Review

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The role of telomeres and vitamin D in cellular aging and age-related diseases

Abstract: Aging is a complex biological process characterized by a progressive decline of organ functions leading to an increased risk of age-associated diseases and death. Decades of intensive research have identified a range of molecular and biochemical pathways contributing to aging. However, many aspects regarding the regulation and interplay of these pathways are insufficiently understood. Telomere dysfunction and genomic instability appear to be of critical importance for aging at a cellular level. For example, age-related diseases and premature aging syndromes are frequently associated with telomere shortening. Telomeres are repetitive nucleotide sequences that together with the associated sheltrin complex protect the ends of chromosomes and maintain genomic stability. Recent studies suggest that micronutrients, such as vitamin D, folate and vitamin B12, are involved in telomere biology and cellular aging. In particular, vitamin D is important for a range of vital cellular processes including cellular differentiation, proliferation and apoptosis. As a result of the multiple functions of vitamin D it has been speculated that vitamin D might play a role in telomere biology and genomic stability. Here we review existing knowledge about the link between telomere biology and cellular aging with a focus on the role of vitamin D. We searched the literature up to November 2014 for human studies, animal models and in vitro experiments that addressed this topic.

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Introduction

Aging is a multifactorial biological process characterized by a progressive decline of organ functions leading to an increased risk of age-associated diseases and death. Deterioration of genomic integrity and genomic instability are critical aspects in aging at a cellular level. Telomeres appear to be of critical importance for genomic stability and cellular aging. Telomeres are the end caps of chromosomes and were first identified in 1938 by Hermann Müller [1, 2]. Since then telomere biology has been widely investigated and numerous studies indicate an involvement of telomeres in the process of aging [3]. Telomere shortening and dysfunction have been proposed as indicators of cellular aging and are associated with age-related diseases including cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), cancer or chronic obstructive pulmonary disease [3]. Micronutrients, such as vitamins and trace elements play an important role in cell metabolism and some studies suggest a direct effect of these micronutrients on telomere biology and cellular aging [4]. Vitamin D, for example, is a steroid hormone with genomic and non-genomic activities that is involved in the regulation of cell proliferation, differentiation and apoptosis [5]. As a result of the multiple functions of vitamin D it has been speculated that vitamin D might play a role in telomere biology and genomic stability. Here we review existing knowledge about the potential link between vitamin D and telomere biology with a focus on age-related diseases.

Cellular aging

Aging and senescence

Aging is a physiological condition characterized by a progressive decline of organ function ultimately leading to death [6]. Several molecular and biochemical pathways contribute to aging and one of the most important of these is cellular senescence [7]. Cellular senescence is an irreversible arrest of cell proliferation that can be induced in different ways including genomic damage, toxins, irradiation, oxidative stress, oncogene expression, tumor suppressor gene activation and epigenomic alterations [8]. The state of senescence is established and maintained by at least two major tumor suppressor pathways: the p53/p21 and the p16INK4a/pRB pathways [8]. The p53/p21 pathway is activated by genomic or epigenomic stressors through the activation of the DNA damage response (DDR) [8]. The DDR is a network of cellular pathways that sense, signal and repair DNA lesions [9]. It prevents the generation of potentially deleterious mutations and avoids genomic instability and dysfunction [9]. Stress that does not entail direct genomic damage can induce p16INK4a expression, which activates the pRB tumor suppressor, that in turn silences certain pro-proliferative genes [7, 8]. Activation of both, p53/p21 and p16INK4a/pRB, triggers a signaling cascade that induces apoptosis and/or senescence [8]. The nature and degree of stress as well as the cell type, the balance between pro-senescent and pro-apoptotic pathways also decide cell fate [10].
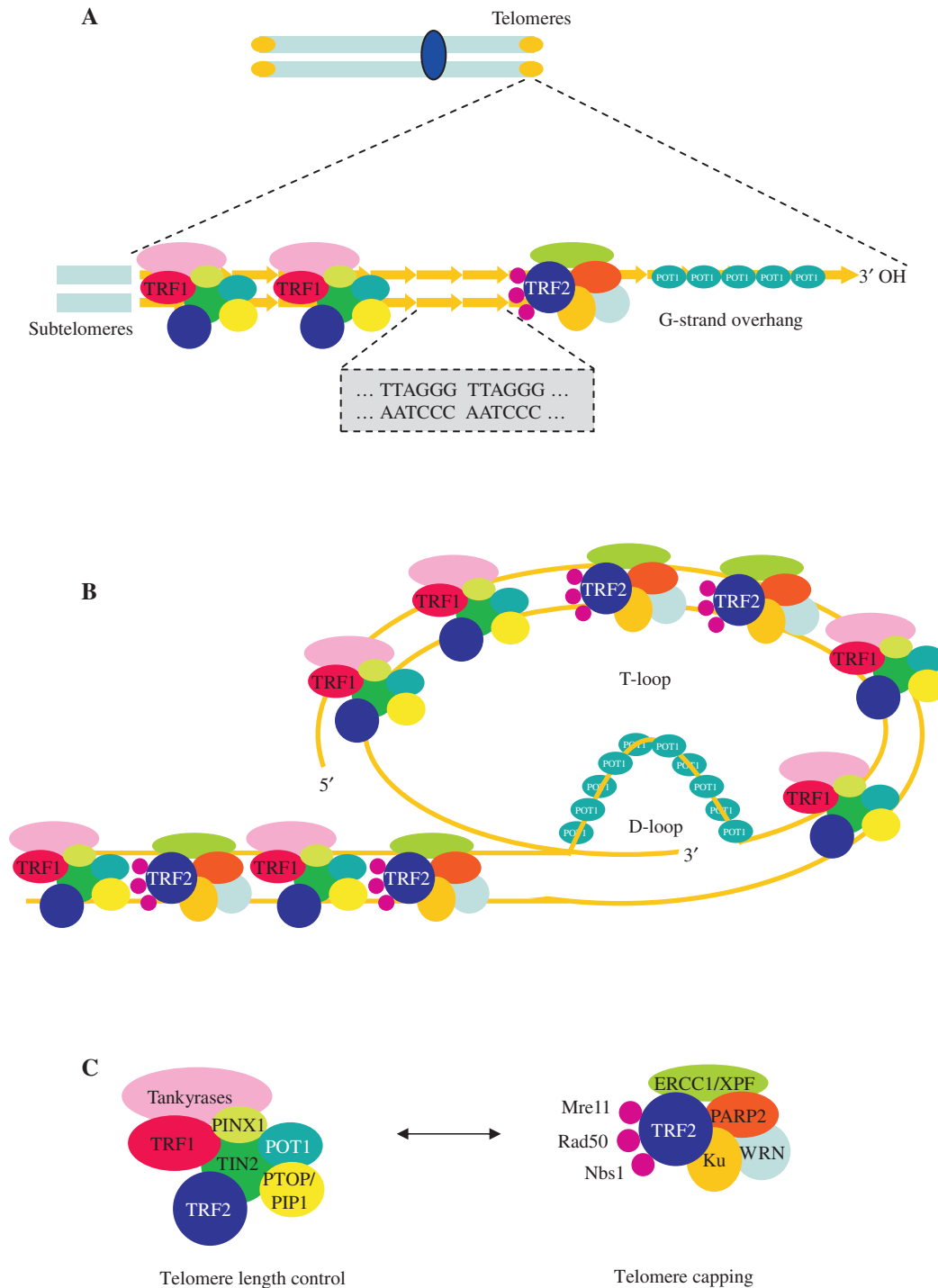
A range of biochemical features characterizes senescent cells: they are metabolically active, relatively resistant to apoptosis and also secrete pro-inflammatory cytokines, chemokines and proteases leading to a chronic inflammatory condition [7, 8]. This phenotype is known as senescence-associated secretory phenotype (SASP) [8]. Proteins that are associated with SASP are tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), matrix metalloproteinases (MMPs), monocyte chemoattractant protein-1 (MCP-1) and insulin-like growth factor binding proteins (IGFBPs) [8]. In addition, an intracellular IL-1a/miR-146a/b/IL-6/CCAAT/enhancer binding protein (C/EBP-b) loop as well as related p38/nuclear factor κ-light chain enhancer of activated B cells (NF-κB) – and mammalian target for rapamycin (mTOR) – mediated pathways appear to contribute to the SASP phenotype [8]. Moreover IL-6 and IL-8 are able to stimulate or inhibit Wnt (wingless, Drosophila segment polarity gene and abd integrated, vertebrate homolog) signaling and cell proliferation, respectively, depending on the physiological context [8]. The

