Instituto Juan March
de Estudios e Investigaciones

159 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Telomeres and Telomerase: Therapeutical Targets for Cancer and Aging

Organized by

S. Neidle, J. W. Shay and M. A. Blasco

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INDEX

PAGE

PAGE

Introduction
María A. Blasco

Mortal and immortal stories

On one hand, telomere dysfunction has been proposed to be causal of the aging process as indicated by several human premature aging syndromes that are characterized by a faster rate of telomere loss, as well as, by the study of several mouse models with dysfunctional telomeres. Restoring the functionality of telomeres (ie., by re-introduction of telomerase) in cells with compromised viability due to telomere dysfunction is envisioned as a putative Gene Therapy of age-related diseases.

On the other hand, it is now generally accepted that the ability of tumor cells to grow indefinitely is sustained by activation of telomerase. Indeed, detection of telomerase activity can be used as a marker for tumor growth. In addition, both telomerase activity and telomeres are envisioned as potential targets for new tumor therapies.

There has been a significant amount of research on the role of telomerase in aging and cancer over the past five years. The current Juan March meeting has brought together some of the leading scientists in the telomere and telomerase field, who have presented their most recent discoveries. The current meeting has been also focused on how to advance the most promising areas into translational research.

Therapeutical approaches: relevance for Biomedical research

Cancer

Telomerase is involved in telomere maintenance in 80-85% of human tumors, whereas it is inactive in somatic cells. It is thus a potential target for therapeutic interventions. Proof-of-principle experiments have shown that selective inhibition of telomerase leads to progressive telomere shortening and ultimately to cancer cell apoptosis, and have provided strong support for the concept of telomerase as an anticancer target.

There are a number of distinct approaches to telomerase inhibition that are currently being studied, notably:

- direct inhibition of the catalytic active site
- antisense inhibition of the telomerase RNA template
- induction of higher-order structure in the telomerase substrate/primer
- mutation of the RNA domain.
- telomerase immunotherapy and hTert-promoter oncolytic viruses

The Juan March meeting has provided a forum for evaluating the progress in achieving therapeutic outcomes from these different approaches.

Aging

Telomere loss with normal aging, as well as, in the context of premature aging diseases, is the primary cause of pathological states characterized by a decrease in the proliferative and renewal potential of tissues. Re-introduction of telomerase activity in the affected tissues has been envisioned as a putative Gene Therapy of age-related diseases.

Several speakers at the Juan March meeting have addressed the impact of telomerase deficiency and telomere shortening on aging in the human organism, which opens the possibility of using telomerase in Gene Therapy of age-related diseases and premature aging syndromes.

Session 1: Structure and function of telomeres
Chair: Jerry W. Shay

Positive and negative regulators contribute to the cell cycle limited activity of *Saccharomyces cerevisiae* telomerase

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The ability of telomerase to lengthen telomeres is cell cycle regulated, occurring preferentially in late S/G2 phase but not in G1 phase. Nonetheless, the catalytic subunit of telomerase, Est2p, is telomere associated throughout most of the cell cycle, including in the G1 phase [1]. The association of Est2p with telomeres is telomerase RNA (*TLC1*) dependent. The heterodimeric Ku complex, which binds yeast telomeres *in vivo*, has recently been shown to associate with telomerase RNA [2]. Absence of Ku results in elimination of the G1 bound Est2p but had only modest effects on Est2p binding in late S phase. *TLC1* mutations that prevent telomerase RNA from binding Ku have a similar effect on Est2p's association with telomeres. These data suggest that Ku promotes telomerase activity by increasing the local concentration of core telomerase at telomeres. The Pif1p DNA helicase is a negative regulator of telomerase-mediated telomere lengthening [3]. Because Pif1p is telomere associated only in late S/G2 phase, its activity does not explain the lack of telomerase action in G1 phase. Over-expression of Pif1p results in reduced Est2p association with telomeres, and this reduction requires the ATPase/helicase activity of Pif1p. These data support a model in which Pif1p limits telomerase action by catalytically removing telomerase from chromosome ends in late S/G2 phase.

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Separation of silencing from perinuclear anchoring functions in yeast Ku80, Sir4 and Esc1 proteins

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The positioning of chromosomal domains within interphase nuclei is thought to contribute to the establishment and maintenance of epigenetic controls. Although this is particularly well characterized for telomeres of budding yeast, the molecular basis of their specific subnuclear organization is poorly understood. Using GFP-tagged chromosomal domains and live microscopy, we investigated mechanisms through which chromatin can be anchored to the nuclear periphery. Interphase positioning of telomeres can be achieved through two partially redundant mechanisms. One requires the heterodimeric Ku complex; the second requires Silent Information Regulators (SIRs) and correlates with transcriptional repression. Excised silent chromatin rings are able to bind the nuclear envelope in a Sir-dependent manner.

To determine what is protein domains are directly implicated in anchoring chromatin to the nuclear periphery, we fused candidates to LexA and targeted them to a GFP-tagged chromosomal locus. A shift from a random subnuclear distribution to a perinuclear one was monitored for the tagged locus by live fluorescence microscopy. A mutant allele of yku80 and a 312-aa domain of Sir4 ($Sir4^{PAD}$) serve as minimal anchoring elements, each able to relocalize an internal locus to the nuclear periphery in the absence of transcriptional repression or an intact SIR complex. Chromatin tethering by $Sir4^{PAD}$ requires either the Ku complex or Esc1, a Sir4 ligand that sits at the inner face of the nuclear envelope. Esc1 localization is not influenced by loss of Yku or Sir4, nor by the position of nuclear pores. Parallel anchoring pathways requiring Yku and Esc1, respectively, are also shown to mediate natural telomere anchoring *in vivo*. Although anchoring to Ku and Esc1 via the Sir4PAD domain is independent of silencing, its anchoring pathways cooperate to generate a high concentration of SIR proteins at the nuclear periphery which is critical for promoting repression by perinuclear tethering.

RPA activates telomerase by loading Est1p to chromosome ends

Vera Schramke, Pierre Luciano, Vanessa Brevet, Sylvine Guillot, Yves Corda Maria Pia Longhese, Eric Gilson and Vincent Géli

Replication protein A (RPA) is a highly conserved single-stranded DNA binding protein involved in DNA replication, recombination, and repair. We show here that RPA is present at telomeres of the budding yeast *Saccharomyces cerevisiae* with a maximal association in S-phase. A truncation of the N-terminal region of Rfa2p (rfa2 delta 40 allele) results in a severe telomere shortening due to a defect in the in vivo regulation of telomerase activity. Furthermore, in cells carrying the rfa2 delta40 allele, the binding of Est1p is impaired and normal length regulation can be restored by expressing a Cdc13-Est1p hybrid protein. These findings indicate that RPA activates telomerase by loading Est1p to telomeres during S-Phase. We propose a model of in vivo telomerase activation that involves the synergistic action of RPA and Cdc13p at the G-rich 3' overhang of telomeric DNA.

Multiple roles for telomeres in protecting genomic stability

Kyle Miller, Miguel Godinho Ferreira, Julia P. Cooper

Taz1p, the fission yeast ortholog of human TRF1 and TRF2, binds telomeres and regulates diverse telomere functions including telomerase activity, position effects on nearby transcription, and meiotic chromosome movements. In addition, taz1D-telomeres are treated as DNA double strand breaks and thereby subjected to the prevailing mode of DNA break repair; either homologous recombination (HR), which is innocuous at telomeres, or nonhomologous end-joining (NHEJ), which elicits end-fusions. We find that NHEJ is favored under conditions of low Cdc2 kinase activity, leading to lethal fusions between taz1D telomeres under conditions in which the G1 phase of the cell cycle is extended. Thus, the dangers to 'unprotected' telomeres vary with the cell cycle.

taz1D cells also have particular problems with growth in the cold (20°C), a condition that induces cell cycle delay and chromosome missegregation. Under these conditions, chromosomes lacking Taz1 do not undergo covalent end-fusion, but do appear to become entangled with each other, and chromosome breakage occurs throughout the genome. The spindle assembly checkpoint and a checkpoint-independent function of Rad3p (the ATR homolog) are required for taz1D cells to survive at 20°C. Intriguingly, a mutation in topoisomerase II (Top2p) suppresses the cold sensitivity defects of taz1D cells, suggesting a scenario in which unprotected telomeres become substrates for Top2p, perhaps requiring its activity to resolve stalled telomeric replication forks. Furthermore, cycling taz1D cells are hypersensitive to treatments that induce DNA breakage (e.g. g-irradiation) at all temperatures, despite having functional checkpoint pathways and lacking end-fusions. These observations signal roles for functional, Taz1-containing, telomeres in both preventing and repairing DNA breaks throughout the genome.

Heterogeneity among human ALT cell lines

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In view of the evidence that telomerase-negative yeast survivors [Lundblad and Blackburn, 1993] may be subdivided into two classes [Teng and Zakian, 1999; Le et al., 1999], we have been searching for differences among human cell lines that have an Alternative Lengthening of Telomeres (ALT) mechanism.

We showed previously that a minority of ALT cell lines do not express detectable levels of the telomerase RNA subunit, hTR [Bryan et al., 1997]. Further Northern analyses have shown that 9/38 ALT cell lines (24%) have undetectable hTR expression. If hTR expression status reflects distinct ALT mechanisms, it would be predicted that cell lines would be either hTR-positive or -negative from the time that ALT is activated. Timing of the loss of hTR expression was therefore examined in two ALT lines. In one (JFCF-6T.1R), loss of hTR expression occurred at approximately the same time as emergence from crisis and activation of ALT, but in another (JFCF-6T.1L) hTR expression was not lost until more than 30 population doublings later. This suggests that the functions of hTR in normal cells (e.g., templating telomerase-mediated maintenance of the telomeric single-stranded overhang [Masutomi et al., 2003]) are not required for the survival of ALT cells, so there is no selective disadvantage if hTR expression is switched off in a stochastic manner (e.g., by methylation of its promoter [Hoare et al., 2001]).

