A role of LINE-1 in telomere regulation

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1. ABSTRACT

Long interspersed nuclear elements (LINE-1) are well known as retrotransposons. A number of reports indicate that down-regulation of LINE-1 substantially affects growth of malignant cells and epithelial mesenchymal transition, which is difficult to be explained by its function as retrotransposon. More recent data indicate that LINE-1 is broadly involved in the regulation of telomere maintenance. This explains the essential role of LINE-1 for survival of malignant cells and further supports a global function of active LINE-1 elements in cell proliferation. We further discuss the implications of LINE-1-associated telomere regulation on evolution of telomeric structures, on embryogenesis and on therapy of malignancies.

2. INTRODUCTION

2.1. Evidence for multiple functions of LINE-1

LINEs, long interspersed nuclear elements, are self-propagating retrotransposons consisting of two open reading frames ORF1 and ORF2 (1). ORF1 codes for an RNA binding protein capable of binding LINE-1-specific RNA while ORF2 codes for an endonuclease that opens DNA and a reverse transcriptase (RT) ensuring the production of progeny DNA that can be re-integrated into the genomic DNA of the host. A small fraction of LINE-1 sequences - estimated 5-6 LINE-1 elements throughout the genome - code for full length highly active LINE-1. These highly active LINE-1 subsets are responsible for 84% of LINE-1 related retro-transposition (2).

LINE-1 transcription is regulated on an epigenetic level as well as by premature polyadenylation, endogenous anti-sense promoter producing antisense-derived siRNA and miRNA. Hypomethylation of the promoter allows transcription of otherwise silent genes. On a physiological level genome-wide hypomethylation occurs in primordial germ cells and after fertilisation of oocytes (3). At this stage LINE-1 retrotransposon activation has the potential to contribute to genome rearrangement. Thus, LINE-1 can contribute to evolution of individual genotypes. This function fits well with its canonical role.

However, enhanced LINE-1 expression is not only seen early in embryogenesis but remains active up to the morula stage (4). As retro-transposition and subsequent genetic rearrangement is no longer desired in multiple cell stages of embryogenesis we suggest that LINE-1 might have other physiological functions. This notion is supported by the fact that the inhibition of LINE-1 by specific antisense oligonucleotides in mice leads to embryologic arrest at the 4-cell stage (5). A lack of retro-transposition would hardly explain such a detrimental phenotype after LINE-1 down-regulation. LINE-1 is also activated in human embryonic stem cells and in cells following reprogramming (6, 7) further supporting a role of LINE-1 in embryogenesis.
With respect to pathophysiology it has been shown that LINE-1 is often re-activated in cancer due to epigenetic deregulation. LINE-1 protein expression has been detected in most types of epithelial cancer (8). In pancreatic cancer it was found that LINE-1 core related with the de-repression of satellites that are responsible for chromosomal integrity (9). Moreover, the localization of LINE-1 coding proteins seems to influence prognosis. These proteins can be detected either in cytoplasm or the nucleus. Nuclear detection of LINE-1 specific protein was associated with reduced survival due to local recurrence and distal metastasis (10, 11) suggesting the influence of LINE-1 on gene regulation. Given the potential retrotransposon activity of these elements it is evident that LINE-1 can contribute to oncogenesis by activation of oncogenes (12) or down-regulation of tumour suppressors (13). However, a number of observations also suggest retro-transposition independent functions of LINE-1 in cancer development. For example down-regulation of LINE-1 by siRNA reduces tumour growth (14). Growth reduction can hardly be explained by a lack of active retro-transposition but may, for example, result from reduced expression of c-myc, a factor relevant for tumour growth (15). Moreover, Rangasamy and colleagues found that silencing of LINE-1 expression in tumour cell-lines reduces parameters relevant for epithelial mesenchymal transition (EMT) (16). EMT is an essential step in tumour progression where cancer cells gain migratory functions to metastasize. A role of LINE-1 in EMT might explain the fact that LINE-1 expression has been associated with poor clinical outcome and the occurrence of metastases (17, 18).

However, immunohistochemical data indicate that LINE-1 is already up-regulated in early tumour development as LINE-1 protein has been found to be broadly expressed in dysplastic colon polyps and early stages of breast cancer (11, 19). This early expression of LINE-1 in tumours might further suggest other functions of LINE-1 in tumour development different from retro-transposition or EMT.