Wnt signaling pathway regulates crucial aspects of cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development [11]. Cellular aging is also influenced by endocrine factors, like insulin-like growth factor 1 (IGF-1), Klotho and fibroblast growth factor 23 (FGF-23) [12]. Reduced IGF-1 expression in mice dramatically prolongs the lifespan, probably due to the regulation of forkhead box transcription factor 1 (FOXO1) activity [12]. The Klotho-FGF23 axis is a well known aging network; in fact, overexpression of Klotho in mice extends lifespan [13–15].

Telomere biology

Telomere dysfunction is one of the most important oncogenic stimuli able to activate a senescence response [8]. Telomeres (from Greek telos 'end' and meros 'part') are nucleoprotein structures that are highly conserved and are the end caps of chromosomes [2]. In humans, the telomeric DNA sequence is a tandem repeat of six nucleotides –TTAGGG– that extends approximately 10–15 kilobases [16, 17]. Telomeric DNA is double-stranded for most of its length with a 3'G rich single-stranded overhang of several repeats at the very end (150–200 bp) [16–20] (Figure 1A). The G-strand overhang can also fold back and anneal with the double-stranded region of telomeres forming a looped structure known as a T-loop [18–20] (Figure 1B). The closed configuration of the T-loop provides a protective cap that defines the natural end of the chromosome [20]. Telomeres participate in the maintenance of genomic and cellular stability and replication; in fact, they protect the genome from degradation, unwanted recombination and chromosomal fusion [18–20]. Chromosomal fusion can lead to chromosome imbalance, gene amplification, non-reciprocal translocations and altered gene expression [21]. In addition, the folding of the telomeric sequence in a three-dimensional (3D) structure protects telomeres from the recognition of the 3'OH as a double strand break-like (DSB) [19]. In the presence of telomere dysfunction all these conditions would activate the DDR cascade leading to cellular senescence [21].

Telomere repeats are bound to telomere-specific proteins, as shown in Figure 1A, B and C. Some of these proteins are associated exclusively with telomeres and compose the telomeric core complex known as telosome or sheltrin [22, 23]. The telomere sheltrin is composed of six telomere-specific proteins [22, 23]: Telomeric Repeat binding Factor 1 (TRF1), Telomeric Repeat binding Factor 2 (TRF2), Repressor/Activator Protein 1 (RAP1),
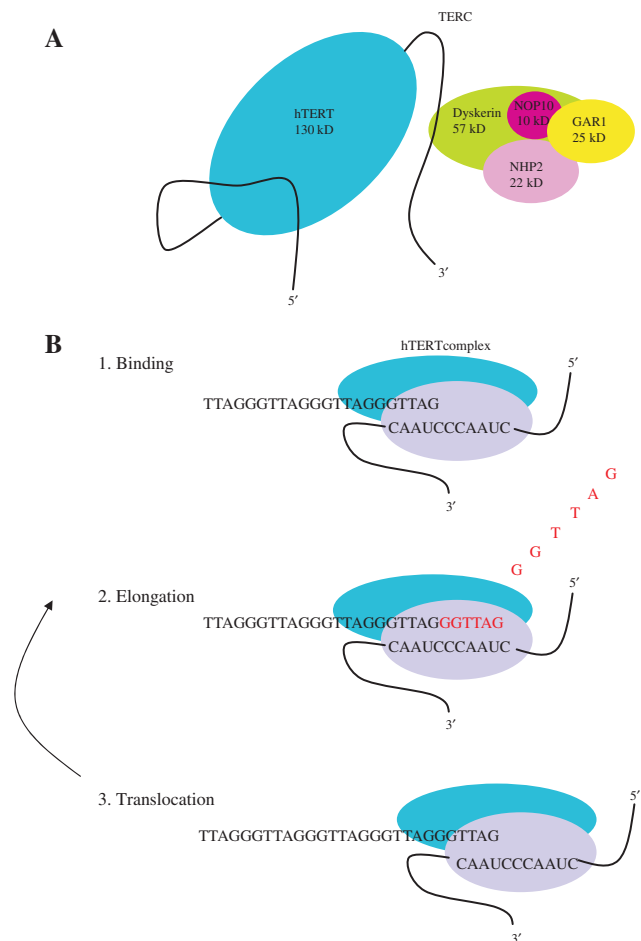
Figure 1: Telomere structure.
(A) Telomers are composed by a double strand region of −TTAGGG− repetitions and by a single strand region called G-strand overhang. Two protein complexes are bound to telomeres, the telomere repeat binding factor 1 (TRF1) complex and the telomere repeat binding factor 2 (TRF2) complex. (B) The G-strand overhand can fold back and invades the double strand region leading to the formation of T-loop and D-loop structures. The resulting 3D conformation protects the 3'OH end of the chromosome. (C) Composition of the two main telomere-associated protein complexes. The TRF1 complex is involved in telomere length control, whereas the TRF2 complex functions as protective end cap of telomeres. Modified from Blasco et al. [18, 19].

TRF1 Interacting protein 1 (TIN2), TINT1/PIP1/PTOP 1 (TPP1) and Protection Of Telomeres 1 (POT1). The TRF1 complex is involved in telomere length control regulating the access of the telomerase to the telomere sequence [24]. The TRF2 complex is thought to have a fundamental role in protecting the G-strand overhang from degradation, as well as in preventing telomeric fusions [22] (Figure 1C).