We previously found that a subset of cells within every ALT cell line examined had subnuclear structures that contain PML protein and other proteins characteristic of PML nuclear domains, together with telomeric chromatin (telomeric DNA and telomere-specific binding proteins) [Yeager et al., 1999]. AG11395, an SV40-immortalized, telomerase-negative Werner syndrome cell line, appears to lack this characteristic. Fluorescence in situ hybridization (FISH) analysis showed that SV40 sequences are present in most AG11395 telomeres. For this cell line it is therefore not possible to determine terminal restriction fragment (TRF) size using the usual combination of HinfI and RsaI restriction enzymes because these enzymes recognise many sites within the SV40 sequence. Use of other enzymes, however, showed that the TRFs of AG11395 cells have the extreme heterogeneity of length that is characteristic of all human ALT cell lines examined to date. To examine whether the mechanism of telomere maintenance in these cells is similar to that in other ALT cell lines, we targeted a plasmid tag into a telomere and showed that it was copied into other telomeres, as demonstrated for other ALT cells [Dunham et al., 2000]. In addition, we transferred an AG11395 chromosome into another ALT cell line (GM847 cells), and showed that the SV40 sequences were copied into other GM847 telomeres. Thus, despite the

presence of extensive amounts of non-telomeric DNA in the telomeres of these cells, there are no clear indications that the telomere maintenance mechanism differs from that of other telomerase-negative cell lines. However, in AG11395 cells (but not in other ALT Werner syndrome cells and tumors) there was no significant colocalization of telomeric (or SV40) DNA and PML protein.

Although we have not found evidence for the existence of a second ALT mechanism in human cells, these data indicate that the telomeres of telomerase-negative cells can be maintained in the absence of ALT-associated PML bodies. At present, it is not clear whether another type of nuclear aggregate is required to substitute for APBs. The presence of large amounts of non-telomeric sequence within the telomeres of ALT cells may somehow disrupt the localization of telomeric chromatin to APBs. The data suggest the possibility that examining tumors for the presence of APBs might underestimate the number that utilize the ALT mechanism.

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Session 2: Structure and function of telomerase
Chair: Titia de Lange

A telomerase holoenzyme

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The direct roles of telomerase-associated proteins other than telomerase reverse transcriptase (TERT) remain ambiguous. To gain a more comprehensive understanding of stable telomerase ribonucleoprotein composition, we performed affinity purification of endogenously assembled, TERT epitope-tagged *Tetrahymena* telomerase ribonucleoproteins. We identified four new proteins in roughly equivalent stoichiometry with TERT and telomerase RNA. Genes encoding all four proteins have been cloned, and the two that were cloned first have been characterized extensively.

The p45 and p65 subunits are associated with the vast majority of telomerase RNA and telomerase activity in cell extract, as judged by immunodepletion experiments. Like telomerase RNA and TERT, both p45 and p65 appear to be telomerase-specific. Genes encoding the two proteins are essential for vegetative growth, and strains genetically depleted for each protein are compromised for telomere length maintenance. Reduced expression of p65 results in reduced accumulation of telomerase RNA, while p65 over-expression increases telomerase RNA accumulation. These findings demonstrate that telomerase holoenyzme proteins other than TERT are required for physiological telomerase ribonucleoprotein biogenesis and function.

The sequence of p65 suggests the presence of a divergent N-terminal La motif and also a putative second RNA binding motif. Recombinant p65 binds with high specificity and affinity to telomerase RNA, and p65 enhances telomerase RNA assembly with recombinant TERT. However, p65 does not affect recombinant enzyme specific activity: the p65-TERT-RNA complex still fails to recapitulate endogenous holoenzyme properties of DNA primer interaction and elongation processivity. The role of the additional *Tetrahymena* telomerase holoenzyme proteins in this regard remains to be investigated. These results suggest that *in vitro* as well as *in vivo*, telomerase holoenzyme proteins have specialized, non-redundant, critical roles in telomerase ribonucleoprotein assembly and catalytic activation.

Dyskeratosis congenita

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(i) Clinical aspects

Classical dyskeratosis congenita (DC) is an inherited bone marrow (BM) failure syndrome characterized by the triad of abnormal skin pigmentation, nail dystrophy and mucosal leucoplakia. Since its first description in 1906 by Zinsser, a variety of noncutaneous (dental, gastrointestinal, genitourinary, neurological, ophthalmic, pulmonary and skeletal) abnormalities have also been reported. BM failure is the principal cause of early mortality with an additional predisposition to malignancy and fatal pulmonary complications. X-linked recessive, autosomal dominant and autosomal recessive forms of the disease are recognised.

Clinical manifestations in DC often appear during childhood but there is considerable variability from patient to patient. The skin pigmentation and nail changes typically appear first, usually by the age of 10 years. BM failure/aplastic anaemia usually develops below the age of 20 yrs; 80-90% of patients will have developed BM abnormalities by the age of 30 yrs. In some cases the BM abnormalities may appear before the mucocutaneous manifestations and the patients may be categorized to have "idiopathic aplastic anaemia".

Oxymetholone (anabolic steroid) can produce an improvement in haemopoietic function in many (>50%) patients for a variable period of time. Transient successful responses to haemopoietic growth factors (GM-CSF, G-CSF and erythropoietin) have also been reported. The use of oxymetholone and growth factors can be synergistic in some patients. However, the definitive treatment for severe BM failure is haemopoietic stem cell transplantation (SCT) and there is some experience using both sibling and alternative stem cell donors. Unfortunately because of early and late fatal pulmonary/vascular complications following SCT the results of conventional transplants have been unsatisfactory. SCT using fludarabine-based protocols, which avoid radiotherapy and busulphan, appears to be giving encouraging preliminary results. The genetic studies described below suggest that treatments based on correction of telomerase activity might also benefit DC patients.

(ii) Cell and molecular biology

Although the occasional DC patient may show some evidence of chromosome breakage, in general there is no significant difference in chromosomal breakage between DC and normal lymphocytes with or without the use of bleomycin, diepoxybutane, mitomycin-C and γ-irradiation. This observation enables DC patients to be distinguished from related disorders such as Fanconi anaemia (FA).

Primary DC skin fibroblasts are abnormal both in morphology and in growth rate. Furthermore, they show unbalanced chromosomal rearrangements (dicentrics, tricentrics, translocations) in the absence of any clastogenic agents. In addition, PB and BM metaphases from some patients show unbalanced chromosomal rearrangements in the absence of any

clastogenic agents. These studies provide evidence for a defect, which predisposes DC cells to developing chromosomal rearrangements.

Haemopoietic progenitor studies have shown reduced numbers of all progenitors compared to controls and there is usually a downward decline with time. The degree to which progenitors are reduced can vary from patient to patient and they can be reduced even when the PB count is normal. The demonstration of abnormalities of growth and chromosomal rearrangements in fibroblasts suggests that the BM failure is likely to be a consequence of abnormalities in both haemopoietic stem and stromal cells.

Carriers of X-linked DC show complete skewing in X-chromosome inactivation patterns (XCIPs). The presence of the extremely skewed pattern of X-inactivation in PB cells suggests that cells expressing the defective gene have a growth/survival disadvantage over those expressing the normal allele. Furthermore a skewed XCIP provides important information about carrier status for use in the counselling of families at risk of DC. In addition XCIPs data allow us to distinguish an inherited mutation from a *de novo* event in sporadic male DC cases, as well as autosomal from X-linked forms of the disease.

Initially, through linkage analysis in one large family with only affected males it was possible to map the gene for the X-linked form to Xq28. The availability of polymorphic genetic markers from the Xq28 and additional X-linked families facilitated positional cloning of the gene (*DKC1*) that is mutated in X-linked DC. Identification of the *DKC1* gene in 1998 made available a genetic test that can be used to confirm diagnosis in suspected cases. It also led to the demonstration that another rare syndrome, the Hoyeraal-Hreidarsson (HH) syndrome, is due to mutations in the *DKC1* gene. HH is a severe multi-system disorder characterized by severe growth failure, abnormalities of brain development (usually cerebellar hypoplasia), BM failure and immunodeficiency (T+B-NK- severe combined immunodeficiency). The recognition that HH is a severe variant of DC has further highlighted the considerable variability of the DC phenotype.

In addition to providing an accurate diagnostic test this genetic advance has provided insights into the pathogenesis of DC. The *DKC1* gene is expressed in all tissues of the body indicating that it has a vital "house keeping function" in the human cell. This correlates well with the multi-system phenotype of DC. The *DKC1* gene and its encoded protein, dyskerin, are highly conserved throughout evolution. Dyskerin is a nucleolar protein that is associated with the H/ACA class of small nucleolar RNAs (snoRNAs) and is involved in pseudouridylation of specific residues of ribosomal RNA (rRNA). This step is essential for ribosome biogenesis and therefore initially suggested that DC arises largely because of defective ribosome biogenesis.

Subsequent studies have shown that dyskerin also associates with the RNA component of telomerase (hTERC) that too contains a H/ACA consensus sequence. Telomerase is an enzyme complex that is important in maintaining the telomeres of chromosomes. The precise composition of the telomerase complex is unknown but two essential components, the RNA component (hTERC) and the catalytic reverse transcriptase (hTERT), have been well characterized. Telomerase activity can be reconstituted *in vitro* using just the hTERC and hTERT. In patients with X-linked DC it was initially demonstrated that the level of hTERC

was reduced and that telomere lengths were much shorter than in age matched normal controls. Subsequently it was found that telomeres are also shorter in cells from patients with autosomal forms of DC. This therefore suggested that DC might principally be a disease of telomere maintenance rather than ribosomal biogenesis. Further clarification came from linkage analysis in one large DC family, which showed that the gene for autosomal dominant DC is on chromosome 3q, in the same area where the gene for *hTERC* had been previously mapped. This led to *hTERC* mutation analysis in this and other DC families and the demonstration that autosomal dominant DC is due to mutations in the *hTERC* gene.

Since the *DKC1* encoded protein dyskerin and hTERC are both components of the telomerase complex it now appears that DC arises principally from an abnormality in telomerase activity. Affected tissues are those that need constant renewal, consistent with a basic deficiency in stem cell activity due to defective telomerase activity. The demonstration of *DKC1* and *hTERC* mutations in DC families provides an accurate diagnostic test in a significant subset of cases. For DC patients this now also provides the basis for designing new treatments. For the wider community it provides the first direct link between a human disease that is characterized by features of premature ageing and short telomeres. Therefore unravelling the biology of this rare disease has important implications not only for patients with DC but also for the more common disorders such as ageing, cancer and AA which too are associated with abnormal telomeres. It is noteworthy, *hTERC* mutations have been identified in patients with AA but who lacked diagnostic features of DC. Furthermore, AA patients with *hTERC* mutations had significantly shorter telomeres than age-matched controls. These data indicate that, in a subset of patients with AA the disorder is associated with a genetic lesion in the telomere maintenance pathway. They also highlight the diverse manifestations of DC: its severe form as the HH syndrome, its classical form and its mildest form as " aplastic anaemia".

