage protection of telomerase. Alternatively, ALT cells may be more resistant to DNA damage as they are able to form clinically significant tumors in a background of high genome instability. ALT cells, due to the lack of active telomerase, are expected to be entirely refractory to treatment by telomerase inhibitors. The association between telomere integrity and sensitivity to DNA damaging chemotherapeutic agents, together with the evidence implicating telomerase in the cellular response to DNA damage, is consistent with the possibility that TMM may be predictive of tumor response to chemotherapy.

Current treatments for liposarcomas act through specific over-expressed proteins; for example, doxorubicin response correlates with levels of topo2a (Mitchell et al., 2010), and nutlin acts as an antagonist to MDM2, a protein highly overexpressed in de-differentiated liposarcomas (Muller et al., 2007; Singer et al., 2007). Trabectidin has enhanced activity in myxoid/round cell liposarcomas due to the fusion protein (FUS/CHOP) characteristic to these malignancies (Conyers et al., 2011). These current treatments are thus expected to be independent of TMM. Although studies to date have failed to identify an ALT-specific expression profile, we expect that future studies will identify molecular targets specific to ALT that will enable more directed treatments. We expect that future treatments will be able to leverage knowledge about TMM for improved patient outcome.

5. Conclusions

Cancer treatment is steadily moving forward into the exciting realm of personalized medicine, targeting unique characteristics of each patient's disease for improved efficacy. Identification of the TMM active in a particular tumor provides a path to this personalization, indicating the potential benefit, or lack thereof, for telomerase inhibitors and DNA damage-inducing agents. Continuing research into the molecular mechanisms of ALT, accounting for inherent histological biases within liposarcoma, will identify molecular pathways for ALT-specific anti-cancer treatment that will increase value of TMM information in designing patient treatment.

6. References

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Soft tissue tumors include a heterogeneous group of diagnostic entities, most of them benign in nature and behavior. Malignant entities, soft tissue sarcomas, are rare tumors that account for1% of all malignancies. These are predominantly tumors of adults, but 15% arise in children and adolescents. The wide biological diversity of soft tissue tumors, combined with their high incidence and potential morbidity and mortality represent challenges to contemporary researches, both at the level of basic and clinical science. Determining whether a soft tissue mass is benign or malignant is vital for appropriate management. This book is the result of collaboration between several authors, experts in their fields; they succeeded in translating the complexity of soft tissue tumors and the diversity in the diagnosis and management of these tumors.

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