In vitro culture of somatic cells provides evidence of a limited replicative potential. This phenomenon was originally described by Hayflick in human fibroblasts, serially passaged in culture [25]. The Hayflick limit of cellular division is the maximum number of population doublings cells may undergo and is mediated by telomere erosion. Due to the inability of the DNA polymerase to maintain the length of the 3′ overhang, somatic cell replication is accompanied by a loss of 50–200 bp of telomeric sequence at every cell division [26]. The consequence of this phenomenon is that a somatic cell can undergo a defined number of doublings before telomeres become critically short, lose their protective properties and send cells into senescence, or cause cell death [23]. In contrast to most somatic cells, hematopoietic stem cells, keratinocytes in the basal layer of the epidermis, uterine endometrial cells, germ cells and various tumors avoid telomere shortening by activation of telomerase [26, 27]. Telomerase is a ribonucleoprotein with reverse transcription activity, which adds de novo telomere hexanucleotide repeats to the chromosome ends [18, 19, 28, 29] (Figure 2A and B). Telomerase contains a highly conserved reverse transcriptase [human telomerase reverse transcriptase (hTERT)], an associated template RNA [telomerase RNA component (TERC)] and a key auxiliary protein known as Dyskerin [28, 29]. Exogenous expression of telomerase in primary human fibroblasts is sufficient to reconstitute telomerase activity and to counteract telomere erosion. The resulting telomere maintenance immortalizes most human cell types [24].

Telomere length – analytical aspects

To date a range of different methods for the measurement of telomere length exists. Each method has advantages and disadvantages. The first method that has been developed is based on the Southern blot technique [30]. It relies on the absence of restriction enzyme recognition sites within the TTAGGG tandem repeat sequence. Genomic DNA is digested with cutting restriction endonucleases, like *HinfI* and *RsaI* to degrade the non-telomeric sequence. The terminal restriction fragments (TRFs) are separated by gel electrophoresis and transferred to a membrane. A radioactively-labeled telomere-specific

Figure 2: Telomerase structure and activity.
(A) The telomerase enzyme is composed by the human telomerase reverse transcriptase (hTERT), the telomerase RNA component (TERC) and the key auxiliary protein like Dyskerin, NOP10 (novel nucleolar protein 10), GAR1 (glycine and arginine rich domain) and NHP2 (non-histone protein 2). (B) Telomerase adds de novo telomere hexanucleotide repeats to the ends of the chromosome in a three-stage process: 1) recognition and binding of the hTERT complex; 2) elongation by adding complementary nucleotides; 3) translocation of the hTERT complex. Stages 2 and 3 are then repeated. Modified from Smogorzewska et al. and Marrone et al. [24, 29].

probe is then used for hybridization and telomere length is evaluated by densitometry. The result of this analysis indicates the distribution of telomere length over all chomosomes and cells present in the sample [30]. It does not provide information about telomere length in individual cells or individual chromosomes. The method requires a substantial quantity of DNA (i.e., μg) is time consuming and not suitable for large population-based studies. The analytical principle of this hybridization-based method implies a decreasing signal as telomere

become shorter. There is a threshold telomere length below which no detectable signal will be generated. The inability to detect very short telomeres limits the utility of this method in studies of cellular aging, where short telomeres are of particular interest [30]. Although the TRFs assay is considered the gold standard, the above mentioned disadvantages have led to the development of other methods that overcome the above mentioned limitations. Quantitative fluorescence in situ hybridization (Q-FISH) is an alternative method that was initially described by Lansdorp et al. [30]. In this method dividing cells are arrested in metaphase and are then fixed, permeabilized and stained with a fluorescent probe that binds specifically to the telomeric sequence. The intensity of the fluorescent signal at each telomere is a measure of telomere length. The main advantage of this method is the capability to measure telomere length of individual cells. The combination of Q-FISH with conventional FISH using chromosome-specific probes, allows not only the analysis of telomere length of individual cells, but also of individual chromosomes [30]. Similarly to the Southern blot-based method described above, Q-FISH is also a hybridization technique, which implies the existence of a threshold telomere length below which no detectable signal is produced. As Q-FISH is performed on cells in metaphase, the analysis is limited to cells that proliferate in vitro. Consequently, not all cells are suitable to be analyzed with this method. Recently, a confocal telomere quantitative fluorescence in situ hybridization method (Telomapping) has been described [31]. This method allows the quantification of telomere length in tissue samples or biopsies and gradients of telomere length within the tissue can be studied. The longest telomeres have been found in the stem cell compartment. Canela et al. described another Q-FISH-based method [32]. This automated high-throughput Q-FISH (HT Q-FISH) method combines the Q-FISH technology of telomeres in interphase nuclei with an automated high-throughput (HT) microscopy. It allows the quantification of telomere length as well as the percentage of short telomeres in large sample sets [32]. Another powerful variant of Q-FISH is the flow-FISH, where interphase telomere FISH is coupled with flow-cytometry [30]. Flow-FISH measures the total telomeric signal of a cell, which is the sum of all telomeres in each cell. As it is a flow-cytometry-based technique, telomere length on a large number of individual cells can be performed allowing the analysis of telomere length of defined populations or subpopulations of cells. As this method requires vital cells, it is widely used for the analysis of telomere length in hematopoietic cells. Finally, a quantitative-polymerase

chain reaction (Q-PCR) based assay for telomere length analysis has been described [33]. This method requires the amplification of two DNA regions by PCR. The first region is the telomeric sequence (telo) that is recognized by specific primers. The second region is a single copy gene (SCG) that is used as control for amplification. The most frequent SCG used is the 36B4 gene, which encodes the acidic ribosomal phosphoprotein P0. After amplification, telomere length is quantified as the amount of telomeric sequence compared to SCG and is expressed as a relative telomere length or T/S ratio [33]. This method allows a relative quantification of the telomeric sequence present in all cells of the sample. As for TRFs assay, it is not possible to evaluate telomere length of individual cells or chromosomes. However, the Q-PCR-based technique is fast, highly sensitive and requires low amounts of DNA (i.e., nanograms). As a result of these characteristics it is often chosen for large population-based studies. Further adaptations of this method are the multiplex-based assay, in which both DNA regions are amplified in the same reaction, reducing also the amount of DNA [34]; or the absolute telomere length analysis, in which a synthetized oligomer standard for both DNA regions is co-amplified allowing the expression of telomere length as an absolute value [35].

The intra- and inter- laboratory variability, the lack of a common standard reference and the absence of reference ranges, are limiting the application of telomere length analysis in clinical laboratories. Recently, an international collaborative study group has been developed in order to harmonize telomere length analysis between laboratories [36].

Telomeres and age-associated diseases

With increasing age most human somatic tissues and adult stem cells undergo telomere attrition, as they do not express sufficient amounts of telomerase to maintain telomere length indefinitely [37]. Dysfunctional telomeres may also arise by an independent mechanism called telomere uncapping [38]. In this alternative process, there is interference between the telomeric sequence and telomere-binding proteins, frequently the result of mutations, which leads to immediate uncapping of telomeres without telomere shortening [38]. Both critically short telomeres and uncapped telomeres impair cell viability and lead to senescence or apoptosis [37, 38].

A number of age-related conditions, like CVD, T2DM, neurodegenerative diseases and premature aging syndromes (e.g., congenital dyskeratosis), are characterized

by a faster-than-normal rate of telomere shortening [3, 7, 39–41]. However, the association between telomere length and age-associated diseases is still a matter of debate. Some prospective studies have shown that short telomeres are associated with increased all-cause mortality [42–46], whereas other studies have not found such an association [47–51].