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Telomere length homeostasis is achieved *via* a switch between telomerase extendible and non-extendible states

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Telomerase counteracts telomere erosion that stems from incomplete chromosome end replication and nucleolytic processing. According to the 'protein counting model' of telomere length control (Marcand et al. 1997), telomerase-mediated telomere extension is regulated by the number of telomere-bound Rap1p molecules. At least two not mutually exclusive models could provide a mechanistic basis for this increased activity of telomerase on shorter telomeres. First, the elongation efficiency of telomerase, i.e. the number of nucleotides added per elongation event could be regulated as a function of telomere length. Second, the productive association of telomerase with the telomere 3' end could be regulated in a length-dependent manner. To distinguish between these models, we have developed an assay to measure telomere elongation at nucleotide resolution in *Saccharomyces cerevisiae*. We find that telomerase does not act on every telomere in every cell cycle. Instead, it exhibits an increasing preference for telomeres as their lengths decline. The number of nucleotides that are added to a telomere in a single cell cycle varies between a few to more than 100 nucleotides and is independent of telomere length. Deletion of the telomeric protein Rif1p gives rise to longer telomeres by increasing the frequency of elongation events. Thus, by taking a molecular snapshot of the result of a single round of telomere replication, we demonstrate that telomere length homeostasis is achieved *via* a switch between telomerase extendible and non-extendible states.

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Telomere length and telomerase regulation in normal skin cells

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Telomerase, the ribonucleoprotein complex able to stabilize telomere length in germ line and tumor cells is also expressed in regenerative tissues such as the epidermis of the skin. *In vitro* proliferation studies had suggested that telomerase was too low in order to be active and, therefore, it is largely ignored that telomerase may also be an important enzyme for normal cells. We now show that telomerase is easily detectable in keratinocytes *in situ* from several specimen of different-age donors and that accordingly the mean telomere length in old donors is not significantly (if at all) reduced as compared to young donors. This is quite in contrast to peripheral blood lymphocytes which show a statistically significant age-dependent decline. We further show that *in vitro* propagation is correlated with a rapid downregulation of telomerase activity and that this correlates with a proliferation-dependent telomere shortening similar to that seen in telomerase-negative fibroblasts. While the addition of growth factors (including EGF) has no effect on telomerase upregulation in the keratinocytes, tissue-like growth in organotypic cultures is correlated with reappearance of telomerase activity suggesting that telomerase in the epidermal cells is not expressed constitutively but is regulated in a tissue-dependent manner. This control seems crucial for the normalcy of the cells, because ectopic expression of hTERT in fibroblasts induces longevity which is correlated with changes in the expression profile - including growth-controlling and differentiation-related genes, as well as chromosomal aberrations. A potential mechanism for the induction of chromosomal aberrations will be discussed.

Erosion of the telomeric single-strand overhang at replicative senescence

Sheila A. Stewart, Ittai Ben-Porath, Vincent J. Carey, Benjamin F. O'Connor, William C. Hahn and Robert A. Weinberg

Cultured primary human cells inevitably enter a state of replicative senescence, for which the specific molecular trigger is unknown. We demonstrate that the single-strand telomeric overhang, a key component of telomere structure, is eroded at senescence. Expression of telomerase prevents overhang loss, suggesting that this enzyme prevents senescence through the maintenance of proper telomere structure. In contrast, progressive overhang loss occurs in cells that avoid senescence through inactivation of p53 and Rb, indicating that this erosion is the result of continuous cell division and not a consequence of senescence. We thus provide the first evidence for a specific molecular alteration in telomere structure, and suggest that this change, rather than overall telomere length, serves as a trigger for senescence.

Altered mitochondrial membrane potential in TERT transgenic mice

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Telomerase maintains telomere length and architecture in eukaryotic cells, thus protects dividing cells from replicative senescence in vitro. A few reports have shown that telomerase can promote cellular survival and suppress apoptotic cell death, independent of its activity. Here we show that expression of TERT mRNA is induced in the ipsilateral cortical neurons following occlusion of the middle cerebral artery in adult mice. Transgenic mice that overexpress TERT showed significant resistance to ischemic brain injury even though telomerase activity was not detected. NMDA receptor-mediated excitotoxicity was reduced in forebrain cell cultures overexpressing TERT. NMDA-induced accumulation of cytosolic free Ca2+ was reduced in forebrain neurons from TERT transgenic mice, which was attributable to the rapid flow of cytosolic free Ca2+ into the mitochondria from the cytosol without change in Ca2+ influx and efflux through the plasma membrane. Strikingly, mitochondrial membrane potential was markedly increased in neurons from TERT transgenic mice. These results provides evidence that TERT is inducible in postmitotic neurons following ischemic brain injury and prevents NMDA neurotoxicity through shift of the cytosolic free Ca2+ into the mitochondria, and thus plays a protective role in ameliorating ischemic neuronal cell death, implying a novel function of TERT in cytoplasm.

Session 3: Mechanisms of telomere dysfunction
Chair: Susan M. Gasser

Protection of human telomeres: the role of TRF2 and ERCC1/XPF

Titia de Lange

Human telomeres are protected by TRF2. Loss of TRF2 leads to a DNA damage response that is partially ATM dependent and results in either apoptosis or senescence. TRF2 inhibition also results in partial loss of the telomeric 3' overhang and chromosome end fusions formed through non-homologous end-joining (NHEJ). We have found that ERCC1/XPF-deficient cells retained the telomeric overhang after TRF2 inhibition, identifying this nucleotide excision repair endonuclease as the culprit in overhang removal. Furthermore, these cells did not accumulate telomere fusions, suggesting that overhang processing is a prerequisite for NHEJ of telomeres. ERCC1/XPF was also identified as a component of the telomeric TRF2 complex. ERCC1/XPF-deficient mouse cells had a novel telomere phenotype, characterized by Telomeric DNA containing Double Minute chromosomes (TDMs). We speculate that TDMs are formed through the recombination of telomeres with interstitial telomere-related sequences and that ERCC1/XPF functions to repress this process. Collectively, these data reveal an unanticipated involvement of the ERCC1/XPF NER endonuclease in the regulation of telomere integrity and establish that TRF2 prevents NHEJ at telomeres through protection of the telomeric overhang from ERCC1/XPF.

Chromosome instability and telomeres

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Current models for telomere deprotection suggest that uncapping leads directly to end-fusions and chromosome instability[1]. However, in the presence of factors that block cell cycle checkpoint activities cells are able to divide beyond senescence (M1) for 10-15 doublings until they reach crisis (M2)[2]. If telomeres are sufficiently short to become deprotected at M1 and induce end-fusion events that lead to chromosome disruption, it is not clear how cells would be able to divide for an additional 10-15 doublings before significant apoptosis begins at M2. In order to address this issue, we followed the evolution of chromosomal abnormalities, apoptosis and DNA damage foci between M1 and M2. We find matching quantitative increases in the frequencies of DNA damage foci and telomere associations between M1 and M2, consistent with the previous demonstrations that at senescence or following deprotection these foci correspond to signals emanating from telomeres[3,4]. However, there is a large discordance between the number of DNA damage foci and the cellular phenotype, including the number of chromosomal abnormalities/rearrangements, changes in cell growth rate or an increase in apoptosis. We show that telomere associations (TAS) are unstable since they are much more abundant in G1 than in metaphase spreads. Their quantity in G1 corresponds to the number of observed γ-H2AX DNA-damage complexes, and they are disrupted by the phosphodiesterase and ATM/ATR inhibitor caffeine, suggesting that TAS might represent weak interactions such as arrested DNA repair intermediates rather than actual covalent end-to-end fusions. M2 occurs once telomeres become sufficiently short to produce breakage-fusion cycles and a dramatic increase in apoptosis.

These results suggest that there may be a qualitative difference in the packaging/structure of short telomeres that leads to end-associations in M1 versus end-fusions, dicentric chromosome formation, and apoptosis during M2. One model consistent with the present results is that non-canonical telomeric sequence variants at the base of the telomeres could produce mismatches with the invading G-rich overhangs and destabilize t-loop formation. The unfolded telomeres would still have intact G-rich 3' overhangs and sufficient double-stranded telomeric sequences to provide partial masking from the DNA repair apparatus. A partial DNA damage response might initiate end-associations with another telomere that are "transient" (i.e., either do not involve actual covalent ligation of the ends, or where breakage is preferentially induced within the short stretch of telomeric repeats so that some protective sequences still remain). A transition to breakage-fusion events would occur when telomeric sequences became so limited that ineffective masking occurred. The

implications of non-canonical sequences for pathologies involving premature replicative aging will be discussed.

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Shortened telomeres join to DNA breaks interfering with their correct repair

Laura Latre, Laura Tusell, Marta Martín, Rosa Miró, Joseph Egozcue, María A. Blasco, Anna Genescà

Telomeres cap chromosome ends, preventing end-to-end fusions. Short telomeres tend to fuse other chromosome ends with short telomeres forming a dicentric chromosome. By irradiating the telomerase-knockout mouse model, we have identified a new type of rejoining. Here we report that short telomeres may not only fuse to other chromosomes with short telomeres but also to broken DNA ends. This new type of rejoining may also contribute to chromosome instability by the formation of non-reciprocal translocations and other rearrangements after the breakage of dicentric bridges at anaphase. In addition, telomerase knockout mice have increased radiation sensitivity. We conclude that, besides a possible slower rate of repair, the major mechanism for the chromosomal sensitivity observed in these mice is the presence of uncapped chromosomes with critically shortened telomeres, joining to radiation-induced breaks, and therefore interfering with the correct rejoining of broken chromosomes. It remains to be established whether the interference effect reported here has consequences on the radiation sensitivity associated with aging in humans, and whether this may account for the important inter-individual differences to the cytotoxic effects in radiation therapy.

Consequences of telomere dysfunction: Making ends meet

Susan M. Bailey

Telomeres are highly specialized nucleoprotein structures that maintain genomic stability by stabilizing and protecting the ends of linear chromosomes; this is an essential function as inferred cytogenetically from the end-to-end fusions that result when telomeric end-capping fails or is lost. In striking contrast to natural chromosomal termini, broken chromosome ends produced by DNA double strand breaks (DSBs) are highly recombinogenic, and represent a major threat to the integrity of the genome due to their potential for leading to chromosomal rearrangements that contribute to genomic instability and tumorigenesis.

