REVIEW

Telomeres and telomerase in adrenocortical tissue maintenance, carcinogenesis, and aging

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Abstract

Telomere dysfunction and telomere maintenance mechanisms contribute to major steps of carcinogenesis. Dysfunctional telomeres lead to the generation of genomic aberrations, such as amplifications and deletions. Telomere maintenance mechanisms, such as telomerase activity and alternative telomere lengthening, provide the basis of malignant cell expansion independent of telomere shortening-induced apoptosis or senescence, ensuring tumor survival. Recent advances highlight the importance of these mechanisms in adrenocortical carcinogenesis. In this review, we will summarize the main models of telomere physiology and their impact on adrenocortical tissue maintenance, aging, and carcinogenesis.

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Overview

Mammalian chromosomes end in a stretch of TTAGGG nucleotide repeats known as telomeres. There are two key challenges inherent to these structures. First, telomeres need to be protected from being recognized as a form of damaged DNA by the DNA surveillance and from being processed by the DNA repair machinery. Second, the semi-conservative DNA replication machinery does not entirely copy linear chromosomes, leading to a loss of terminal sequences and telomere shortening over consecutive cell divisions in the absence of a specific mechanism to restore these sequences. Physiological processes have evolved to meet these challenges. The recognition of telomeres as double strand breaks (DSBs) is prevented by a specialized DNA structure and a dedicated complex of proteins bound to the telomere. Cells reaching a critically short telomere length or harboring otherwise dysfunctional telomeres are effectively removed from the pool of proliferating cells through induction of apoptosis or senescence. Dysregulation of either of these protective processes can lead to acquisition and maintenance of a malignant phenotype. Malignant transformation as well as ongoing acquisition of malignant characteristics can be initiated by the accumulation of genomic alterations caused by telomere dysfunction-induced breakage fusion bridge

cycles (BFBs). An indefinite potential of expansion of malignant cells can be secured by the acquisition of telomere length maintenance mechanisms (TMMs), which prevent telomere shortening, either telomerase activity (TA)-dependent or TA-independent alternative mechanisms of telomere lengthening (ALT).

Here, we will give insight into the current understanding of how these problems are solved physiologically and how they are exploited by malignant cells, specifically those of adrenocortical origin. We will focus on how telomere-protective mechanisms under normal physiological circumstances prevent the accumulation of chromosomal aberrations, how defects in these processes lead to the acquisition of a pro-cancer genome, and how TMMs ensure tumor survival. We will summarize what implications these mechanisms bear for adrenocortical cancer (ACC) and discuss implications of telomere physiology in tissue maintenance and aging of the adrenal cortex.

Telomere physiology, telomere protection, and telomere dysfunction

It has long been known that human fibroblasts cease to divide after ~50 cell divisions, a limit known as the Hayflick limit (Hayflick & Moorhead 1961, Hayflick 1965). One reason for this is believed to be the

Figure 1 Model of telomere shortening and telomerase activity. (A) Telomere shortening. A 3'-overhang is created by the lagging strand synthesis after excision of the RNA primer resulting in a shortened telomere. 3'-synthesis of the leading strand can be carried to completion by the DNA polymerase. A 3'-overhang is created by a yet unidentified mechanism, most likely a 5'-exonuclease. After a certain number of cell divisions, telomeres become critically short and induce cellular senescence or apoptosis. (B) Telomere maintenance. Telomerase, consisting of the protein (TERT) and RNA (TERC) subunit extend telomeres by adding telomeric repeats (TTAGGG) in the 3'-direction. The DNA polymerase can then synthesize the lagging strand. This process can maintain telomere length or lead to telomere lengthening.