Summing up, the impact of LINE-1 on cancer progression strongly points out to multiple roles of LINE-1 in cell regulation. Recently we have gained experimental evidence that LINE-1 also supports the telomere maintenance mechanism (TMM), another feature shared by tumorigenesis and embryogenesis (20). LINE-1 induced TMM might explain the contribution of LINE-1 in early tumour stages as enhanced TMM is an early feature in tumour development. The current review focuses on the specific role of LINE-1 in telomere regulation.

2.2. Telomere structure and function

As the human genome is organized in linear chromosomes their termini have to be protected. To prevent chromosomes from degradation they contain terminal repeat elements known as telomeres. In human cells telomeres are hexameric DNA repeats 5’-TTAGGG- 3’ (21) and end with a single strand overhang. Thus, they have to be protected from DNA damage response (DDR). The shelterin is a specific protein complex, which binds telomeres and protects their G-tails from being misinterpreted as DNA double strand breaks. This complex consists of 6 proteins, TRF1, TRF2, TIN2, RAP1, TPP1, and POT1. TRF2 makes telomeres build a t-loop to protect them from being tethered by DNA damage sensing molecules. Furthermore, the components of shelterin suppress pathways of cell cycle arrest normally mediated by DNA damage. TRF2 wraps 90bp of telomere DNA and thereby prevents activation of ataxia telangiectasia mutated kinase (ATM) (22) while POT1 protects against activation of ataxia telangiectasia and Rad3 related kinase (ATR) (23). ATM and ATR are promoters of DNA-repair pathways (24). Thus, lack of shelterin results in unprotected telomeric structures and leads to the activation of DNA repair pathways, which is associated with cell cycle arrest. Deregelation of shelterin proteins also causes anaphase bridges between dividing nuclei disabling normal cell division (25) and therefore facilitating the formation of multi-karyotypic cells in malignancies. Interestingly, shelterin proteins like TRF2 also appear to have further functions such as down-regulation of immune cells (16) possibly leading to a privileged immunological microenvironment of cells with protected telomeres.

Telomere maintenance is essential for long-term cell survival. Due to the mechanism of the DNA replication machinery each replication cycle is accompanied by loss of nucleotides at the chromosomal ends (26). If a critical length is reached cell cycle arrest and senescence are induced. To counteract the constant telomere attrition, the so-called “end replication problem”, eukaryotes possess an enzyme called telomerase that preserves telomere length. The human telomerase (hTERT) is a reverse transcriptase, which uses hTER as an RNA template and primer for telomere elongation. In higher organisms TMMs are physiologically active in stem cells but inactivated in differentiated cells. Pre-malignant and malignant cells have adopted this mechanism ensuring their prolonged survival. Generally, reactivation of TMM is listed as one of the hallmarks of cancer (27). Consequently, inhibition of TMM is thought to be a target for cancer therapy. Therefore a detailed understanding of molecular mechanisms in TMM is relevant to define these possible targets.

Telomerase activity (TA) is the classical TMM-pathway used by stem cells and 80% of tumours. Thus, inhibitors of human telomerase such as GRN163L have been developed and are currently tested in phase II clinical trials (28, 29).
However, a number of tumours, preferentially sarcomas and gliomas, do not necessarily express telomerase and can elongate their telomeres by the alternative mechanism of telomere maintenance (30, 31). A hallmark of alternative lengthening of telomeres (ALT) (31) is a highly increased mean length of telomeres up to 20 kb, compared to telomere length of TA positive cells (6-8 kb) (32). Based on several observations ALT seems to result from homologous recombination (HR) (33) that involves the protein rad51 and rad52 (34). ALT is associated with mutations in the ATRX-gene (35). ATRX has an important role prohibiting cohesion of two telomere strands impeding sister chromatid exchange. Thus, loss of ATRX function facilitates recombination, which can lead to telomere elongation (36). Another feature of ALT cells is the presence of ALT-associated promyelocytic leukemia bodies (APB) and c-circles (34). C-circles are extra-chromosomal circular DNA sequences consisting of telomeric repeats (30). Their function is currently unknown but they can be used as a marker for ALT.