We have demonstrated that effective end-capping of mammalian telomeres has a seemingly paradoxical requirement for proteins more commonly associated with DNA double-strand break (DSB) repair (Bailey et al., 1999). Ku70, Ku80, and DNA-PKcs (the catalytic subunit of DNA-dependent protein kinase) all participate in DSB repair through non-homologous end-joining (NHEJ). Somewhat surprisingly, mutations in any of these genes cause spontaneous chromosomal end-to-end fusions that maintain large blocks of telomeric sequence at the points of fusion, which are not a consequence of telomere shortening, nor are they telomeric associations (Gilley et al., 2001; Goytisolo et al., 2001; Samper et al., 2000). We have also shown that nascent telomeres produced via leading-strand DNA synthesis are especially susceptible to these end-to-end fusions, suggesting a crucial difference in the postreplicative processing of telomeres that is linked to their mode of replication (Bailey et al., 2001; Espejel et al., 2002).

It was originally thought that telomeric DNA sequence alone could stabilize chromosome ends. However, it is now recognized that a variety of proteins that bind either directly to telomeric DNA, or indirectly to other proteins that are themselves bound to telomeric DNA, are also required to form a protective nucleoprotein higher order chromatin structure that serves to "cap" the end of the chromosome and prevent deleterious terminal rearrangements (de Lange, 2002). The goal of our genetic analyses of chromosome end capping has been to identify the genes and the proteins they encode that enable the protective function of telomeres. Cytogenetic analysis has proven to be an invaluable tool for assessing the integrity of chromosome end protection. I will present data examining the dual roles played by DNA repair proteins.

Cellular responses to unprotected chromosome ends include triggering of cell-cycle checkpoints, and induction of senescence or apoptosis (Karlseder, 2003; Karlseder et al., 1999). These studies indicate that the cell treats dysfunctional telomeres as if they were DSB ends. Critically shortened telomeres associated with telomerase deficiency have recently been

shown to misjoin with DSBs in yeast (Chan and Blackburn, 2003), and in mice (Latre et al., 2003). We find that uncapped mammalian telomeres due to DNA-PKcs deficiency not only fuse to one another, but also inappropriately fuse to DSB ends produced by ionizing radiation, demonstrating that these novel rearrangements, which significantly contribute to the instability seen in these backgrounds, result without requisite telomere shortening.

The three proteins comprising DNA-PK have each been found at mammalian telomeres (Bianchi and deLange, 1999; d'Adda di Fagagna et al., 2001; Hsu et al., 1999), suggesting that telomere protection requires the holoenzyme. However, exposure of wild type cells to a highly specific DNA-PKcs inhibitor designated IC86621 (ICOS; manuscript in press) for a single cell cycle, resulted in the induction of numerous chromatid-type telomeric fusions (manuscript in press). The absence of chromosome-type fusions provides further evidence supporting a post-replication role for DNA-PKcs, most likely in reconstructing functional telomeres on newly replicated chromosome ends. Its precise role, however, remains elusive. Nevertheless, we can conclude that the kinase activity of DNA-PKcs is required for both of its roles, i.e., preserving natural ends and joining of unnatural DSB ends (Kurimasa et al., 1999), and does not serve to distinguish between them.

Phosphorylation of the variant histone H2AX occurs in megabase-sized domains about the sites of DSBs (Rogakou et al., 1999). These domains are cytologically visible with immunofluorescence, and allow DSBs to be accurately quantified. By this means it has been shown that the background level of DSBs in genetically normal cells is quite low, ~0.05 DSBs/cell (Rothkamm and Lobrich, 2003). This implies that NHEJ proteins are rarely called upon to join DSBs in a natural setting. In contrast, with every cell cycle the protective telomere structure disassembles to allow replication, and must be reconstructed afterwards. In normal human cells, NHEJ proteins participate in capping 92 new leading-strand telomeres. This number of chromosome ends is equivalent to 46 DSBs, far in excess of the naturally occurring number of DSB ends. This prompts us to speculate that the primary function of NHEJ proteins is not to join ends, but to preserve them.

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Role of chromatin modification activities at mammalian telomeres

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Telomeres are capping structures at the ends of eukaryotic chromosomes composed of TTAGGG repeats bound to an array of specialized proteins. Telomeres, together with centromeres, are considered heterochromatic regions. Yeast and flies defective for activities that modify the state of chromatin, also show abnormal telomere function. In mammals, however, the putative role of chromatin modifying activities on regulating telomeres is unknown. Here, we have studied telomere length and function in mice doubly deficient for the Suv39h histone methyltransferases (HMTases), Suv39h1 and Suv39h2 (Suv39DN), which govern histone H3 lysine 9 (H3-K9) methylation at heterochromatic regions. We show that primary cells derived from Suv39DN mice have a percentage of telomeres that are abnormally long compared to those of wild-type controls. Using chromatin immunoprecipitation (CHIP), we show that telomeres contain methylated H3-K9 (H3-K9M), as well as heterochromatin protein 1 (HP1), and that these chromatin modifications are decreased at Suv39hDN telomeres. These results indicate that mammalian telomeric chromatin contains H3-K9M, and that this histone modification at telomeres depends on the activity of the Suv39h HMTases. In addition, we show that altering the methylation state of H3-K9 in Suv39hDN cells results in decreased HP1 binding at telomeres and deregulation of telomere length.

Session 4: Regulation of mammalian telomerase in cancer
Chair: Virginia A. Zakian

The role of telomeric chromatin in length regulation and during malignant transformation

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We have shown that, in *Saccharomyces cerevisiae tel1Δ* cells, vertebrate telomeric DNA repeats are taken into account for length setting in a Rap1-independent fashion (1). Tethering several N-terminal domains of either Tbf1 or Reb1 adjacent to a telomere results in a reduction of its length that is proportional to the number of targeted molecules. Furthermore, the regulation of vertebrate-repeats is slightly impaired in cells carrying a mutated allele of *TBF1*. These data suggest an important role for Tbf1 and Reb1 in a Rap1-independent pathway of telomere length setting. Interestingly, Reb1 is known to facilitate transcriptional activation and insulation, probably through chromatin remodeling. These findings suggest an intriguing parallel between the yeast Tbf1 and the human TRF1, which share very homologous DNA binding domains and which appear to cis-inhibit telomerase in a Rap1-independant way. They also strengthen the importance of the chromatin in the mechanisms of telomere length regulation.

In order to study the structure of telomeric chromatin in B-cell Chronic Lymphocytic Leukemia (B-CLL), we assayed the expression profile of the genes encoding hTERT, TRF1, TRF2 and associated proteins by real-time quantitative RT-PCR. In parallel, the expression of telomerase was analyzed by TRAP assay. We found in a cohort of 55 patients that B-CLL cells contain a very low level of telomerase mRNA and enzymatic activity as compared to normal B cells from tonsils or peripheral blood. Interestingly, this telomerase decline correlates with an elevated level of the mRNAs corresponding to factors suspected to *cis*-inhibit telomerase activity (*TRF1*, *TIN2* and *PINX1*). By contrast, there is no detectable change in the expression of *TRF2*, Overall, it seems that B-CLL cells are committed to block telomerase expression and activity. These results suggest that specific telomeric chromatin dysfunctions are associated with B-CLL and that the inhibition of telomerase can participate to the malignant transformation of B cells.

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Role of Pin2/TRF1 in telomere maintenance and cell cycle control

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Telomeres are essential for preserving chromosome integrity during the cell cycle and have been implicated in mitotic progression, but few clues are known about the signaling molecule(s) involved. Telomerase plays a crucial role in maintaining telomere length and its ability to elongate telomeres is regulated by other factors, including telomeric DNA-binding proteins. Since these telomere proteins do not directly regulate telomerase activity, it is not known how they regulate telomere elongation. Similarly, it is unclear whether telomere proteins play any role in the cell cycle. During our study of cell cycle control, we previously isolated three human proteins, Pin1-3, that are involved in mitotic regulation (1). Pin1 is a conserved peptidyl-prolyl isomerase that specifically isomerizes only the phosphorylated Ser/Thr-Pro bonds in certain proteins, thereby catalytically inducing conformational changes to regulate protein function following phosphorylation (1, 2). Pin2 is identical to TRF1 with the exception of a 20 amino acid internal deletion but is more abundant than TRF1 in the cell (3); they are likely generated from the same gene *PIN2/TRF1* (4). Consistent with a role in mitotic regulation, we have previously shown that Pin2/TRF1 levels are tightly regulated during the cell cycle and its deregulation specifically affects mitotic regulation (3, 5). To elucidate how Pin2/TRF1 is involved in telomere maintenance and cell cycle control, we have been using biochemical approaches and yeast two-hybrid screen to identify Pin2/TRF1-interacting proteins. Characterization of the Pin2/TRF1-interacting proteins confirms the dual roles of Pin2/TRF1 in telomere maintenance and cell cycle control, as suggested by how they were originally identified, and also provides new insights into the regulation of telomerase and mitotic checkpoints.

Biochemical approaches led us to find that Pin2/TRF1 interacts with ATM and microtubules (6-9). Significance of the Pin2/TRF1 and ATM interaction has been demonstrated by our findings that following DNA damage, ATM phosphorylates Pin2/TRF1 and suppresses its ability to induce abortive mitosis and apoptosis (6). Moreover, direct inhibition of Pin2/TRF1 in ATM-negative cells is able to bypass the requirement for ATM in specifically restoring telomere shortening, the G2/M checkpoint defect and radiosensitivity (7). In addition, we also find that Pin2/TRF1 is specifically localized to the mitotic spindle during mitosis and inhibition of endogenous Pin2/TRF1 is also able to restore the mitotic spindle checkpoint defect in response to microtubule-affecting drugs in ATM-negative cells (8, 9).

These results demonstrate a crucial role for Pin2/TRF1 in the ATM-dependent regulation of telomeres and DNA damage response.

The yeast two-hybrid screen led us to identify six known and four unknown genes (PinX1-4), whose products interact with Pin2 (10). PinX1 binds the telomerase catalytic subunit hTERT and potently inhibits its activity. Overexpression of PinX1 and its TID (telomerase inhibitory domain) inhibited telomerase activity, shortened telomeres and induced crisis, whereas depletion of endogenous PinX1 significantly increased telomerase activity and lengthened telomeres. Significantly, both PinX1 and its TID domain bound hTERT *in vivo* and *in vitro*, and also potently inhibited its activity *in vitro*. In contrast, neither PinX1 nor its TID induced telomere shortening or crisis in telomerase-negative cells. Interestingly, human *PINX1* is located at 8p23, a region with frequent loss of heterozygosity in a number of human tumors. Moreover, depletion of endogenous PinX1 in HT1080 cells increased their tumorigenicity, whereas overexpression of PinX1 or PinX1-C inhibits their ability to form tumors in mice. These results indicate that PinX1 represents a novel class of proteins that can regulate telomerase activity directly, and is a strong candidate for a tumor suppressor (10).