progressive shortening of telomeres, a mechanism first theoretically proposed by Olovnikov in the early 1970s of the last century (Olovnikov 1973). Due to the inefficiency of the semi-conservative replication carried out by DNA polymerases using RNA primers and the action of putative telomere-processing nucleases, short stretches of DNA at the chromosome ends get lost with each replication and eventually reach a critically short, dysfunctional state (Fig. 1A; Harley *et al.* 1990, Levy *et al.* 1992). Cells that must continue to divide for many generations, such as stem cell compartments within the skin, hematopoietic system, and male germ cells, prevent this shortening by the activity of the ribonucleoprotein enzyme telomerase (Taylor *et al.* 1996, Weng *et al.* 1996, Wright *et al.* 1996). Telomerase consists of two subunits, a protein component (TERT) that has reverse transcriptase activity and a ribonucleotide subunit (TERC) that harbors a template for TTAGGG telomeric repeats (Greider & Blackburn 1987, Morin 1989). This enzyme adds telomeric TTAGGG sequences to the end of the telomere in the 5'-3' direction and subsequently DNA polymerases synthesize the lagging strand (Fig. 1B). TA is also an important marker of human embryonic stem cells (Thomson *et al.* 1998). Early in human adrenocortical development, *TERC* expression is restricted to the very outer zone of the

emerging organ, probably marking the (prenatal) stem cell compartment (Yashima *et al.* 1998).

It is worthwhile to mention that murine cells display considerable differences in TA and telomere physiology. Rodent cells have much longer telomeres, TA is almost ubiquitously present in mouse organs, murine mouse embryonic fibroblasts (MEFs) undergo a stage of crisis as early as after eight to ten population doublings, and the progression to senescence is believed to be mainly driven by the accumulation of ongoing DNA damage as it can be prevented by low oxygen culture conditions (Kipling & Cooke 1990, Prowse & Greider 1995, Parrinello *et al.* 2003, Itahana *et al.* 2004).

Telomeres, the outer ends of chromosomes, by definition harbor an open end of DNA. This open end needs to be protected from being recognized by the very efficient DNA surveillance and repair machinery, which is charged with the tasks of ensuring integrity of the genome and removing cells with damaged DNA from a population. Indeed, eukaryotic cells have the ability to distinguish the end of a linear chromosome from a DSB by two interdependent mechanisms: 1) telomeres form a specialized DNA structure and 2) telomeres are tightly bound by a protein complex, the shelterin complex (Fig. 2A and B). Telomeres form a loop structure (T-loop) in

Figure 2 (A) Multiple shelterin complexes can be found at the telomere DNA T-loop and most likely exist in different compositions to serve the multiple functions of the telomere, protect it from recognition and processing by the DNA surveillance machinery. (B) Model of the telomere cap complex (shelterin complex) bound to the end of the telomere. (C) Data from a gene expression array (IDs refer to the HG-U133 Plus 2 array) of adrenocortical tumors. Fold change is expressed as ACC versus ACA and NL as one group. *TERT*, *POT1*, *TPP1/ACD*, and *TIN2* are significantly differently expressed in malignant versus non-malignant adrenocortical tissues. Official gene symbols are used in the array: *POT1*, protection of telomeres 1; *ACD*, adrenocortical dysplasia homologue (*TPP1/ACD*); *TERT*, telomerase reverse transcriptase; *TERF2IP*, TERF2-interacting protein (*RAP1*); *TERF2*, telomeric repeat-binding factor 2 (*TRF2*); *TERF1*, telomeric repeat-binding factor 1 (*TRF1*); *TINF2*, TRF1-interacting nuclear factor 2 (*TIN2*).

which the 3'-overhang inserts into double-stranded telomeric DNA (Griffith *et al.* 1999). Six different core proteins binding either to telomeric DNA or serving as interconnectors between DNA bound proteins form the shelterin complex (de Lange 2005). TRF1 and TRF2 bind to double-stranded telomeric TTAGGG repeats, while POT1 also binds to the single-stranded 3'-overhang (Chong *et al.* 1995, Broccoli *et al.* 1997, Baumann & Cech 2001). The remaining three components of this complex bind to DNA-bound factors and potentially form several possible configurations, which are proposed to serve different functions (Liu *et al.* 2004a). RAP1 binds to TRF2, TIN2 binds to TRF1 and TRF2, and TPP1/ACD serves as an interconnector between TIN2 and POT1 (Kim *et al.* 1999, Li *et al.* 2000, Houghtaling *et al.* 2004, Liu *et al.* 2004b, Ye *et al.* 2004a,b). The telomeric DNA-protein complex has a dual function: it protects telomeres from being recognized as DSBs and regulates telomerase access to the telomere (de Lange 2005).