3. LINE-1 AND TELOMERES

3.1. Effect of LINE-1 on telomere maintenance mechanism

Recently, we could demonstrate for the first time that inhibition of highly active LINE-1 sequences was associated with telomere attrition in telomerase positive cell-lines (20). Correlating with the notion that LINE-1 exerts multiple functions the regulation of telomeres by LINE-1 probably occurs at multiple levels. We observed that a knock down (KD) of highly active LINE-1 reduced hTERT on a transcriptional level (20). We and others (15) also demonstrated that a reduction of active LINE-1 correlated with a reduced transcription and protein expression of KLF-4 and c-myc (15), two relevant transcription factors for hTERT. Thus, the regulation of telomerase by LINE-1 might be mediated indirectly via those two proteins.

Moreover, we have shown that LINE-1 KD is associated with the reduction of shelterin with respect to mRNA- and protein-expression. With the exception of RAP1 all shelterin components were reduced. It should be noted that KD of neither c-myc nor of KLF-4 or hTERT correlated with a reduction of shelterin. This suggests that the induction of shelterin is specific for LINE-1.

The LINE-1 KD induced reduction of the shelterin correlated with an induction of double strand breaks at telomere sequences named telomere induced foci (TIF). LINE-1 KD induced double strand breaks primarily at the telomeric sequences but not throughout the genome, again supporting a specific role of LINE-1 in telomere integrity. The reason for TIF appearance is unlikely caused by failure to elongate telomeres due to decreased human telomerase activity. A reduction in hTERT activity would rather lead to senescence induction after multiple passages. We therefore suggest that increased TIF observed in our experiments result from reduced protection by the shelterin complex. The deranged shelterin complex might also be the reason for an increased anaphase bridging observed in LINE-1 KD cells. The TIF might also cause the fast slowdown of tumour cell growth and G2 arrest observed in LINE-1 siRNA treated cells. It is well established that unprotected telomeres that have double strand breaks induce ATM-mediated senescence (37). Similar to LINE-1 KD c-myc KD also correlated with a halt in cell growth and proliferation (38). However, as LINE-1 appears to affect the expression of i) shelterin complex ii) c-myc and iii) KLF-4 LINE-1 might have a regulatory role up-stream of these transcription factors (Figure 1).

We further suggest that the induction of c-myc and hTERT by LINE-1 could be the link to LINE-1-induced EMT activation in tumour cells as has been observed by Rangasamy and co-workers (16). On the one hand c-myc has been shown to play a role in TGF-beta mediated SNAIL expression and EMT (39), on the other hand hTERT was associated with the activation and induction of EMT markers (40). Summing up, the role of LINE-1 i) in telomere maintenance, ii) in induction of stem cell- and iii) EMT-associated transcription factors indicates that LINE-1 mediates a pattern of regulatory pathways promoting cell growth and migration rather than the induction of a single protein (Figure 1).

Supporting this idea, the group of Spadafora has shown that LINE-1 globally modulates cell signalling by the formation of RNA:DNA hybrids (31). They suggested that LINE-1 encoded reverse transcriptase is involved in this RNA:DNA hybrid formation. In normal cells dsRNA is processed by an endoribonuclease Dicer cutting it into small regulatory RNAs that regulate heterochromatin formation and gene expression. In LINE-1 expressing malignant cells LINE-1 associated reverse transcriptase produces DNA:RNA hybrids. These hybrids prevent small regulatory RNA formation leading to globally altered transcription (Figure 1). Alternatively, LINE-1 itself could lead to modulation of proteins such as DNMT1 or DNMT3b that are involved in global epigenetic regulation (TA, MB unpublished). Whatever mechanism is more dominant it appears to be evident that LINE-1 is a global regulator of pathways controlling cell proliferation and migration. Such a global regulation might be mediated by epigenetic regulations leading to the loss of heterochromatin in a set of defined gene loci as suggested in Figure 1.

As LINE-1 expression appears to be relevant for shelterin expression we suggest that LINE-1 will also be substantial for the integrity of telomeres in ALT.
cells. Preliminary data support the hypothesis of this mechanism as LINE-1 KD appears to reduce growth of ALT cells (41, 42). Apart from the induction of the shelterin complex it might be interesting to investigate the role of LINE-1 related to other features of ALT cells such as c-circles.

3.2. Evolutionary aspects

There are only three reverse transcriptases encoded by the human genome: i) telomerase, ii) RT encoded by non-long terminal repeat (non-LTR) retrotransposons such as LINE-1 and iii) RT encoded by retroviral elements such as LTR retrotransposons and endogenous retroviruses. Given the observation that LINE-1 induces hTERT it is interesting that retrotransposon, endogenous retroviral encoded RT and telomerase are evolutionary related and might have a common ancestor (43, 44).