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Telomere-initiated cellular senescence is a DNA damage checkpoint-mediated response

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Telomere-initiated cellular senescence is triggered when telomeres, the ends of linear chromosomes, cannot fulfil their normal protective functions. Although changes in several cellular markers have been shown to be associated with senescence, the mechanisms that control it are largely unknown. In order to gain a better molecular understanding of this phenomenon, we studied the molecular markers associated with senescence in primary human MRC5 and BJ fibroblasts. Since their life span can be extended by the expression of telomerase, senescence is telomere-initiated in these cells. Our analysis reveals that senescent human fibroblasts display molecular markers of DNA damage checkpoint activation characteristic of cells bearing DNA double-strand breaks. These markers include the accumulation of nuclear foci of several activated DNA repair and DNA damage checkpoint factors. A post genomic approach (ChIP on CHIP) revealed the direct involvement of eroded telomeres in the activation of the DNA damage response. We also show that many of these DNA damage response proteins, but intriguingly not all, associate with uncapped telomeres. Finally, we reveal that interfering with the actions of DNA damage checkpoint kinases can restore the DNA replicative ability of senescent cells. These data demonstrate that the DNA damage checkpoint apparatus is activated in senescent cells, it plays a causative role in senescence and that senescent cells can be induced to re-enter the cell cycle by interfering with the DNA damage checkpoint functions of the cell.

Telomerase and Ku86 act together at the telomeres of germ cells, a comparative transcriptome analysis of unmodified mouse cells

Sonia Franco and María Blasco

Introduction: Mouse models of telomere dysfunction include telomerase-deficient mice with critically short telomeres (G3 Terc-/-) and Ku86-deficient mice with impaired telomere capping. Using meiosis as a working model, we have previously shown that both G3 Terc-/- and Ku86-/- germ cells show decreased proliferative potential, resulting in hypogonadism and infertility. Doubly deficient mice generated by us (G3 Terc-/-/Ku86-/-, Ref. 1) show further reduction in meiotic function. In addition, and despite the fact that telomere fluorescence intensity of G3 Terc-/-/Ku86-/- meiocytes is similar to that of G3 Terc-/- meiocytes, the short telomere-induced apoptosis previously observed in G3 Terc-/- germ cells is prevented in the absence of Ku86, suggesting a role for Ku86 upstream of p53 in cellular responses to dysfunctional telomeres. We have used genomic profiling to identify the molecular pathways triggered by telomere dysfunction in mouse cells.

Materials and methods: Testicular cell suspensions were obtained from 2-4 mo-old male wild-type, G3 Terc-/-, Ku86-/- and G3 Terc-/- /Ku86-/- mice. Total RNA was hybridized to oligonucleotide arrays (Affymetrix, MG-U74).

Results: In all three models of telomere dysfunction (Terc-/-, Ku86-/-, G3 Terc-/-/Ku86-/-), derepression of gene expression was predominant over gene repression. Although some pathways were specific of either short telomeres (G3 Terc-/-) or Ku86 deficiency, there was significant overlap in the transcriptional response to telomere dysfunction of any ethiology. Genes involved in extracellular matrix deposition, response to oxidative stress and response to chronic inflammation were induced in all three genotypes studied. The number of genes activated and the fold-change for a given gene generally followed the gradient: G3 Terc-/-/Ku86-/- >> G3 Terc-/- > Ku86-/-, which grossly correlates with the degree of organ dysfunction. More detailed analysis of selected gene subsets is currently underway.

Conclusions: Gene activation pattern in response to telomere dysfunction shares features of previously described profiles for aging, response to oxidative stress and chronic tissue damage, strengthening the notion that telomeres play an essential role in these processes.

Telomeres and telomerase: therapeutic targets for cancer and aging

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It has been reported that there is a heritable component to telomere length (1). In addition, there is mounting correlative evidence that telomere shortening may be an important aspect of tissue dysfunction in chronic diseases (2-5). Because telomerase activity is not detected or is present in reduced amounts in somatic tissues, telomeres shorten with each cell division in almost all cells including proliferative cells of the skin, gastrointestinal system and blood. It is possible that inflammation associated with chronic disease may accelerate telomere-shortening leading to age-related diseases. For example, in addition to effects on epithelial cells of the airway passages, chronic exposure to tobacco smoke may result in increased cell divisions and turnover of immune cells resulting in enhanced telomere shortening. Recently we found that short telomeres in peripheral blood lymphocytes correlated with a 6-fold increased likelihood of development smoke-related cancers (1).

Since the expression of hTERT reconstitutes telomerase activity and circumvents the induction of senescence, we have used hTERT to "telomerize" a variety of human cell types (6) such as human corneal fibroblasts/epithelial cells, skin keratinocytes/dermal fibroblasts, and bronchial epithelial cells/lung fibroblasts and used these to form organotypic cultures. Such organotypic cultures express differentiation-specific proteins, suggesting that hTERT does not inhibit normal differentiation functions of cells. hTERT preferentially elongates the shortest telomeres (7), so transiently expression of telomerase could have profound effect on cell lifespan. Since telomerase in the absence of other alterations is not oncogenic (8), the production of transiently or reversibly "telomerized" engineered cells offer the possibility of treating a variety of chronic diseases and aged-related medical conditions that are due to telomere-based replicative senescence.

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Exploiting telomerase gene expression for targeted genetic therapies

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Research Interests

Nicol Keith is Head of the Telomerase Therapeutics Group at the Cancer Research UK Beatson Laboratories, University of Glasgow. The overall objective of the group is to carry out translational research extending the identification of basic mechanisms of gene regulation into validated targets for new therapeutics. The focus is on understanding telomerase promoter specificity to develop novel cancer therapeutics including small molecule transcriptional modulators, signal transduction inhibitors and gene therapy solutions.

Cancer Therapeutics

Many of the new generation cancer therapeutics target one of the four key features of cancer cells that distinguish them from normal cells; Loss of cell cycle control; Loss of apoptosis control; Loss of control over invasion and metastasis; Loss of senescence control with gain of cellular immortality. A central cause for loss of senescence and the consequent immortality of cancer cells is now known to reside in an enzyme called *telomerase*.

Telomerase as an anticancer strategy

A major challenge facing cancer therapy is to generate tumour-specific treatment strategies. The level and frequency of telomerase activity and component gene expression in cancers reinforces the notion that telomerase has potential within anticancer strategies. Telomerase expression is in general much higher in malignant tissue compared with normal tissue, and this differential may be greater than that for classical enzymatic targets of chemotherapy such as thymidylate synthase, dihydrofolate reductase or topoisomeraseI/II. Indeed, the inhibition of telomerase in cancer cells in tissue culture leads to cell death, which strongly supports efforts to develop telomerase inhibitors for clinical use. Numerous potential therapeutic strategies have been proposed with the telomere or telomerase enzyme as the molecular target. One potential drawback of these strategies is the phenotypic lag, that is the number of cell divisions necessary before inhibition of telomerase can lead to sufficient shortening of the telomere to initiate cell death. However, careful consideration of the relevant clinical situation and the appropriate timing of therapy should overcome this hurdle. Moreover, recent data using cultured tumour cells indicate the response in terms of induction of cell death by telomerase inhibition can indeed be quite rapid. However, an alternative strategy is to exploit tumour-specific telomerase gene expression to target suicide gene therapy vectors to cancer cells, thus causing rapid cell kill.

Telomerase-directed gene therapy

Suicide gene therapy aims to selectively target cancer cell without harming normal cells thus reducing the toxicity often associated with conventional therapies. In order to achieve this

selectivity, gene therapy approaches require mechanisms to regulate and limit the expression of therapeutic genes to cancer cells. Achieving this aim remains a challenge for the development of clinically useful suicide gene therapy. Tumour-specific gene promoters can be used for transcriptional targeting to improve selectivity and increase therapeutic index. However until recently this has been difficult and disappointing as many tumour specific promoters show weak transcriptional activity and are unable to drive efficient expression of the therapeutic gene. In addition, the promoter activity is often restricted to one tumour type for example HER2/NEU positive is limited to use with breast cancer.

As a solution we have developed the telomerase hTR and hTERT promoters as highly efficient, cancer specific transcriptional regulators capable of targeting suicide gene therapy constructs to a broad range of cancer types. Proof of concept has been achieved using telomerase targeted adenoviral suicide gene therapy vectors, using the promoter sequences to regulate expression of the bacterial NTR gene within a replication defective adenoviral delivery system. *In-vitro / In-vivo* data demonstrates the benefits of these telomerase vectors to effectively regulate the expression of genes to activate cytotoxic compounds in cancer cells and not normal cells.

Conclusions

In its brief history, telomerase has already made a major impact in our understanding of cellular immortality and cancer progression. It has also provided us with a most intriguing and attractive therapeutic target. Yet we must remain cautious and realistic. It is unlikely that telomerase inhibition will be a blockbuster therapy curing all cancers. However, optimism is justifiable, and given careful consideration of both tumour biology and the appropriate clinical scenarios, the introduction of telomerase-based therapeutics into the clinic is awaited with great anticipation.