Dysfunctional telomeres, either critically short or lacking shelterin components, activate DNA damage-signaling pathways that ultimately lead to cellular senescence or apoptosis (Bodnar *et al.* 1998, Lee *et al.* 1998, Karlseder *et al.* 1999). These pathways exploit the same mechanisms that recognize DNA breaks and induce stalling of the cell cycle and potentially repair of the lesion (Artandi & Attardi 2005). Deprotected telomeres can be visualized in cells as telomere dysfunction-induced foci (TIFs) by co-staining for

factors such as γ H2AX and p53BP together with telomere *in situ* hybridization using telomere-specific peptide nucleic acid probes (Takai *et al.* 2003). The recruitment of these immediate DNA damage factors leads to the activation of ATM/ATR, which in turn leads to p53 activation and increased levels of p21 (Brown *et al.* 1997, Karlseder *et al.* 1999). A parallel pathway leads to the expression of *p16/INK4A*, which in human but not murine cells leads to a cell cycle arrest (Jacobs & de Lange 2005). These pathways ultimately lead to the withdrawal of cells harboring critically short or damaged telomeres from the pool of proliferating cells via apoptosis or senescence. Therefore, telomere damage signaling-induced senescence and apoptosis can be regarded as a cellular defense mechanism against malignant transformation, preventing the acquisition of genomic aberrations (Artandi & Attardi 2005, Ju & Rudolph 2006, Deng *et al.* 2008). Although the mechanism that determines *in vivo* the preference for induction of apoptosis or senescence is not well understood, there is evidence for cell type dependence, e.g. some epithelial cells and lymphocytes tend to preferably undergo apoptosis and fibroblasts preferably progress to senescence (Karlseder *et al.* 1999).

Telomere dysfunction-induced apoptosis and senescence function to prevent the accumulation of genomic aberrations. Telomere dysfunction in *p53* proficient cells leads to cell cycle arrest, senescence, and apoptosis (Karlseder *et al.* 2003). By contrast, in the setting of *p53* deficiency, dysfunctional telomeres are processed by

Figure 3 Model of genomic shuffling by breakage fusion bridge (BFB) cycles. Dysfunctional telomeres (absent red circles) fuse and form dicentric chromosomes. During cell division, the fused chromosomes are pulled to the two different poles of the emerging daughter cells. During anaphase these can be observed as anaphase bridges, chromosomal material spanning from one daughter cell to the other. Eventually, a break occurs and creates another open end, which can serve as a new starting point for a subsequent BFB. Ultimately, this process leads to genomic amplifications and deletions.

the DNA repair machinery (Attardi 2005). Dysfunctional telomeres are processed via end-to-end fusions to form dicentric chromosomes. In accordance with this mechanism, dicentric chromosomes have been found in telomerase-deficient cells as well as in MEFs from *Pot1*-deficient animals and from adrenocortical dysplasia (*acd*) mice, which harbor a mutation in the *Tpp1/Acd* gene (Hande *et al.* 1999, He *et al.* 2006, Hockemeyer *et al.* 2006, 2007, Wu *et al.* 2006, Else *et al.* 2007). Dicentric chromosomes lead to an oncogenic mechanism of genome shuffling, which is known as breakage fusion bridge (BFB) cycles (Fig. 3; Artandi *et al.* 2000, Lo *et al.* 2002, Murnane & Sabatier 2004, Murnane 2006). Over subsequent cell divisions, the two free ends of a dicentric chromosome are pulled to the different poles of the emerging daughter cells. In tissue sections and cell culture, these structures can be observed as anaphase bridges, chromosomal material spanning the nuclei of the two emerging daughter cells. Interestingly, this morphological correlate was observed in malignant tumors as early as 1891 (Hansemann 1891). These bridges of chromosomes will eventually break and newly expose open ends that can serve again as starting points for a BFB. The break does not necessarily take place at the former fusion site, but can take place anywhere in the chromosome, although it is likely that specific ‘weak’ areas exist, which are predisposed to serve as a breakage site. This process leads to translocations, regional genomic amplifications, and genomic losses that can