This close relationship might be the reason why LINE-1 can even take over functions of telomerase throughout evolution. An example is seen in drosophila. This organism lacks telomerase but its DNA encodes three non-LTR elements to protect its chromosomal termini. More precisely, drosophila telomeres consist of three tandem repeats of the non-LTR retrotransposons HeT-A, TAHRE and TART (45-47). It should be noted that retrotransposons appear to be part of the general DNA damage response in this organism filling the gap of a double strand break (48). Thus, drosophila might have adopted those retrotransposons to prevent the induction of DNA damage program due to the end-replication problem at the telomeres. Interestingly, HeT-A does not even code for a reverse transcriptase but has to rely on the enzyme of the other retrotransposons for its retro-transposition. Importantly, the protein expressed by HeT-A was found to be localized at the chromosomal ends implying that not only the presence of the sequence is relevant but also its encoded protein. This again suggests that retrotransposons should be seen as a general mechanism to ensure TMM. The drosophila proteins HOAP and HipHop, which bind to the telomeres, have no homologies to human shelterin proteins.

Even more striking is a co-evolution of telomeres and telomeric repeat-specific non-LTR retrotransposons in insects. Chromosomal ends of the silkworm Bombyx mori possess a hybrid structure of telomeric repeats and autonomous non-LTR retrotransposons SART and TRAS (49, 50). The proteins encoded by the retrotransposon can exert endonuclease activity at telomeric repeats and possess an active reverse transcriptase.

Given the evolutionary proximity between retroviral elements and telomeric structure it is
interesting to note that ALT cells are characterized by the presence of c-circles. C-circles might also be seen as a remnant of viral structures as viruses often have circular genomes.

Telomeric regulations have also been adopted by a number of DNA virus. For example Ebstein Barr Virus employs protection caps of its ends involving shelterin protein TRF1 and TRF2, telomeric repeat containing RNA (TERRA) and chromatin (51). This again highlights the close proximity of genetic elements in the eukaryotic and the viral universe making it more likely that elements have been exchanged in different species throughout evolution.

Another observation could support the idea that LINE-1 has a specific affinity and possibly protective function for telomeres. The group of Moran generated cells with functional defects in non-homologous end joining (NHEJ) by mutations in the DNA-dependent protein kinase. Those cells appear to be specifically prone for LINE-1 retro-transposition at the telomeric ends (52). Thus, in eukaryotic cells with dysfunctional telomeres LINE-1 integrates more easily. This can be interpreted as ancient mechanism of telomere protection that can be reactivated.

3.3. Implications for stem cells and embryogenesis

Understanding the regulatory aspects of stem cells has always facilitated the understanding of malignant cell biology as malignant cells adopt them and other mechanisms to ensure their survival. As already mentioned LINE-1 is expressed early after fertilization of the oocyte. Moreover, LINE-1 appears to induce stem cell associated factors such as c-myc and KLF-4. KLF-4 and c-myc are two of four factors sufficient for stem cell reprogramming. SOX or NANOG were not induced by LINE-1 (TM, MB, unpublished). Thus, LINE-1 appears to be involved in parts of the program relevant for reprogramming and might have a function in this cell regulatory pattern. This hypothesis is supported by the finding that reprogramming of inducible stem cells leads to LINE-1 up-regulation (6). The fact that LINE-1 also supports TMM and EMT nicely fits to a role of LINE-1 in embryonic and stem cell development.

Interestingly, at the very early stage of embryonic development telomerase is not highly expressed (53). At this stage TMM appears to depend on sister chromatid exchange involving recombination mediated by the protein rad50. Therefore, an ALT-like mechanism can be anticipated. ALT might be the evolutionary older mechanism compared to telomerase-related TMM. This might be mirrored in early ontogenesis as an ALT related TMM is followed by a telomerase associated TMM at the blastocyst stage. The contribution of retrotransposons to ALT still has to be investigated. However, given the evolutionary role of LINE-1 in telomere maintenance mechanisms and the high expression of LINE-1 at the early embryo (4) it could be envisioned that LINE-1 most likely also supports an telomerase independent ALT-related TMM at the early embryonic stages. LINE-1 certainly ensures telomere stability by supporting the expression of the shelterin.