CVD is among the most frequent age-related disease and the number one cause of death. There is substantial evidence linking CVD with telomere biology [52]. Several studies have shown that a high rate of telomere attrition is associated with an elevated risk of coronary artery disease, myocardial infarction (MI) and heart failure [52–61]. For example, in the West of Scotland Primary Prevention study (WOSCOPS) mean telomere length of peripheral blood leukocytes (LTL) was shorter in patients with severe triple vessel coronary artery disease than in individuals with angiographically normal coronary arteries [58]. In addition, associations between a reduced telomere length and the severity of CVD have been reported [56]. Cardiovascular risk factors like hypertension seem also to be related to telomere biology. In fact, both reduced telomere length and telomere uncapping were found in patients with hypertension [59, 60]. So far only two prospective studies have been published. In both studies telomere length was an independent predictor of MI and stroke [62, 63].

T2DM is another important cardiovascular risk factor and early evidence suggests that altered telomere biology may contribute to the development of the disease [64–70]. A recent meta-analysis performed on nine cohorts with a total of 5759 cases and 6518 controls indicated that shortened telomere length is significantly associated with T2DM risk [67]. Furthermore, telomere shortening seems not only to be associated with the incidence of T2DM but also with progression of the disease and the number of diabetic complications, such as retinopathy, nephropathy, neuropathy and peripheral vascular disease [70]. However, not all studies have been able to show a prospective relationship between telomere length and incident T2DM [65].

Alzheimer's disease (AD) is the most common neurodegenerative disease associated with aging. The association between LTL and the incidence of AD is still debated. Shorter LTL were found in AD patients [71], but no correlation was found between AD and telomere length of cerebral cells [72]. Moreover, in a longitudinal study LTL was not associated with changes in cognitive status of AD patients after 2 years of follow-up [73].

Cancer can also be considered an age-related disease, as its risk increases with aging. The potential link between telomere length and malignancies has been extensively studied in various types of tumor tissue and peripheral blood leukocytes. As the dynamics of telomere length differs between tissue and blood cells it is important to distinguish between these two approaches [74]. Reduced telomere length and poorer survival were observed in breast and prostate cancer cells as well as in sarcoma cells [74]. These findings could be explained by the Hayflick limit: telomeres become shorter at each cell division until a critical telomere length is reached. Cells with critically short telomeres undergo senescence and/or apoptosis. However, if the check-point is bypassed, cells continue to proliferate, which leads to genomic instability, accumulation of mutations and development of malignancies [75]. Additional factors like oxidative stress and chronic inflammation can aggravate this phenomenon and accelerate tumor formation [75]. However, to date it is not clear if telomere shortening is a cause or a consequence of tumor development and further studies are needed to clarify this important aspect.

Long telomeres have been shown to be associated with worse prognosis in carcinoma of the liver, colon, esophagus, head and neck [74]. Several explanations for this finding have been proposed. For example, estrogen-dependent anti-oxidant effects could contribute to telomere maintenance in breast cancer and other hormone-related malignancies [75]. It has also been speculated that cells with longer telomeres have an increased telomerase activity. Telomerase-stimulating factors, such as interleukines 2-4-6-7-10 and 13, may induce and maintain telomerase activity in these cells [75]. The presence of longer telomeres may delay senescence so that cells with long telomeres have a prolonged life span and consequently may encounter more situations, where DNA damaging stimuli can cause genetic abnormalities and chromosomal instability that ultimately lead to a malignant transformation of the affected cell.

The majority of existing studies indicate that alterations of telomere length in tumor tissue are associated with a worse prognosis. In addition, it has been speculated that tumor etiology and the stage of tumor progression may play a pivotal role for the development of alterations in telomere length [74]. For example, in clear cell renal cell carcinoma no association between telomere length in tumor tissue and patient survival was observed [74]. In any case the predictive value of telomere length measurement in tumor tissue is largely limited by the fact that it can only be obtained once the diagnosis has been established.

Several studies have investigated the relationship between LTL and cancer risk or prognosis and results

are conflicting [75]. Shorter LTL were found in different cancer types, including head/neck, lung, kidney, bladder, ovarian, breast, gastric, skin, esophagus, osteosarcoma and non-Hodgkin lymphoma [75, 76]. In contrast, numerous studies indicate that cancer risk is associated with longer LTL [75]. This was found in cancers of the skin, breast, lung, kidney, hepatocellular carcinoma and non-Hodgkin lymphoma [75]. Finally, non-associations between LTL and cancer risk have been found in breast, prostate, colon and endometrial cancers [75].

These observations suggest that the timing of sample collection is an important factor that may explain some of the discordant results in previous studies that investigated the association between LTL and cancer risk. It is also possible that LTL lengthening could be the consequence of an activation of the immune system during tumor formation [75]. An alternative explanation could be that when LTL becomes critically short, compensatory mechanisms, such as hTERT activation and alternative non-telomerase-based mechanisms that maintain telomere integrity, are switched on [75]. In addition, LTL may also be modified by cancer treatment [75]. Finally, there are differences in telomere length between subtypes of blood leukocyte that further limit the interpretation of LTL results [75]. Differences in study design, cancer type, sample processing, LTL measurement and patient characteristics may be contributing factors to contradictory results in telomere length association studies [75]. Although a number of studies have investigated the association between telomere length in tumor cells or in peripheral blood leukocytes and cancer progression or survival this relationship remains insufficiently understood and further studies are needed.

Another frequent condition of aging is osteoporosis. There are conflicting reports in the literature regarding the association of telomere shortening and age-related bone loss. In the TwinsUK cohort study, LTL was independently associated with a decrease in bone mineral density (BMD) and longer LTL was also associated with reduced risk of clinical osteoporosis [77]. In contrast, in the Health Aging and Body Composition Study (Health ABC), LTL was not associated with BMD, change in BMD over 5 years, osteoporosis or fractures at baseline or after 7 years of follow-up [78].

Although there is evidence for association between telomere length and age-related diseases, neither a conclusive causative link nor a predictable association can be established. Longitudinal studies as well as assessment of other markers of telomere biology are needed to further clarify the role of telomeres in aging and the development of age-related diseases.