Further reading

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Session 5: Therapeutical approaches based on telomeres and telomerase
Chair: Victoria Lundblad

Telomerase-based cancer therapy

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Telomerase is an enzyme required for maintenance of telomeric DNA "capping" the ends of eukaryotic chromosomes. It is a unique ribonucleoprotein reverse transcriptase for which no redundant enzyme nor efficient alternative pathway is known. Without telomerase, telomeres shorten to a critical length, chromosomes become unstable, and cell cycle arrest or death ensues. Human telomerase is tightly repressed in most normal somatic cells, transiently inducible in certain stem or progenitor cells, and constitutively activated in germline and tumor cells. Multiple academic groups and biotechnology and pharmaceutical companies have targeted telomeres or telomerase for potential therapeutic products over the past 5-10 years. Although there are thousands of publications, including a number of positive cell- and animal-based efficacy studies, currently there are only a small number of drug candidates in development. The first clinical studies based upon telomerase target the catalytic protein component (hTERT) as an antigen for therapeutic vaccines against cancer, and show preliminary safety and signs of efficacy. An additional strategy to target telomerase-positive tumor cells for destruction utilizes the hTERT promoter to drive conditional replication of an oncolytic virus. This approach is in preclinical development. The more conventional, drug-like strategy of enzyme inhibition is the focus of this presentation. We are conducting IND-enabling studies of GRN163 and GRN163L, two N3'-P5' thio-phosphoramidate oligonucleotide inhibitors of telomerase. GRN163L is a lipidated version of GRN163 with increased potency compared to the parent compound. Both oligonucleotides tightly bind to the template region of the RNA component of telomerase, hTR, blocking the active site of the enzyme. GRN163 and 163L inhibit telomerase activity and prevent tumor cell growth in culture and *in vivo* in multiple model systems, with few signs of toxicity to normal cells or tissue. Pharmacokinetic and biodistribution data support intermittent systemic dosing of either compound. In conclusion, the data suggest that telomerase will provide multiple, relatively safe approaches to pan-cancer treatment, including immunotherapy, oncolytic virus, and enzyme inhibitors.

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Telomerase immunotherapy of cancer

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High-level expression of the telomerase reverse transcriptase (hTERT) in >85% of human cancers, in contrast to its restricted expression in normal adult tissues, points to hTERT as a broadly applicable target for anti-cancer immunotherapy. We have previously shown that cytotoxic T lymphocytes (CTL) recognize peptides derived from hTERT and kill hTERT+ tumor cells of multiple histologies *in vitro*. Moreover, because survival of hTERT+ tumor cells requires functionally active telomerase, hTERT may serve as a prototypic immune target for which mutation or loss as a means of escape may be incompatible with sustained tumor growth. Here, we report results of a two phase I studies of hTERT vaccination in cancer patients. In the first study (now closed), seven HLA-A2 pts with advanced cancer (5 with prostate cancer, 2 with breast cancer) were vaccinated up to 6 times subcutaneously every other week with autologous monocyte-derived dendritic cells loaded *ex vivo* with the hTERT HLA-A2 binding peptide I540. As measured by peptide/MHC tetramer, ELISPOT, and cytotoxicity assays, hTERT specific T lymphocytes were induced in four of seven patients. Tetramer-guided high-speed sorting and polyclonal expansion achieved highly enriched populations of hTERT-specific cells that killed tumor cells in an MHC-restricted fashion. Despite concerns of telomerase activity in rare normal cells, no significant toxicity was observed, including in the bone marrow. Partial tumor regression in one patient with breast cancer was associated with the induction of CD8+ tumor infiltrating lymphocytes. In a second study (ongoing), patients with advanced breast cancer are vaccinated subcutaneously with I540 peptide emulsified in adjuvant with GM-CSF. Among 5 patients treated thus far at the first dose level, no serious adverse events have been observed. Injection site reactions and tumor pain following I540 peptide vaccination have occurred (in contrast to our first trial). In one patient, flow cytometric analysis of serial tumor biopsies revealed marked induction of hTERT-specific CD8+ T cells following vaccination. These results demonstrate the immunological feasibility of vaccinating patients against telomerase and provide rationale for telomerase vaccine trials in healthier cancer patients more likely to respond clinically.

Unusual base pairs and drugs in telomere sequences

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We have studied complexes of telomere sequences with anthraquinone drugs. Only in some cases co-crystals were obtained. With the d(TGGGGT) sequence new crystal structures were found, similar to the one described by Phillips (1), but no drug was present in the crystals. A remarkable feature of the new structures is the versatility of the terminal thymines. In particular a thymine quartet was found intercalated between two G-quartets. It appears that such intercalation of thymine tetrads into guanine sequences might be available for TG_{1-3} yeast telomere sequences.

An opposite result was found in co-crystals of the partial human telomere sequence d(TAGG), which had been previously described to form G-quartets in solution (2). Here a stack of anthraquinone drugs was present into which unusual G·G base pairs were intercalated, no G-tetrad was found. The TA part of the sequence formed one WC base pair and one reverse Watson-Crick pair; it did not stack with the drugs. The reverse WC base pair adds to the versatility of AT sequences, which we have found (3) to be able to also acquire the Hoogsteen conformation. The finding of a DNA oligonucleotide intercalated into a drug crystal may be of interest for drug/DNA delivery systems.

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Unusual DNA structures and telomerase inhibitors

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Telomerase is expressed in tumour cells but not in most somatic cells and thus telomeres and telomerase may be proposed as attractive targets for the discovery of new anticancer agents. Telomeric DNA is prone to structural polymorphism: its three-dimensional structure can differ markedly from the classical double helix. The 3' G-rich telomeric overhang may adopt an intramolecular G-quadruplex structure *in vitro* which blocks telomerase. Agents that stabilize G-quadruplexes have the potential to interfere with telomere replication by blocking the elongation step catalysed by telomerase and can therefore act as antitumor agents. We have identified by Fluorescence Resonance Energy Transfer several series of G-quadruplex ligands that also exhibit potent and specific anti-telomerase activity with IC_{50} in the nanomolar concentration range (1,2). A DNA nanomolecular machine has been designed based on a duplex-quadruplex equilibrium (3). Specific recognition of a G-quadruplex conformation by small molecules was demonstrated by equilibrium dialysis and electrospray mass spectrometry using a specific set of nucleic acid structures (4). Long term treatment of tumor cells at sub-apoptotic dosage with triazines induces a delayed growth arrest that depends on the initial telomere length. This growth arrest is associated with telomere erosion and senescence.

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Targeting telomeres via DNA quadruplexes

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The two functions of the telomerase enzyme complex in cancer cells, are to catalyse the synthesis of telomeric DNA repeats onto the 3'end of telomeres, and to physically protect these single-stranded ends. Inhibition of these functions leads to exposure of the 3'ends, although there are major differences in their time-scales.

Folding of single-stranded telomeric DNA into four-stranded quadruplex structures can be achieved by small-molecule ligands. These result in rapid inhibition of proliferation and replicative senescence, consistent with telomere uncapping as a consequence of quadruplex formation. G-quadruplex ligands have also been found to have a significant effect on proliferation in combination with established anticancer agents such as flavopiridol and cis platinum.

POSTERS

Hypoxic regulation of telomerase gene promoters

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Telomerase activity has been shown to be up regulated under hypoxic conditions in human solid tumour cells (Biochem and Biophys Res Comm 260:365-370 (1999)). Using Dual Luciferase Assays for co-transfection of telomerase promoter containing vectors with a HIF-1α expression vector we have shown an increase in expression from both hTR and hTERT promoters. Although steady state levels of hTR remained unchanged, as shown by northern blot, semi-quantitative PCR revealed that levels of hTERT wild type splice variant increased in relation to time in hypoxia. In order to investigate whether chromatin remodelling could play a role in telomerase gene regulation under conditions of hypoxia we used Chromatin Immuno-Precipitation (ChIP) to look at modification of the histone N-terminal tails. An increase in methylation on Lysine 9 of H3 following hypoxic treatment was revealed, which correlated with an increase in levels of the K9 specific SUV39H1 and the K9/K27 specific G9a methyl-transferases. Consistent with the known fact that HP1 associates with methylated K9 we revealed an increase in HP1γ but not HP1α levels in response to hypoxia. Whilst methylation of K9 has previously been associated with gene repression it has recently been reported that HP1γ can enhance transcription of certain genes (Biochem and Biophys Res Comm 293:1217-1222 (2002)) and that HP1 is involved in heat shock induced expression of HSP70 (J. Cell Biol 161(4):707-714 (2003)). We propose that in this case this methylation complex is acting in a positive manner to enhance telomerase gene expression, perhaps as part of a stress response pathway.

TRF2 dysfunction and transformation of human cells

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To explore the role of telomeres in human cell transformation, we have generated retroviruses expressing different alleles of the TTAGGG repeat factor gene (TERF2) and used them to transduce fibroblasts immortalized by the combined expression of hTERT and SV40 T/t proteins (Hahn et al., Nature 400, 464-8, 1999). By comparing the phenotypes of cells expressing the various alleles, we noticed a significant correlation between the level of chromosome instability and growth defects. This is in agreement with previous data from the T. de Lange laboratory (reviewed in De Lange, Oncogene 21, 532-40, 2002). Noteworthy, chromosomal abnormalities are detectable in long-term cultures of cells that have lost the expression of the TERF2 allele. This suggests that the burst of chromosomal instability induced by TRF2 dysfunction at early passages results in a set of stable chromosomal rearrangements in late cultures. Importantly, these late cultures are able to form colonies in semisolid medium at a higher frequency than corresponding cultures transduced with retrovirus that do not express TERF2 or that expressed TERF2 alleles with no apparent effect on chromosomal stability. These data suggest a model in which TRF2 dysfunction can generate mutations involved in the transformation of human cells.

Activation of telomerase through VEGF signaling triggers angiogenesis following hind-limb ischemia

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In mammals, age-dependent telomere dysfunction may contribute to a reduction in cell viability, altered differentiation functions and impaired regenerative/proliferative responses. Recent advances indicate that sustained level of telomerase activity in endothelial cells and precursors may confer a pro-angiogenic phenotype. We have investigated whether telomerase contributes *in vivo* to tissue regeneration following hind-limb ischemia and adenovirus-mediated VEGF165 treatment in young and aged rats. Unilateral hind-limb ischemia was induced by surgical dissection of the femoral artery in young (3 months) and aged (22 months) male Fisher rats. Concomitantly, animals were injected i.m. with saline solution, AdCMV.Null or AdCMV.VEGF165 (5X107 pfu). Evaluation of angiogenesis by capillary count and of telomerase by RT-PCR, immunoistochemistry and TRAP assays were performed on adductor and quadricep muscles at 3, 8 and 14 days upon treatment (n = 6 animals/point). VEGF delivery to ischemic tissues induced angiogenesis and a significant increase of TERTmRNA, TERT protein and telomerase activity in skeletal muscles, vascular endothelial, smooth muscle and satellite skeletal muscle cells in young and, to a lower extent, older animals, presumably as a consequence of tissue remodeling. To explore the molecular mechanisms underlying this phenomenon, VEGF-dependent induction of TERT expression and activity was reproduced *in vitro* in differentiated murine myoblasts C2C12. Such induction was abrogated by addition of 7-Nitroindazole, a Nitric Oxide Synthases inhibitor, indicating that VEGF-mediated synthesis of nitric oxide is involved in this process. In addition, adenovirus-mediated hTERT gene transfer in young rats induced angiogenesis 3 and 8 days after ischemia with an efficiency similar to that elicited by VEGF165, as assessed by capillaries density. Thus TERT appears to directly contribute to angiogenesis *in vivo*, suggesting an extracurricular telomerase function.