be visualized by spectral karyotyping and estimated by comparative genomic hybridization (Artandi *et al.* 2000, O’Hagan *et al.* 2002, Else *et al.* 2007). BFBs can serve as a mutagenic mechanism leading to the amplification of oncogenes and the loss of tumor suppressor genes. It is important to keep in mind that under normal circumstances cells harboring dysfunctional telomeres, which can serve as initiators of BFBs, are efficiently removed by apoptosis or senescence. Most models examining the sequelae of dysfunctional telomeres, therefore, make use of cellular and animal models deficient in the major components of the DNA damage-signaling machinery, such as p53 (Fig. 4). It is worthwhile mentioning that TA or TMMs (as opposed to their function described below) can act as a cancer preventive mechanism in this model. At least in the case of telomere shortening-induced telomere dysfunction, TMMs can obviate the occurrence of dysfunctional telomeres as starting points for BFBs (Hornsby 2007).

Dysfunctional telomeres in carcinogenesis and ACC

As described, dysfunctional telomeres, either short or decapped, can result in genomic alterations through BFBs over multiple cell divisions. Evidence that dysfunctional telomeres lead to increased carcinogenesis stems from animal models, mainly using late generation *TERC*-deficient mice, which develop

Figure 4 Telomere-focused model of tumorigenesis. Dysfunctional telomeres, either as a consequence of telomere decapping or resulting from telomere shortening, will be recognized by the DNA surveillance machinery and induce senescence or apoptosis (telomeres are shown as red circles). In the setting of checkpoint deficiency, such as *p53* deficiency, chromosomes may fuse and start BFBs, leading to a shuffling of the genome and genomic amplifications and deletions. This first step generates a pro-cancer genome. In a second step, chromosomes are stabilized by telomere length maintenance mechanisms (either TA or ALT) and the emerging cancer cells acquire independence of telomere shortening-induced senescence or apoptosis.

critically short telomeres due to the absence of TA (Blasco *et al.* 1997). Breeding these mice to a *p53*-deficient background results in a significant increase in tumor incidence compared with *p53* deficient, but telomerase-proficient mice (Chin *et al.* 1999). Two human syndromes underscore the role of telomere dysfunction in human carcinogenesis: dyskeratosis congenita (DC) and Li-Fraumeni syndrome. DC patients whose genome harbors mutations in genes encoding telomerase (*TERC* or *TERT*), shelterin components (*TIN2*), or a telomerase-processing protein (*DKC1*) are predisposed to develop early cancers, presumably induced by critically short dysfunctional telomeres (Heiss *et al.* 1998, Vulliamy *et al.* 2001, Armanios *et al.* 2005, Savage *et al.* 2008). The majority of Li-Fraumeni syndrome is caused by *p53* mutations (Malkin *et al.* 1997). It is noteworthy that ACC is one of the common syndrome-defining malignancies (Li *et al.* 1988). A recent study links carcinogenesis in Li-Fraumeni syndrome to telomere shortening (Tabori *et al.* 2007). In family analyses of affected and unaffected *p53* mutation carriers, telomere length was significantly shorter in patients who develop malignancies, arguing for an involvement of telomere dysfunction in carcinogenesis in Li-Fraumeni syndrome (Tabori *et al.* 2007, Tabori & Malkin 2008).

Some recent reports suggest the participation of genes encoding shelterin complex components in spontaneous human malignancies. Studies focusing on B-CLL found expression levels of *TRF1*, *RAP1*, and *POT1* significantly decreased and of *TPP1/ACD* significantly increased (Poncet *et al.* 2008). In gastric cancer, *POT1* expression levels correlate with tumor stage (Kondo *et al.* 2004). While these studies, as well as those focusing on other malignancies, do not display a common trend of up- or downregulation of single shelterin components (Bellon *et al.* 2006, Lin *et al.* 2006, Salhab *et al.* 2008), they show a common scheme of dysregulation of the complex. Recently, several series of gene expression arrays have been published, including some of ACC. In the dataset generated by Giordano *et al.* *POT1* and *TPP1/ACD* are expressed at significantly higher and *TIN2* at significantly lower levels in ACC vs ACA and normal adrenocortical tissue (*POT1* 1.6-fold, *TPP1/ACD* 1.6-fold, and *TIN2* 0.75-fold; all $P < 0.001$; Fig. 2C, Giordano *et al.* 2009).