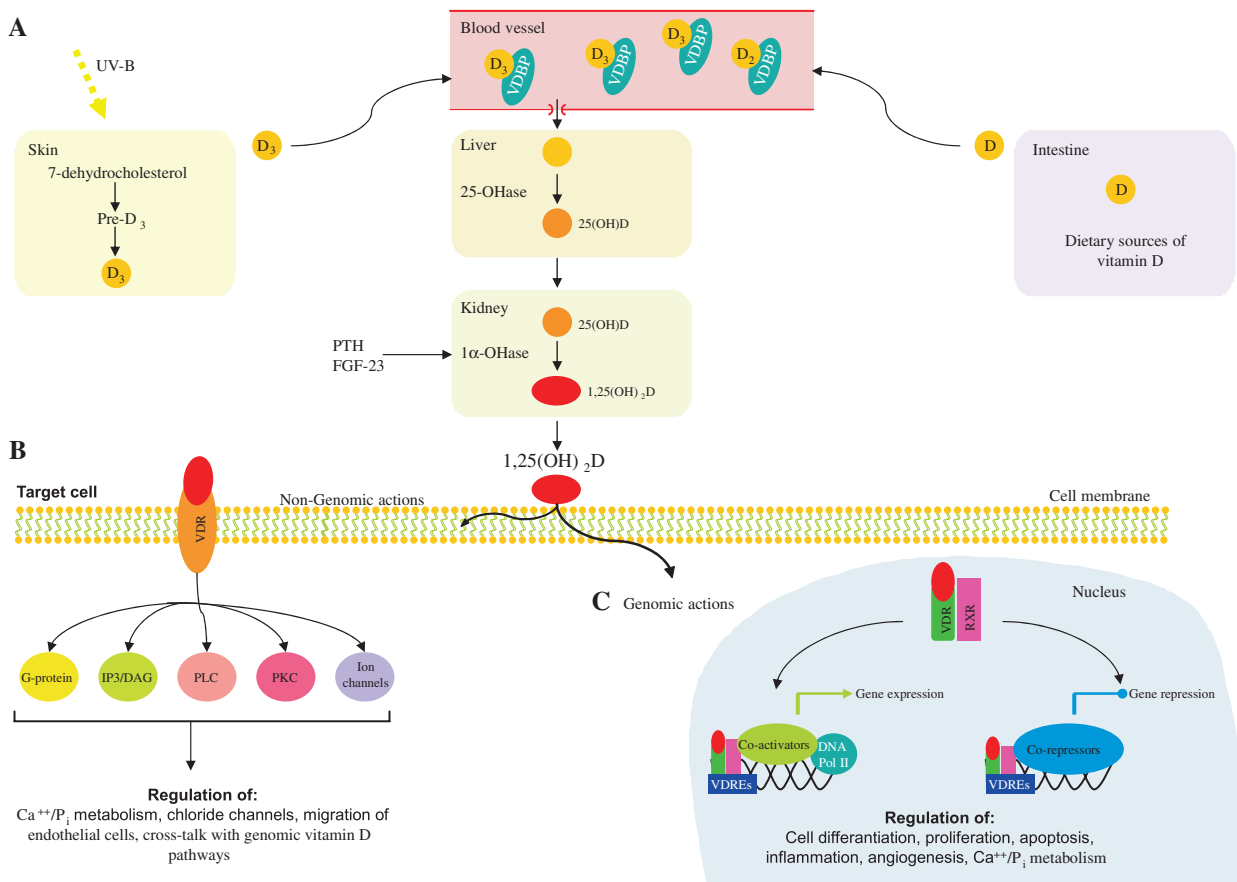# Vitamin D

Metabolism and physiologic aspects of vitamin D

Vitamin D metabolism is a complex process involving the action of UV radiation and hydroxylation steps (Figure 3A). Vitamin D_3 is primarily produced in the skin through the action of UV-B light on 7-dehydrocholesterol [80]. In most individuals, dietary intake, from sources such as wild-caught fatty fish, provides only a small additional contribution to total vitamin D levels [81].

Vitamin D requires two hydroxylation steps to reach its active form. The first hydroxylation, occurs in the liver and produces 25-hydroxyvitamin D_3 (25-OHD$_3$), the predominant form of the vitamin found in the circulation. 25-OHD$_3$ undergoes further hydroxylation at the C1 position in the proximal tubule of the kidney [82]. The 1,25-dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] thus formed is responsible for most, if not all, of the biological actions of the vitamin. A number of extra-renal tissues have demonstrated the ability to convert 25-OHD$_3$ to 1,25(OH)$_2$D$_3$. The 1,25(OH)$_2$D$_3$ produced by these tissues appears to act locally in an autocrine or paracrine fashion and does not contribute significantly to the circulating 1,25(OH)$_2$D concentration [83].

Genomic actions

1,25(OH)$_2$D$_3$ primarily exerts its effects on target tissues through genomic actions via the vitamin D receptor (VDR) (Figure 3C). After binding 1,25(OH)$_2$D$_3$, VDR forms heterodimers with one of three retinoid X receptors (RXRα, RXRβ, RXRγ) The VDR-RXR heterodimers interact with specific enhancer elements in DNA, known as vitamin D response elements (VDREs), as well as co-activators, and induce gene transcription [84, 85]. The VDR-RXR complex may also suppress the expression of certain genes, such as those encoding PTH or CYP27B1 [86, 87]. This may be mediated by the specific VDRE causing the VDR to interact with co-repressor molecules, via epigenetic mechanisms or by the modulation of specific microRNAs [88, 89]. There are thousands of VDREs, which are responsible for the cell-specific regulation of more than 200 hundred genes [5, 90].

The vitamin D receptor has been located in many cell types, including enterocytes, myocytes, immune cells (including activated T and B lymphocytes, macrophages and dendritic cells) as well as neurons and glial cells of

Figure 3: Vitamin D metabolism.
(A) Vitamin D$_3$ is produced in the skin through the action of UV-B light on 7-dehydrocholesterol. A small amount of vitamin D can also be obtained from dietary sources, such as wild-caught fatty fish, mushrooms and milk. Circulating vitamin D is bound to Vitamin D Binding Protein (VDBP). In the liver vitamin D is hydroxylated in 25-hydroxy-vitamin D [25(OH)D]. Subsequently, 25(OH)D is hydroxylated in the bioactive 1α,25-dihydroxy-vitamin D [1,25(OH)$_2$D] by the enzyme 1α-hydroxylase (1α-OHase), predominantly in the kidney. 1,25(OH)$_2$D exerts its biological functions via non-genomic (B) and genomic (C) actions. DNA Pol II, DNA polymerase II; IP3/DAG, inositol triphosphate/dia-cylglycerol; PKC, protein kinase C; PLC, phospholipase C; RXR, retinoid X receptors; VDR, vitamin D receptor; VDREs, vitamin D response elements. Modified from Deeb et al. [79].

the central nervous system, among many others [5, 91, 92]. Vitamin D therefore exerts numerous genomic actions, including control of calcium and phosphate exchange across the cell membranes of enterocytes, renal tubular cells and skeletal muscle myocytes [92, 93]. Such genomic pathways facilitate calcium absorption from the gut, renal reabsorption of calcium and normal skeletal muscle function [5].

Vitamin D is also involved in immune system regulation via genomic actions. It causes up-regulation of cytokine signals, antimicrobial peptides and the development of regulatory T cells [94]. Vitamin D also appears to exert genomic signals regulating cell proliferation, differentiation and apoptosis [95], while also inhibiting angiogenesis [96]. 1,25(OH)$_2$D$_3$ also appears to have a role

in the central nervous system in detoxification, protection against free radicals, neuronal calcium regulation, immunomodulation and enhanced nerve conduction [97]. In the cardiovascular system, vitamin D down-regulates renin transcription [98] and also prevents impairments in cardiac relaxation and contractility [99] and the development of left ventricular hypertrophy [100]. The role of the VDR in telomere biology is discussed in the section 'Vitamin D and telomere biology'.