Telomere anchoring pathways in yeast

Florence Hediger, Frank R. Neumann, Griet Van Houwe, Karine Dubrana and Susan M. Gasser

The positioning of chromosomal domains within the interphase nucleus facilitates transcriptional repression in simple organisms like yeast, and helps coordinate the timing of DNA replication with gene expression in more complex organisms. Using rapid time-lapse microscopy of GFP-tagged chromosomes in interphase yeast cells, we show that natural telomeres are highly dynamic, yet move within a restricted volume adjacent to the nuclear envelope. As chromosomes segregate during mitosis, this anchorage is lost. Live microscopy of telomeres in appropriate mutant strains shows that perinuclear attachment requires the heterodimeric Ku complex, but not Myosin-like proteins Mlp1 and Mlp2. Using *yku*-deficient strains, we detect a second, S-phase specific, Sir-dependent pathway for tethering natural telomeres at the nuclear periphery, mechanistically linking repression and anchorage. Positional analysis of different telomeres show that the efficiency of association with the nuclear envelope is variable, perhaps correlating with inherent repression level at each particular end. At all telomeres analysed so far, anchoring dependency is restricted to the yKu and the Sir pathways, although their relative importance can be different at each particular end.

Telomerase activity and p16 expression as prognostic indicators in Dukes stage C colorectal tumours

Cristina García-Aranda, Rosa González-Quevedo, Alberto Morán, Carmen de Juan, Antonio Díaz, Andrés Sánchez-Pernaute, Antonio Torres, Eduardo Díaz-Rubio, José Luis Balibrea, Manuel Benito, Pilar Iniesta

Background: Telomerase activity and p16 expression can be considered two of the most important molecular markers implicated in the development of human tumours. We have previously detected a positive interaction between these parameters in Non-small cell lung cancer (NSCLC), with p16 expression as a protective variable in patients who had developed tumours that showed positive telomerase activity.

Aim: Our purpose consists of investigating the usefulness of this association to evaluate prognosis of patients affected by other relevant pathologies, such as colorectal cancer.

Patients and Methods: We have determined telomerase activity and p16 expression in a series of 78 prospectively collected colorectal adenocarcinomas obtained from patients who had undergone surgery without other treatment. Telomerase activity was investigated by a telomeric repeat amplification protocol enzyme-linked immunoabsorbent assay-based procedure. p16 expression was examined by Western-blot, and promoter methylation was analysed by a Methylation Specific PCR method . Association with survival were evaluated.

Results: Positive levels for telomerase activity were detected in 81% of cases, this variable showing a significant association with tumour recurrence. Negative p16 expression was found in 25 (31%) samples, 6 of which showing p16 promoter methylation. Survival distributions for telomerase, adjusted for p16, indicated a positive interaction between both parameters. According to our data, p16 expression can be considered as a significant protective variable in patients with Dukes' stage C tumours showing telomerase activity.

Conclusion: Telomerase activity and p16 expression analyses may be considered prognostic factors in Dukes' stage C colorectal tumours. Our results suggest that combined study for both parameters could be useful for the selection of patients with unfavorable outcomes, in order to establish adjuvant therapy protocols.

Telomere maintenance and chromosome instability in human cells

Pirzio L., Pottier G., J. Murnane and Sabatier L.
(presented by Marie-Anne Marrière)

The consequences of telomere loss were studied in human cell models tagged with plasmids containing telomeric repeat sequences and integrated on end of a "marker" chromosome. Such models permit to establish the relation between telomere dynamics and chromosome instability. The consequence of spontaneous telomere loss is a cascade of events, dramatically mutagenic for the cell. Chromosomes lacking one telomere remain unstables until they are capped. We observed : chromosome instability, gene amplification via breakage/fusion/bridge (B/F/B) cycle, chromosome imbalances (gain and loss of chromosome arms) (Lo et al, Neoplasia 2002).

Anti-cancer treatments aim to kill tumor cells. However, the consequences of the treatment-induced chromosome breaks could be dramatic for the stability of the genome of the surviving cells. In the present study, we analysed how chromosome lacking one telomere could interact with radiation-induced chromosome breaks. We show that radiation-induced chromosome breaks interact with the instability generated by spontaneous telomere loss inducing a huge instability. Such instability could permit the emergence of treatment-resistant subclones.

Telomerase deficiency promotes premature signs of renal aging in mice

G. Pérez-Rivero, M.P. Ruiz-Torres, M. Blasco and D. Rodríguez-Puyol

Renal aging is characterized by renal dysfunction, oxidative damage and fibrosis in mice. Mice deficient for telomerase RNA component, Terc(-/-), lack telomerase activity and show progressive telomere shortening with increasing mouse generations, eventually leads to telomere dysfunction and appearance of age-related pathologies from the third generation (G3) onwards. Renal function, oxidant/antioxidant balance in renal cortex and arterial pressure were evaluated in age-matched six-month old G1, G3 Terc(-/-) mice and wild type (WT) mice. Our findings show that G3 Terc(-/-) mice develop hypertension and renal dysfunction (low creatinine clearance, high level of plasmatic urea and low sodium fractional excretion) at 6 months of age. Oxidant/antioxidant balance was analysed in renal cortex by measuring the activity and mRNA expression of antioxidant enzymes, catalase, superoxide dismutase (both Cu-Zn and Mn-dependent isoforms) and gluthatione peroxidase, as well as the hydrogen peroxide production and the total oxidant capacity. G3 and G1 Terc(-/-)mice show decreased catalase activity and catalase mRNA expression, coincidental with higher SOD activity and SOD mRNA expression than WT mice. Hydrogen peroxide production and total oxidant capacity were increased in G1 and G3 Terc(-/-) mice compared to WT mice. As the result of this pro-oxidant status, oxidative damage was detected in proteins extracted from the renal cortex of G1 and G3 Terc(-/-) mice. Finally, TGB-beta1 and collagen I expression were increased in Terc(-/-) mice compared with WT mice. In conclusion, mice lacking telomerase activity show a premature renal dysfunction, characterized by increased oxidative damage and pro-fibrotic sings.

Human hematopoietic CD34-positive cells comprise different subpopulations that differ in their telomere length

Petriz J, García-Escarp M, Martínez-Muñoz, Barquinero J, Marín P

OBJECTIVE: Telomeres are nucleoprotein structures at the chromosomal ends, essential to maintain chromosomal integrity and genomic stability, and are critical for the proliferative activity of cells. Recent data point to the existence of different classes of human stem cells with variable self-renewal potential and short- or long-term repopulating capacity. These observations led us to investigate the telomere length in highly purified human hematopoietic CD34+ cells, obtained from bone marrow, cord blood, and from mobilized peripheral blood of healthy donors (n=14), and patients with multiple sclerosis (MS) undergoing autologous stem cell transplantation (n=6).

DESIGN AND METHODS: We have used flow cytometry-based fluorescent *in situ* hybridization (Flow-FISH) for the measurement of relative telomere length in highly purified preparations of CD34+ cells.

RESULTS: Analysis of the CD34+ cell samples showed a surprising heterogeneity in the relative telomere length. Strikingly, a very small fraction of cells with very large telomeres (median 0.39%; range 0.07-7.16%; n = 20) of the cells was systematically identified in all samples of purified CD34+ cells, but not in those containing non-selected cells. Mean values for five different CD34+ cell subpopulations on the basis of the telomere length increase were: 1/ 32.75 ± 24.99 kb, 2/ 2.39 ± 1.02 kb, 3/ 0.41 ± 0.13 kb, 4/ 0.10 ± 0.02 kb, and 5/ baseline. Average telomere length is 7.4 kb in peripheral blood CD34+ cells. The highest estimated telomere length of CD34+ cells was approximately 85 kb.

CONCLUSIONS: Our results lend support to previous observations on the heterogeneity of the stem cell compartment. Here we show that CD34+ cells comprise different subpopulations that differ in their telomere length. The finding of CD34+ cells with very long telomeres could provide new clues to the biology and the pathology of these intriguing cells.

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Short telomeres protect from diet-induced atherosclerosis in apolipoprotein E-null mice

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By imposing a replicative defect in most somatic cells, gradual telomere attrition during ageing is thought to progressively impair cellular function and viability and may contribute to age-related disease. Immune cells play important roles in all phases of atherosclerosis, a multifactorial disease that prevails within the elderly. Because shorter telomeres have been found in circulating blood leukocytes of human patients with advanced coronary atherosclerosis, it has been suggested that telomere shortening may predispose the organism to atheroma development. In this study, we assessed the impact of telomere attrition on atherogenesis induced by dietary cholesterol in apolipoprotein E (apoE)-deficient mice, a well-established model of experimental atherosclerosis that recapitulates important aspects of the human disease. Our study shows that late-generation mice doubly deficient in apoE and telomerase RNA experience telomere attrition and a substantial reduction of atherosclerosis compared with control mice with intact telomerase, in spite of sustained hypercholesterolemia in response to the atherogenic diet. Short telomeres impaired the proliferation of both lymphocytes and macrophages, an important step in atherosclerosis development. Therefore, telomere exhaustion resulting in replicative immunosenescence may serve as a mechanism for restrictingatheroma progression.

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Resistance to the short-term antiproliferative activity of the G-quadruplex ligand 12459 is associated with telomerase overexpression and telomere capping alteration

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Ligands that stabilize the telomeric G-rich single-strand DNA overhang into G-quadruplex can be considered as potential antitumor agents that block telomere replication. 12459, a potent G-quadruplex ligand that belongs to the triazine series has been previously shown to induce both telomere shortening and apoptosis in the human A549 cell line as a function of its concentration and time-exposure. We show here that A549 clones obtained after mutagenesis and selected for resistance to the short-term effect of 12459 frequently displayed hTERT transcript overexpression (2- to 6-fold). Overexpression of hTERT was also characterized in 2 resistant clones (JFD10 & JFD18) as an increase in telomerase activity, leading to an increase in telomere length. An increased frequency of anaphase bridges was also detected in JFD10 and JFD18, suggesting an alteration of telomere capping functions.