Telomere length maintenance mechanisms in ACC

TMMs are regarded as crucial to ensure tumor survival. Roughly, 90% of human malignant tumors exploit the mechanism of TA to gain potential for indefinite clonal expansion without telomere shortening-induced apoptosis or senescence (Kim *et al.* 1994). Following

the description of this phenomenon, a few malignant tumor entities emerged that did not display any considerable TA, but yet did not undergo crisis or progressed to senescence (Bryan *et al.* 1997). Analyses showed that these cell clones established ALT. The molecular bases of ALT are not well understood, but certain surrogate parameters correlate well with the presence of ALT. One parameter is stable, usually very long, telomeres with a wide range of length distribution. Another one is the immunocytochemical co-localization of very bright telomere *in situ* hybridization signals together with promyelocyte leukemia (PML) bodies (Bryan *et al.* 1997). The underlying mechanism of ALT is currently thought to be based on homologous recombination between telomeres (Muntoni & Reddel 2005).

Most human cancers use TA as the primary TMM, but a significant percentage of certain tumor entities, such as liposarcomas or glioblastomas, have been shown to be telomerase negative (Hakin-Smith *et al.* 2003, Johnson *et al.* 2005, Costa *et al.* 2006). A considerable number of these tumors use ALT as their main TMM and in some tumors signs of both TMMs can be found. The data regarding TMMs in ACCs have been inconsistent, which is most likely due to differences in tissue collection, tissue storage, and analyses methods (Hirano *et al.* 1998, Kinoshita *et al.* 1998, Teng *et al.* 1998, Bamberger *et al.* 1999, Mannelli *et al.* 2000). It seems to be undisputed that a considerable number of ACCs are positive for TA (Orlando & Gelmini 2001). Some studies also found TA in benign adrenocortical adenomas (Mannelli *et al.* 2000, Orlando & Gelmini 2001). Interestingly, the first reports investigating ALT in different tumor cells included some ACCs, but, unfortunately, did not give any further pathological or clinical details (Bryan *et al.* 1997). As a result of our interest in exploring possible ALT mechanisms in ACC, we have recently carried out a survey of TMMs in adrenocortical benign and malignant tissues (Else *et al.* 2008). Tissue samples were obtained by a microdissection technique, collecting areas of morphologically proven tumor cells within frozen embedded tissues. Interestingly, virtually, all malignant samples tested in this study displayed evidence for at least one TMM, with the majority employing TA (79%), a minority using both TA and ALT (8%), and a small number displaying only surrogate parameters of ALT (4%). None of the normal adrenal tissues or benign adrenocortical adenomas showed any signs of TMMs. All of the available ACC cell lines (SW13, RL251, NCI-H295R, and NCI-H295A) are positive for TA (unpublished results). NCI-H295R and NCI-H295A also display surrogate parameters for ALT, indicating a use of both mechanisms (Else *et al.* 2008). Although this study, like other previous reports, was entirely descriptive, one can conclude that the presence of TMMs is a special

characteristic of malignant lesions and might further speculate that ACCs depend on the presence of a TMM to preserve a malignant phenotype.

Serial tumor cell transplantation experiments underscore the importance of TMMs for maintaining malignant behavior (Sun *et al.* 2004). SV40 large T antigen and RAS-transformed bovine adrenocortical cells lose their malignant potential over the course of serial transplantations of tumor cells into immunodeficient mice. Malignant behavior can be restored by introduction of TA (Sun *et al.* 2004).