Non-genomic actions

There are a number of responses produced by 1,25(OH)$_2$D$_3$ that have been shown to occur in a time-frame too rapid

to be mediated by gene transcription (Figure 3B). Furthermore, these actions are not impaired by the presence of inhibitors of gene transcription or translation. It appears that these responses are mediated by the VDR located within cell membranes, in specific locations important in signal transduction pathways, known as caveolae [101]. When the VDR is associated with caveolae, binding of $1,25(OH)_2D_3$ may activate a variety of second messengers, including G protein-couple receptors, inositol triphosphate/diacylglycerol (IP3/DAG), phospholipase C (PLC), protein kinase C (PKC) or the opening of ion channels [102].

$1,25\text{-}OH_2D$ has been found to have a number of effects on transcellular calcium fluxes via non-genomic pathways. This includes the enhanced intestinal absorption of calcium as well as increases in intracellular calcium concentrations in skeletal and cardiac myocytes, fibroblasts and osteoblasts [103–107]. Non-genomic effects $1,25(OH)_2D_3$ have also been demonstrated on calcium signaling in pancreatic β-cell cells [108], the rate of migration of endothelial cells [109], the opening of calcium and chloride channels in Sertoli cells [110] as well as increases in second messenger systems in parathyroid cells, osteoblasts, hepatocytes, chondrocytes and epithelial cells [106, 111, 112]. There appear to be instances of cross-talk between the rapid response and genomic vitamin D pathways. For example, $1,25(OH)_2D_3$ acting via a non-genomic pathway on rat osteosarcoma cells was found to cause an increase in osteocalcin gene transcription [113].

Vitamin D deficiency and related diseases

The classical role of vitamin D is in the maintenance of adequate calcium and phosphate status. Severe vitamin D deficiency causes rickets and osteomalacia, while more moderate deficiency is associated with osteoporosis and increased fracture risk [114]. The discovery that VDR exists in multiple tissues unrelated to vitamin D's classical function, has led to intense interest in the role of vitamin D in diverse aspects of health. Epidemiological data support an association between vitamin D deficiency and numerous conditions. These include various parameters of muscle function, multiple autoimmune diseases, upper respiratory tract infection (URTI), tuberculosis, insulin resistance, T2DM, coronary heart disease, heart failure and peripheral vascular disease and all-cause mortality [115–121]. Observational data suggest an association between hypovitaminosis D and cognitive function, depression, bipolar disorder and schizophrenia [122]. Furthermore, there is an association between vitamin D and a number of cancers, including colorectal and prostate cancer [123].
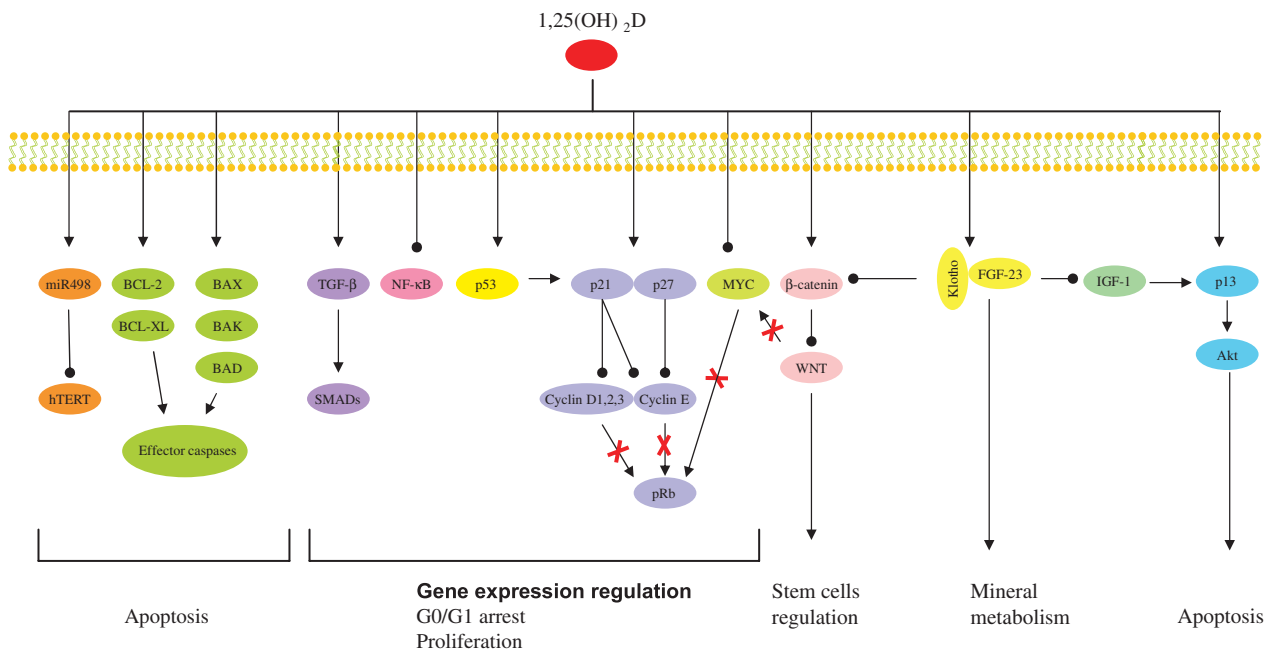
Despite these associations, there are limited data from randomized controlled trials demonstrating that supplementing deficient individuals with vitamin D improves clinical outcomes. However, there is evidence that supplementation improves muscle function, prevents type 1 diabetes and multiple sclerosis, as well as diminishes clinical exacerbations in those with pre-existing multiple sclerosis [124–126]. Supplementation studies have also shown accelerated clinical recovery from tuberculosis, improvement in depressive symptoms and, in males supplemented during the first year of life, a decreased risk of schizophrenia [127–129]. Results from studies addressing domains, such as mortality, and diabetes risk, URTI and cancer have been mixed [130–133].

Vitamin D and aging

Mounting evidence links deficiencies of micronutrients, such as vitamin D, folate and vitamin B_{12} to a series of age-related diseases including neurodegenerative diseases, hypertension, CVD, T2DM and osteoporosis. Deficiencies of these vitamins are common in elderly individuals. Vitamin D deficiency, for example, affects up to 100% of elderly subjects, especially if they are house-bound or live in nursing homes [134]. Considering the role of these vitamins in cell viability, DNA synthesis and repair it can be speculated that deficiencies accelerate telomere shortening and lead to genomic instability.

Vitamin D and senescence

The role of vitamin D in cellular aging and senescence is the consequence of its numerous functions in the regulation of cellular proliferation, differentiation and apoptosis, as illustrated in Figure 4. Vitamin D regulates a range of proteins that are involved in the cell cycle, such as cyclines, cyclin-dependent kinases (CDKs) and the cyclin-dependent kinase inhibitor (CDKIs) p21 and p27. All these proteins are involved in the G1/S phase transition [79]. Once activated, these CDKIs inactivate cyclins D1, 2, 3 and E that also lose their capacity to phosphorylate pRB. Hypophosphorylation of pRB leads the G0/G1 cell cycle arrest and inhibition of proliferation [79]. CDKIs act as negative regulators of cell growth, as they cause G1 arrest. Several genes, including p15, p18, p21 and p27 have also been found to be regulated by vitamin D. For example, a functional VDRE was found in the promoter of the p21 coding gene, suggesting a direct regulation of p21 expression by

Figure 4: Vitamin D involvement in cellular aging and telomere biology.
Vitamin D influences several pathways involved in the regulation of cell growth, proliferation (TGF-β, NF-kB, p53, p21, p27 and MYC), apoptosis (hTERT, BCL-2, BCL-XL, BAX, BAK, BAD and p13), stem cell regulation (Wnt), mineral metabolism (Klotho-FGF-23). Modified from Deeb et al. [79].