As LINE-1 expression is facilitated by promoter hypomethylation the general hypomethylation of stem cells or early embryonic stages ensures its expression. As discussed earlier activation of LINE-1 in stem cells can lead to retro-transposition. Even though retro-transposition contributes in a physiological manner to genomic plasticity after fertilisation it might be unwanted in re-programmed stem cells. Retro-transposition leading to genomic alterations might activate oncogenes, which certainly would limit further use of re-programmed stem cells generated for therapeutic applications in humans. Thus, it might be relevant to investigate i) on the one hand the co-factors necessary for LINE-1 retro-transposition and ii) on the other hand the co-factors that are necessary for LINE-1 induced c-myc activation and TMM, which appear to be desired mechanisms in reprogramming.

In conclusion, the TMM promoting effect of LINE-1 fits well with its activation in reprogramming of stem cells. The definite contribution of LINE-1 to telomere regulation in the early embryo that might be dominated by ALT has to be investigated.

3.4. Therapeutic implications

All the above-described mechanisms implement possibilities for therapeutic interventions in telomeric regulation by inhibition of LINE-1 protein activity. Certainly it is not clear which part of the LINE-1 protein complex is relevant for supporting the telomere maintenance mechanism. As LINE-1 contains a reverse transcriptase reverse transcriptase inhibitors (RTI) are one of the most obvious drugs to be applied. RTIs have initially been developed as anti-proliferative drugs. However, drug-induced unspecific genomic damage resulted in an unacceptable high toxicity and initially prevented further clinical application. The discovery of telomerase promoted further application of RTIs as inhibitors of telomerasers, which lead to telomere shortening in tumour cell lines (54-56). The group of Boeke has suggested that nucleotide-analogue RTIs (N-RTI) might be applied to block the LINE-1 specific reverse transcription (57). Based on this finding we have applied N-RTIs such as DDI and AZT in telomerase positive cell-lines at low levels, which should not be toxic to the genome (58). Using such low concentrations we still could block tumour cell growth. This was associated with telomere attrition and a G2 arrest. However,
in those cells we could not differentiate whether RTIs have an effect on telomerase or on LINE-1. The group of Spadafora and others have also shown that non-nucleotide-RTIs (NN-RTI) such as efavirenz prevented tumour cell growth (14, 59). As non-malignant cells respond less to efavirenz it was proposed that the target of the drug was LINE-1 even though the inhibition of telomerase by NN-RTIs cannot completely be ruled out. Interestingly, ALT cell-lines such as Saos-2 also responded to RTIs by a growth reduction (31, 41). As those cells do not have telomerase an RTI-mediated inhibition of LINE-1 and a role of LINE-1 in ALT could be envisioned.

The observation that LINE-1 KD leads to a growth reduction and G2 arrest in tumour cells certainly calls for the development of specific therapeutic agents acting on LINE-1. Whether inhibition of LINE-1 primarily leads to TIFs or to a reduced telomerase activity might be of less importance.

It should be noted that most chemotherapeutic agents induce TMM leading to longer telomeres. Thus, the induction of TIFs by the inhibition of LINE-1 might be of specific interest as it could sensitize the cells for other cell death inducing drugs. Current data indicate that LINE-1 inhibition leads to growth reduction and possibly cell re-differentiation (59). In addition tumour cells might gain sensitivity to additional treatments such as immunotherapy, cytotoxic antibodies or chemotherapy, which will be subject of further investigations.

It would therefore be reasonable to create LINE-1-specific inhibitors without affecting the activity of physiologically expressed telomerase, which is important for healthy stem cells and tumour ablative immune cells.

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**Abbreviations:** LINE-1: long interspersed nuclear element; LTR: long terminal repeat; RT: reverse transcriptase, EMT: epithelial mesenchymal transition; TMM: telomere maintenance mechanism; DDR: DNA damage response; ATM: ataxia telangiectasia mutated kinase; ATR: ataxia telangiectasia and Rad3 related kinase; hTERT: human telomerase; TA: telomerase activity; ALT: alternative lengthening of telomeres; APB: ALT-associated promyelocytic leukemia bodies; TERRA: telomeric repeat containing RNA; RPA: replication binding protein A; HR: homologous recombination; TIF: telomere induced foci; KD: knock down; NHEJ: non-homologous end joining; RTI: reverse transcriptase inhibitor; N-RTI: nucleotide-analogue RTI; NN-RTI: non nucleotide-analogue RTI.

**Key Words:** Telomere, LINE-1, Shelterin, Telomerase, Retrotransposon, Reverse Transcriptase Inhibitors, Review

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