Shelterin components and tissue homeostasis and development

All attempts to create mice completely deficient in any of the shelterin complex proteins, including Tin2, Trf1, and Trf2, have resulted in lethality early in development (Karlseder *et al.* 2003, Chiang *et al.* 2004, Celli & de Lange 2005). *Pot1* in the murine genome is considerably different as surprisingly two genes, *Pot1a* and *Pot1b*, encoding two homologous proteins were found (Hockemeyer *et al.* 2006, Wu *et al.* 2006). While double knock-out mice share the phenotype of early lethality, *Pot1b*-deficient mice survive to adulthood (Hockemeyer *et al.* 2006). Therefore, most of our knowledge of the consequences of telomere dysfunction in adult murine life stems from *Terc*- and *Tert*-deficient mice, from the conditional *Tif2*-deficient mouse and from one spontaneous mouse mutant, deficient in *Tpp1/Acd*, the so called adrenocortical dysplasia (*acd*) mouse (Lee *et al.* 1998, Liu *et al.* 2000, Lazzerini Denchi *et al.* 2006, Else *et al.* 2007). The *acd* mouse arose spontaneously on the background of the control strain (DW/J) for the pituitary dwarf mutation and it shares many phenotypic features with late generation *Terc*- and *Tert*-deficient mice (Beamer *et al.* 1994, Keegan *et al.* 2005). All three mutant strains display disturbed fur maintenance, are small and subfertile due to severe reduction in male germ cells (Lee *et al.* 1998, Liu *et al.* 2000, Keegan *et al.* 2005). The main difference between these animals is that the *acd* phenotype is apparent in the first generation, while *Terc*- and *Tert*-deficient mice must be bred over at least four to five generations to appreciate this phenotype. The molecular phenotype of dysfunctional telomeres in *Terc*- and *Tert*-deficient mice is acquired over multiple cell divisions and depends on the occurrence of critically short telomeres, while cells from *acd* mice show telomere dysfunction due to a severe deficiency of the shelterin component protein, Tpp1/Acd, already in the first generation. This reflects the two different molecular mechanisms by which telomere dysfunction is thought to be induced in these animal models. In the absence of telomerase telomeres reach a critical length

and become dysfunctional by decreased binding of shelterin components to the telomere, while telomere deprotection in Tpp1/Acd deficiency is immediately evident and does not depend on telomere shortening. A possible explanation for the absence of early embryonic lethality in *acd* mice is the hypomorphic character of the *acd* allele. The *acd* mutation is a splice site mutation and, as often is the case with naturally occurring mutants, either some wild-type protein is produced or some level of function is preserved in the protein leading to an amelioration of the phenotype. While translation of a functional protein from the mutated RNA is very unlikely for the *acd* allele, we were able to detect minimal amount of *wtTpp1/Acd* mRNA in *acd* MEFs. Presumably, this RNA originates from the usage of the 'original' splice site and the amount of translated *wt* protein is sufficient to bypass early lethality and allow some *acd* mice to survive to adulthood (Keegan *et al.* 2005). Although *acd* mice share common features with late generation mice lacking TA, there are some distinct differences. Interestingly, *acd* mice show a peculiar morphology of the adrenal cortex resembling human adrenal hypoplasia congenita (AHC) with cytomegaly. The adrenal cortex and medulla are not as clearly separated as in normal adult mice and, most strikingly, the adrenal cortex consists of large cells with a large eosinophil cytoplasm and cytomegalic pleomorphic nuclei that harbor eosinophilic nuclear inclusion bodies. There are at least two explanations for this particular morphology occurring in *acd* mice, but not in late generation *Terc*-deficient mice. First, as evidenced in the liver-specific inducible knock out of another shelterin protein, *Trf2*, telomere fusions can result in endoreduplication and development of hyperploidy (Lazzerini Denchi *et al.* 2006). This explanation, however, would not explain why this phenotype is not observed in late generation *Terc*-deficient mice unless one assumes that telomere dysfunction in *acd* mice is different or less/more severe than telomere dysfunction induced by critically short telomeres. Another possibility is that cytomegaly may result from lack of an adrenal cortex-specific function of Tpp1/Acd, independent of its role in telomere physiology. At first glance this possibility seems to be unlikely, as no significant functions other than telomere regulation have been shown for shelterin components. However, recent data show that TERT, independent of its role in telomere physiology, participates in cellular signaling pathways and may impact Wnt signaling, a pathway crucial for adrenal development and tissue maintenance (Sarin *et al.* 2005, Choi *et al.* 2008, Kim *et al.* 2008, Venteicher *et al.* 2008).