VDR [135]. CDK activity can also be enhanced by the proto-oncogene c-myc via functional inactivation of p21 and p27. C-myc has been found to be down-regulated by vitamin D in several cell lines, leading also to cell cycle arrest [135]. In addition, vitamin D can have many indirect effects on cell-cycle regulation, due to its cross-talk with other pathways. For example, vitamin D can up-regulate the expression of the transforming growth factor β (TGF-β) signaling cascade and down-regulate the epidermial growth factor receptor (EGFR) signaling pathway [79]. In addition, recently, Strambolsky et al. have identified that vitamin D is able to enhance the physical interaction of VDR with p53 [136], a well known cell cycle regulator. Vitamin D seems also to inhibit the NF-kB pathway in human leukocytes, through the down-regulation of the transcription of c-Rel and p50, two essential subunits of NF-kB [137]. The transcription factor NF-kB is a central component of the cellular response to damage, stress or inflammation [137]. Its chronic activation is observed during aging and in numerous age-related disorders, like T2DM, CVD, osteoporosis [79].

Although the exact mechanism by which vitamin D induces apoptosis is not completely understood, there is evidence that vitamin D controls members of the BCL-2 family [135]. This protein family consists of both anti-apoptotic proteins like BCL-2 and BCL-XL and pro-apoptotic proteins like BAX, BAK and BAD. These proteins in turn form homo- or heterocomplexes and control apoptosis by regulating mitochondrial permeability and the release of cytochrome c, which ultimately leads to the activation of the caspase cascade [135]. Vitamin D exerts also apoptotic functions, as it represses the transcription of anti-apoptotic proteins (BCL-2 and BCL-XL) or inducing the expression of pro-apoptotic proteins (BAX, BAK, BAD) [79]. For example, the down regulation of the anti-apoptotic BCL-2 protein by vitamin D is a well documented phenomenon in several cell lines [135].

Another important function of vitamin D in cellular aging is mediated by the FGF-23-Klotho axis [14]. In fact, defects of FGF-23 or Klotho lead to premature aging phenotypes. Vitamin D through the interaction with VDR induces FGF-23 expression. FGF-23 requires the co-receptor Klotho to activate the Fibroblast Growth Factor Receptor (FGFR) [13]. This interaction leads to the suppression of phosphate reabsorption and vitamin D biosynthesis in the kidney. The mechanisms by which alterations in the mineral ion homeostasis influence aging process are still not clear. The FGF-23-Klotho axis may inhibit insulin-like growth factor 1 (IGF-1) signaling pathway. IGF-1 inhibition is one of the evolutionarily conserved mechanisms

for aging suppression. Evidence of an involvement of the Klotho-FGF-23 axis in the IGF-1 signaling pathway comes from mouse models. Both, Klotho- and FGF-23-deficient mice are characterized by hypoglycemia and extreme sensitivity to insulin [13]. In contrast, transgenic mice overexpressing Klotho are moderate resistant to insulin and IGF-1, without overt diabetes and are long-lived [13, 138]. The mechanism by which secreted Klotho suppresses insulin/IGF-1 is still unknown [138]. Moreover, it is recently reported that Klotho inhibits Wnt signaling pathway [13]. Wnt is essential for stem cell proliferation, in fact, chronic stimulation of Wnt signaling can lead to a rapid exhaustion of stem cells. Stem cell dysfunction contributes to aging processes and the capacity of Klotho to inhibit Wnt signaling could explain the anti-aging effect of Klotho [13]. Finally, Klotho protects cells from oxidative stress, probably regulating nitric oxide (NO) production or superoxide dismutase 2 (SOD) expression [138].

Vitamin D and telomere biology

Studies in humans

A summary of the literature data regarding the link between telomere biology and vitamin D is reported in Table 1. Richards et al. were the first to demonstrate a positive correlation between serum 25-OHD and LTL in humans, which remained significant after adjustment for age [139]. In their study they analyzed 2160 women of the TwinsUK cohort. After multiple adjustments for age, season of vitamin D measurement, menopausal status, use of hormone replacement therapy and physical activity, the difference in telomere length between the highest (124±37.3 nmol/L) and lowest (40.9±11 nmol/L) tertile of 25-OHD concentrations was equivalent to 5 years of telomeric aging [139]. In addition, the authors analyzed a subpopulation of 700 women that used vitamin D supplements. On average, these women had longer telomeres than women who did not use vitamin D supplements. However this difference was not statistically significant. These initial findings were confirmed by a subsequent study performed by Liu et al. [143]. In this study, analyses were performed on 1424 women of the Nurses' Health Study and the results showed a positive correlation between LTL and serum 25-OHD concentrations. Logistic regression analysis indicated a concentration-dependent relationship. However, calcium intake modified this association significantly. 1,25(OH)$_2$D was also measured, but did not correlate with LTL. As single nucleotide polymorphisms (SNPs) in genes involved in vitamin D metabolism (like SNPs in VDR, VDBP CYP2R1 and DHCR7), are reported to affect vitamin D

blood concentrations, Liu et al. analyzed vitamin D-related SNPs in their population. They identified a positive association between rs7041 and rs4588 (both SNPs of VDBP), and 25-OHD levels. However, none of the investigated vitamin D-related SNPs were significantly associated with LTL [143]. Beside these cross-sectional studies, only one interventional study has been performed in order to clarify the effect of vitamin D supplementation on telomere biology [141]. Zhu et al. treated 37 obese Afro-American subjects in a double-blind randomized fashion with either a monthly oral dose of 60,000 IU of vitamin D3 or placebo for a period of 4 months. At the end of the study the serum 25-OHD concentration in vitamin D treated subjects was markedly increased when compared to baseline. The rise in serum 25-OHD was accompanied by a 19.2% increase of peripheral blood mononuclear cell (PBMC) telomerase activity. In the placebo group no such changes were seen. However, LTL was not measured in this study, limiting also the power of their findings. Further support for vitamin supplementation preserving telomere length comes from a recent study in hemodialysis patients [140]. Borras et al. observed longer telomeres in hemodialysis patients treated with calcitriol or analogs for at least 6 months compared to hemodialysis patients without such treatment [140]. Finally, Hoffecker et al. reported a significant correlation between telomere length and serum 25-OHD concentrations in vitamin D deficient (serum 25-OHD <20 ng/mL) patients with systemic lupus erythematosus (SLE) and unaffected controls [142]. Moreover, patients with SLE whose serum 25-OHD concentrations remained insufficient/deficient (<30 ng/mL) after 2.8 years of follow-up had shorter telomeres than patients with a sufficient serum 25-OHD concentration (>30 ng/mL).