Transfection of either hTERT or DN-hTERT cDNAs into A549 cells did not confer resistance or hypersensitivity to the short-term effect of 12459 indicating that telomerase expression is not the main determinant of the antiproliferative effect of 12459. In contrast, transfection of DN-Htert cDNA into resistant JFD18 cells restored sensitivity to apoptotic concentrations of 12459, suggesting that telomerase does participate in the resistance to this G-quadruplex ligand. This work provides evidence that telomerase activity is not the main target for the 12459 G-quadruplex ligand but that hTERT functions contribute to the resistance phenotype to this class of agents.

Identification of putative homologues of telomere end binding proteins in plants

Pascale Rossignol, Peter Shaw and John Doonan

Telomere end binding proteins (TEBPalpha/beta) were first identified in *Oxytricha nova*. Proteins of this family were subsequently found in other organisms due to the presence of a conserved domain in the N terminus similar to TEBPalpha. Even though the level of similarity between the proteins is not really high, the proteins contain a conserved structure, the OB folds that are involved in DNA binding (Peersen et al. 2002). Proteins bound to the extreme end of the chromosome play an important role in telomere biology by protecting the chromosome end, in human and yeast. Deletion of POT1 in *S. pombe* for example, leads to DNA loss and chromosome circularisation (Baumann and Cech 2001).

Telomeric DNA and structure is conserved in plants, and, as in human, length of telomeres is mainly regulated by telomerase (Riha et al., 2001). Proteins involved in others aspect of telomere biology have also recently been characterised in plants and include Ku, ATM, Rad51 or Mre11 proteins (reviewed in Riha & Shippen 2003). Fundamental mechanisms of telomere biology seems to be conserved in plants suggesting that plants could be a model to study telomeres and that the results found could be applied to other organisms. However, many putative telomeric genes appear to have diverged in plants and still little is known on plant telomeric structure and on the role of single strand binding proteins in telomere length and maintenance.

To characterise the role of these proteins in *Arabidopsis thaliana*, we used the conserved domain located in the N-terminus of TEBPalpha to perform blast searches. Two putative homologues of this protein were found in Arabidopsis: AtPOT1;1 and AtPOT1;2. The N-termini of the proteins show a low percentage of identity/similarity (26/47% and 26/42%) when use for a pairwise alignment. Using a 3D-PSSM server, OB folds conserved domains could also be detected. Moreover, 3 domains presenting an homology to the ciliate/non ciliate proteins were determined, 2 in the N terminus overlapping the OB folds of the alpha subunit and 1 in the C terminus overlapping the OB fold of beta subunit of *O. nova*. Since no homologues of the beta subunit have been found in non-ciliate organisms, this suggests that the functions of the ciliate subunits are provided by a single protein in higher eukaryotes (Pitt et al., submitted).

The corresponding AtPOt1;2 gene was cloned in fusion with GFP under the control of a 35S promoter, and the pattern of localisation assessed in plant cells. AtPOT1;2 fused to GFP is localised to both nucleus and cytoplasm in living cells. When treated with detergent, the signal remained in the nucleus. Immuno-localisation should allow us to define the location and the dynamics of the protein. At the same time, RNAi lines are also being constructed to

investigate the function of these proteins in plants. To understand the role of POT1 proteins, members of the complex should be identified, so we have undertaken a screen of a two hybrid library to identify interactors of AtPOT1;2. This study should allow us to identify other proteins involved in telomere maintenance.

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Telomere length is reset during early embryogenesis

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The telomerase enzyme is active in germ cells and during early embryogenesis1-5 and is necessary to maintain telomere length and germ cell viability in successive generations of a species6,7. In this study we demonstrate a telomere lengthening program at the morula-blastocyst transition in mice and cattle that establishes a specific telomere length set point. In studies of cloned embryos, we show that this process restores telomeres to the same length regardless of the telomere length of donor nuclei. We demonstrate that telomere elongation at this stage of embryogenesis is telomerase dependent since it is abrogated in telomerase deficient mice. These data show that the early embryo has a telomerase dependent genetic program that elongates telomeres to a defined length, possibly necessary to ensure sufficient telomere reserves for species integrity.

In silico and biophysical screening of small-molecule databases for compounds binding human telomeric G-quadruplexes

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The activation of the holoenzyme telomerase is one of the key events in the oncological transformation of human cells. In recent years, inhibition of telomerase has thus become established as a promising potential strategy for anti-cancer drug development. The single-stranded, G-rich telomeric DNA substrate of telomerase can fold in a variety of ways to form higher order G-quadruplex structures, which may have an important role in the maintenance and protection of chromosome ends. Compounds designed to interact selectively with quadruplexes have been shown to result in the inhibition of telomerase, and work in our group and others has consequently resulted in the development of compounds that inhibit normal telomerase function in the nanomolar range by the stabilisation of such quadruplexes. The crystallographic determination by us of a novel folding topology for quadruplexes formed from the human telomeric repeat sequence enables the application of rational drug design approaches to the development of small molecules targeting telomeric DNA that will have specificity for the unique loop and other structural features of human quadruplexes. Identification of potential sites of interaction and in silico screening of small-molecule databases has thus yielded compounds that are predicted to possess favourable binding properties. The development of compound series has furthermore allowed the establishment of clear structure-activity relationships describing quadruplex-drug interactions, and the use of the non-cell based TRAP (telomeric repeat elongation protocol) assay has enabled us to gain insights into the extent to which our in vitro assays allow the prediction of enzyme inhibition. We will also report on future plans to characterize the biochemical pathways involved upon treatment with our compounds, thereby making the connection between *in vitro* assays and the cellular response observed in long-term tissue culture experiments.

Repression of telomerase by retinoids, a potential new cancer therapy

Evelyne Ségal-Bendirdjian*, Frédéric Pendino, Charles Dudognon, François Delhommeau, Tewfik Sahraoui, Maria Flexor, Annelise Bennaceur-Griscelli, Michel Lanotte

Natural retinoids currently used to treat various human cancers, including leukemia, skin cancer, cervical cancer, and neuroblastoma, activate gene transcription via nuclear retinoic-acid receptors (RARs) and retinoid-X receptors (RXRs). Their use in differentiation-therapy of acute promyelocytic leukemia (APL) represents a model concept for reprogramming cancer cells. However, they also regulate antiproliferative genes whose functions do not mechanistically concur to this program. We have recently shown that in a maturation-resistant APL cell line, NB4-LR1, pharmacological concentration of ATRA exhibits antiproliferative properties, independent of terminal maturation, through down-regulation of telomerase leading to telomere shortening and cell death. This induction of cell death by telomerase repression requires both a telomerase inhibition and a retinoid signaling threshold.

To further gain insights into the mechanisms by which ATRA represses Htert expression, we examined in this study the effects of receptor selective retinoids, alone or in combination in two different types of maturation-resistant APL cells.

We showed that telomerase repression is obtained only when both RARa and RXR agonists were used in association. Importantly, unlike ATRA, this synergy was obtained at very low agonist concentrations and occurred in other ATRA maturation-resistant APL cells. Although the precise mechanisms remain to be elucidated, these findings provide the first demonstration that dual-liganded RXR and RARa signaling should allow efficient targeting of telomerase in differentiation-resistant tumor cells. Such a combination therapy might hold promise in clinic to avoid side effects of ATRA whose administration can indiscriminately activate all RARs. Given the tissue-specific expression of RARs, a tissue-selective therapy targeting telomerase in tumor cells by synthetic agonists can be envisioned.

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Generation of MEFs that activate the INK4a/ARF locus after ~80 population doublings

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Mouse embryo fibroblasts (MEFs) undergo cellular senescence after the accumulation of cellular doublings under standard *in vitro* culture conditions. The establishment of senescence in MEFs is known to require at least two independent components: oxidative stress, which is intrinsic to the standard oxygen tension; and a functional INK4a/ARF locus, which becomes progressively upregulated with the accumulation of cell doublings. Despite the critical importance of the INNK4a/ARF locus in senescence and tumor suppression, the mechanisms involved in the upregulation of the INK4a/ARF locus are still largely unknown.

We have modified genetically the activity of the INK4a/ARF locus in the mouse germline. In particular, we have generated transgenic mice carrying a large intact genomic segment of about 75 kb containing the entire INK4a/ARF locus. This ectopic INK4a/ARF transgene will be referred to as "tg". After the appropriate crosses, we have obtained mice carrying the "tg" allele in a genetic background that is null for the endogenous INK4a/ARF locus. This has allowed us to analyze the behavior of the "tg" allele in isolation. Interestingly, compared to the endogenous loci, the "tg" allele has a significantly delayed kinetic of upregulation upon serial cultivation *in vitro*. In particular, whereas the normal endogenous locus is fully upregulated at passage ~5 (10-15 doublings), the "tg" allele only reaches full upregulation at passage 30-40 (70-80 doublings). The delayed upregulation of the "tg" allele is probably due to a position effect or to the absence of regulatory elements that are not present in the transgene. In this manner, the "tg" allele provides a unique opportunity to test the role of the INK4a/ARF locus in establishing the timing of senescence.

We will present a complete characterization of the senescence, immortalization, and transformation properties of MEFs INK4a/ARF(-/-;tg/tg). In addition, we have extensively studied the tumor suppression activity of the "tg" allele, thus allowing us to draw conclusions on the correlate between *in vitro* senescence potency and *in vivo* tumor suppression.

Association of RAD51D recombination/repair protein with telomeres

Madalena Tarsounas and Steve West

Homologous recombination (HR) provides an important pathway for double-strand break repair. Additionally, HR may be involved in two aspects of telomere maintenance: (i) an alternative pathway for telomere elongation in yeast cells lacking active telomerase (ALT, for alternative lengthening of telomeres), and (ii) the formation of a specialized telomeric structure called the T-loop.

The RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3) are essential for homologous recombination, and for normal levels of resistance to DNA damaging agents. Using immunofluorescence labelling, we have shown that one member of this family, RAD51D, localizes to the telomeres of both meiotic and somatic cells. Moreover, RAD51D binds telomeric DNA in both HeLa and testis cells, as detected by chromatin immunoprecipitation assays. *In vitro* electrophoretic mobility shift assays show that RAD51D binds to ssDNA and has high affinity for the G-rich strand of telomeric DNA. The inhibition of RAD51D synthesis in cultured cells by RNA interference results in a lethal phenotype. These observations suggest that RAD51D plays a dual cellular role, acting to promote the recombinational repair of DNA damage, and to maintain telomeres through direct interactions with telomeric sequences.

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