AHC with cytomegaly has been well described in human pathologies. It is the pathomorphological hallmark of *DAX1* mutations, Beckwith–Wiedemann syndrome, and IMAGe syndrome, but it also occurs

spontaneously as shown by analysis of pediatric autopsies (~0.8%; Irving 1967, Favara *et al.* 1991, Zanaria *et al.* 1994, Vilain *et al.* 1999, Tan *et al.* 2006). In a recent study of IMAGE syndrome patients, no mutation in *TPP1/ACD* could be found, but other genes encoding shelterin complex members have not been examined (Hutz *et al.* 2006). One could speculate that some of the spontaneous cases of AHC with cytomegaly could be attributed to mutations leading to telomere dysfunction. AHC with cytomegaly may also represent a morphological correlate of cellular senescence. Indeed, cytomegalic cells in the *acd* adrenal cortex stain positive for several senescence-associated markers (unpublished data). AHC with cytomegaly may resemble the common morphological endpoint of several mechanisms, such as stem cell exhaustion.

Telomeres, apoptosis, and senescence in adrenocortical physiology and aging

Apoptosis and senescence can be induced by a myriad of stimuli. Apoptosis occurs under physiological circumstances in the adrenal gland at the border between cortex and medulla. The general paradigm of replenishment of the adrenal cortex starts with cell replacement from a putative peripheral stem cell zone, followed by an inward directed cell displacement and cell death at the innermost part bordering the adrenal medulla (Zajicek *et al.* 1986, Kim & Hammer 2007). Therefore, it is reasonable to regard the observation of apoptosis in this area as a physiological mechanism of a constantly renewing organ. This mechanism is most likely independent of telomere dysfunction or shortening. Senescence in the adrenal cortex is less well understood, mainly due to the absence of reliable markers for senescent cells in general and in the adrenal cortex in specific.

The adrenal cortex undergoes significant age-related changes at the organ level. With age the zona reticularis disappears and dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) production decreases (Hornsby 1995). Cellular senescence may contribute to this process as summarized in a recent review (Hornsby 2002). Assuming absent or insufficient TA in the adult human adrenal cortex, one might expect an age-related decrease in telomere length and, therefore, telomere dysfunction in the adrenal cortex, specifically in the self-renewing stem cell compartment. Recently, it has been shown that telomere length in adrenocortical cells is inversely correlated with donor age (Yang *et al.* 2001). Therefore, over time one would expect that the adrenal cortex would lose its stem or progenitor cells due to critical telomere shortening and dysfunction. These processes would be expected to take place in the peripheral stem cell zone. Under the circumstance of a decreased number of organ-specific

progenitor cells, preservation of the vital functions of the outer zona glomerulosa (=mineralocorticoid production) and zona fasciculata (=glucocorticoid production) may be ensured at the expense of the less vital functions of the innermost zona reticularis (=DHEA production). Senescent cells may also influence the steroidogenic profile through secreted senescence-associated factors. Recent microarray analyses have shown a distinct gene expression profile associated with senescence, including expression of some secreted factors (Shelton *et al.* 1999).

Senescence and stem cell aging are influenced not only by telomere shortening but also by ongoing accumulation of DNA damage, which may be more prominent at the telomere than other DNA sites (Richter & von Zglinicki 2007). Reactive oxygen species have been implicated in age-associated changes in other steroidogenic tissues, such as Leydig cells and one might speculate that free radicals emerging as intermediate products of the process of steroidogenesis contribute to telomere and DNA damage and, in turn, to aging of the adrenal cortex (Hanukoglu 2006, Midzak *et al.* 2009). Overall, it is an interesting theory that the process started by cellular aging (cellular senescence) spreads via organ aging (loss of zona reticularis) to the organismal endocrine level via ceasing DHEA production (Fig. 5).

Senescence in general may also serve as a tumor-defensive mechanism in the adrenal cortex. Benign lesions of the adrenal cortex, nodules, and adenomas are very common, but malignant lesions are extremely rare (Dobbie 1969, Mansmann *et al.* 2004). Recent advances in our understanding of melanocytic lesions have showed that melanocytes in common benign naevi undergo oncogene-induced senescence by the acquisition of *BRAF* mutations (Michaloglou *et al.* 2005). A parallel mechanism may prevent a higher incidence of progression of commonly observed adenomas or nodules to malignant tumors in the adrenal cortex.