Animal studies

So far, animal models have not been used to study the role of vitamin D in telomere biology. However, a limited number of animal studies have investigated the involvement of vitamin D in cellular aging and the results mainly support a significant relationship. VDR knockout mice (VDR −/−), e.g., have been used to study the function of vitamin D in cellular aging processes [144]. Keisala et al. have shown that these mice develop signs of premature aging, such as infertility, muscle atrophy, reduced immune function, and osteoporosis [145]. Furthermore, VDR −/− mice have a shorter life span [145]. The phenotype of premature aging in these animals was accompanied by a reduced expression of NF-κB, FGF-23 and p53 [144, 145]. An alteration of p53 and NF-κB expression by vitamin D is also supported by a two other studies [79, 136].

Table 1: Human studies linking vitamin D to telomere biology.

Author	Study	Study design Cohort (Sample size)	Gender, Male/Female	Age, years	Concomitant pathologies	Effect	Findings	Ref
Richards et al. [139]	Cross-sectional	TwinsUK (2160)	0/2160	49.4±12.9	Age-related diseses	Yes	Negative correlation between LTL and 25-OHD$_3$; The difference in LTL between the highest and the lowest tertiles of 25-OHD$_3$ was equivalent to 5 years of telomeric aging	139
Borras et al. [140]	Interventional: 24 HD patients recieved calcitriol or analogs for 6 months	n.a. (62)	65/52	>50	50 controls, 62 HD patients	Yes	Vitamin D treatment improves LTL compared to untreated patients	142
Zhu et al. [141]	Interventional: 60,000 IU per month oral vitamin D3 or placebo per 4 months	Afro-Americans (37)	13/24	19–50	Overweight	Yes	PBMC telomerase activity increases by 19.2% in the vitamin D group from baseline to study end LTL was not measured	141
Hoffecker et al. [142]	Cross-sectional	SLE Gullah Health (SLEIGH) (118)	0/118	39.86±11.57	59 controls, 59 SLE patients	Yes	No correlation between 25-OHD$_3$ levels and LTL was observed in patients or control group. Patients with vitamin D deficiency had shorter LTL than those with normal vitamin D levels. Higher 25-OHD$_3$ levels were significantly associated with with longer LTL	143
Liu et al. [143]	Cross-sectional	Nurses' Health Study (1424)	0/1424	30–55	n.a.	Yes	1,25(OH)$_2$D$_3$ was not associated with LTL. Vitamin D-related SNPs were not associated with LTL	140

HD, hemodialysis; LTL, leucocyte telomere length; n.a., not available; PBMC, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus; SNPs, single nucleotide polymorphisms.

These pathways are known to play an important role in cellular senescence [8]. Valcheva et al. have also shown that in VDR −/− mice vascular smooth muscle cells produced higher intracellular superoxide anion and thus promoting premature senescence [146].

Beside the VDR −/− mice model, other two mice models (FGF-23 −/− and Klotho −/−) have been utilized for studying the relationship of vitamin D pathway and aging. In fact, both FGF-23 and Klotho deficient mice have increased renal expression of e 1α-hydroxylase, accompanied by significantly elevated serum levels of 1,25(OH)$_2$D, infertility, atherosclerosis, skin atrophy, muscle wasting, T-cell deregulation and short lifespan [144]. A significant rescue of these phenotypes has been achieved providing a vitamin D deficient diet [144]. The hypothesis that hypo- or hyper-vitaminosis D causes accelerated aging is supported by animal studies that show a U-shaped association between serum 25(OH)$_2$D and the risk of cancer [144]. Based on these results it can also be speculated that there is an optimal serum concentration for general health [144].

In vitro studies

Further evidence for a role of vitamin D in telomere biology and cellular aging comes from cell culture studies. Treatment of different cell lines with vitamin D drives cell differentiation and has been shown to dramatically reduce telomerase activity [147]. For example, a reduction of telomerase activity was observed after treating leukemic HL-60 cells for 5 or 7 days with 100 ng/mL of vitamin D$_3$ [147]. However, the significance of this study is limited by the fact that no other vitamin D concentration was tested. Therefore, it remains unclear if there is a concentration-dependent relationship between telomerase activity and vitamin D. A similar phenomenon was observed in human prostate cancer cells [148]. Treatment of these cells with a combination of 1,25(OH)$_2$D and 9-cis-retinoic acid significantly reduced telomerase activity. The authors hypothesized a direct interaction of the VDR/RXR heterodimer with the DR3′ sequence in the human telomerase reverse transcriptase (hTERT) promoter as the underlying mechanism. The DR3′sequence (5′-AGTTCATGGAGTTCA-3′) is a vitamin D response element present in the promoter region of the hTERT gene [148]. Jiang et al. have described an alternative mechanism in an ovarian cancer cell line. Vitamin D downregulates hTERT activity in these cells by decreasing the stability of hTERT mRNA [149]. Kasiappan et al. proposed another appealing mechanism of vitamin D dependent telomerase regulation through small non-coding RNA molecules. They were able to demonstrate that vitamin D can induce microRNA-498 (miR-498) expression, which in turn decreases the mRNA expression of hTERT [150]. A microarray analysis of ovarian cancer cells treated with vitamin D identified miR-498 as the most abundantly induced microRNA. This finding has been subsequently validated in multiple human cancer types [150]. Consistent with this observation, a functional VDRE was identified in the 5-prime regulatory region of the miR-498 genome, which is occupied by the VDR and its co-activators. Furthermore, Kasiappan et al. showed that miR-498 targets the 3-prime untranslated region of hTERT and decreases its expression [150]. The level of miR-498 expression was decreased in malignant human ovarian tumor cells as well as in human ovarian cancer cell lines. The ability of vitamin D to decrease hTERT and to suppress ovarian cancer growth is reduced when miR-498 is depleted [150].

However, all these studies were performed on cancer cell lines that are characterized by genomic instability, chromosome alterations and other features that are associated with transformed cells. Therefore it is not possible to extrapolate the real role of vitamin D in telomere biology in healthy normal cells. Further studies are needed to clarify the role of vitamin D in telomere biology.

Conclusions

In summary, existing evidence supports the concept that vitamin D contributes to cellular aging and telomere biology. For example, human studies show an inverse relationship between serum vitamin D and age-related diseases as well as serum vitamin D and mortality. At a cellular level, vitamin D appears to regulate proliferation, senescence and apoptosis, through genomic and non-genomic pathways. One pathway through which vitamin D can delay cellular aging is the preservation of telomere biology. However, all studies published so far harbor significant limitations and results are sometimes conflicting. Human studies are predominantly of cross-sectional nature and the few longitudinal studies have been too small to draw conclusive findings. Animal and cell culture studies are very heterogeneous as they have used different animal models and cell types. This hampers the comparison of results and makes general conclusions impossible. Further systematic studies are needed to understand the role of vitamin D in telomere biology and cellular aging. In particular, large prospective studies will help to clarify if vitamin D supplementation can delay aging and reduce the risk of developing age-related diseases.

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