Future directions of research: therapeutic and diagnostic application

Telomere dysfunction and TMM can cause initiation and maintenance of a malignant phenotype respectively. Current research has led to a model in which both processes play important roles in major events of carcinogenesis (Fig. 4). As outlined in this review, there is emerging evidence that this model holds true for adrenocortical neoplasms. The understanding and analyses of these mechanisms will further increase our knowledge of the participation of these processes in spontaneous human tumors and hereditary tumor syndromes, and, more importantly, may advance diagnosis and may facilitate the development of new therapies.

Figure 5 Proposed model of the aging adrenal cortex. This hypothesis links cellular aging/senescence to organ and organismal aging. The proposed stem cell compartment of the adrenal cortex (yellow circles) can be found in the periphery of the gland. As the normal adult adrenal cortex does not have any telomere length maintenance mechanisms, telomeres shorten over time and multiple cell cycles and lead to cellular senescence in the stem cell compartment (green circles). In the setting of a diminished stem cell pool, the vital functions of glucocorticoid and mineralocorticoid production in the zona glomerulosa and fasciculata are ensured at the expense of the zona reticularis (orange zone). This results in decreased DHEA/DHEAS secretion and may ultimately relay cellular aging, induced by telomere shortening, via organ aging to aging of the organism.

One hallmark of ACCs is their high genetic diversity, aneuploidy, and the presence of multiple amplifications and losses (Wajchenberg *et al.* 2000). All of these genomic changes could theoretically be explained by telomere dysfunction and repeated BFBs. Indeed, it may be a common pathomechanism in Li-Fraumeni patients who develop short telomeres and potentially telomere dysfunction (Tabori *et al.* 2007). There are also a significant number of DC patients, for whom no genetic mutation has been found: the future may reveal mutations in other genes of the shelterin complex. While at a first glance there is no connection between adrenal pathologies and DC, it is true that there are no thorough functional and morphological analyses of this organ in these patients. Interestingly, a recent publication has described patients with a Fanconi anemia phenotype and adrenocortical insufficiency, and it certainly would be informative to screen these kindred for potential shelterin gene mutations (O’Riordan *et al.* 2008).

The most important development for the future of ACC research is an international effort to collect large numbers of high-quality tissue samples, which then can be subjected to emerging new analytical methods. These methods are clearly not restricted to the analysis of telomere physiology-associated genes, but will give insights into general aspects of adrenocortical carcinogenesis. Bioinformatic methods will help to overcome sample shortage by combining an increasing number of available gene expression data sets. New technologies, such as deep sequencing technologies, will further complement traditional gene expression microarrays in transcriptome analysis and will also make it possible to more efficiently search for novel genomic mutations (Asmann *et al.* 2008). Another promising analytical

method is the evaluation for single nucleotide polymorphisms (SNPs) and copy number variations. Many shelterin complex members harbor SNPs, some of which lead to amino acid changes on the protein level. SNPs may impact protein function or simply be associated with predisposition to cancer development or affect cancer characteristics directly (Engle *et al.* 2006).

It has become clear that TMMs are an important characteristic of ACC, and analyses for TMMs could be used as an adjunct diagnostic procedure to differentiate benign from malignant lesions. Furthermore, TMMs and telomeres may be used as a potential therapeutic target for tumor therapy. Several studies have used telomerase inhibitors as well as substances interfering with the telomere structure in preclinical cell culture and animal systems (Zimmermann & Martens 2007). There are hopes that these strategies may specifically target stem cells within malignant lesions, which theoretically depend on TMMs to a larger extent than non-stem cells.

The last decade of research in the fast moving field of telomere and telomerase sciences has influenced the field of adrenocortical research significantly. The future will show how much of this fertile match between basic sciences and clinically oriented research can be translated from bench to bedside. Our hopes are that it will not only lead to a deeper understanding of disease processes but also to a real advancement in patient care.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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