Medical Hypotheses (2007) **68,** 770—780

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A new molecular model of cellular aging based on Werner syndrome

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Received 6 September 2006; accepted 8 September 2006

Summary In the Hayflick model, a decrease in the number of cells capable of undergoing proliferation constitutes the main criterion of cellular aging and is closely linked to organismal aging.

Evidence suggests that a reduction of DNA replication capacity or a failure in the regulation systems of DNA replication occurs in aging cells, which leads to cellular replicative senescence.

DNA replication depends on two parameters: the number of active replicons and the rate of chain elongation.

Epigenetic parameters, in particular methylation, would be able to, either directly or indirectly, regulate replication origin activity of normal mammalian cells, as well as subsequent DNA replication.

Werner syndrome (WS) is an autosomal recessive disorder that results in premature aging and is considered to be a model system for the study of cellular senescence and aging.

WRN could involve DNA replication initiation, replication foci establishment, and the resolution of stalled replication forks during replication.

In this paper, a molecular model of in vitro cellular aging is presented in which changes in DNA methylation, in particular, global hypomethylation related to methyltransferase Dnmt1 downregulation, and specific hypermethylation related to methyltransferase Dnmt3b upregulation as seen during cellular aging, could be responsible for the inactivation of replication origins or foci and the subsequent documented reduction in DNA replication capacity and increased mutations that are observed in senescent cells.

Thus, Werner syndrome cells could be mimicking what is observed in normal aging in an accelerated form.

Introduction

The length of the G1 phase increases as a function of the age of the culture; the majority of non-cycling cells in aging cultures are in the G1 phase [1]. When cultures approach senescence, the G2 phase is also somewhat lengthened. There is also some lengthening in the S phase in at least a portion of the cycling cells [2,3].

Primary human fibroblasts exhibit an age-related or passage-associated decline in replicative capacity [4,5]. There is a decline in DNA replication capacity [6—8] or a failure of the DNA replication regulation systems during cellular aging. This decline is a possible cause of the lack of cellular proliferation seen at high population doublings.

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0306-9877/$ - see front matter
doi:10.1016/j.mehy.2006.09.024

Werner syndrome (WS), an autosomal recessive disorder, is a premature aging syndrome associated with the early onset of degenerative and neoplastic diseases [9]. The sequential appearance of clinical and biological deterioration in the body systems that is observed in this syndrome suggests that WS is more than a segmental progeroid syndrome [9]. Moreover, the finding that gene expression in WS closely resembles that of normal aging supports the use of WS as a model of aging [10]. WS fibroblasts exhibit an aging phenotype in culture that is characterized by a reduced replicative lifespan; they undergo only 9—11 population doublings (PD), whereas fibroblasts with wild type WRN have the potential for approximately 50—60 PD [11]. In addition, WS cells exhibit genetic instability, manifested by variegated translocated mosaicism [12] and increased mutation rates [13]. WS fibroblasts could be defective in DNA replication initiation [14], as well as foci formation [15]; they could also fail to resolve stalled replication forks or prevent the collapse of replication forks during the replication program [16], leading to a prolongation of the S phase [17]. Therefore, the inability to complete normal replication in WS cells may be a contributory factor in premature aging.

A model of cellular aging is introduced in this paper in which the replication defect in WS mimics the phenomena that occur in normal aging and leads to premature aging. According to the model presented, the defect responsible for reduced replication capacity in the aging cells may be a decline of origin activation in successive PD due to epigenetic changes, particularly changes in DNA methylation driven by methyltransferases (Dnmts).

Werner syndrome: the replication connection

The gene *WRN* is located on human chromosome 8p12 [18].Mutations in this locus in WS patients result in instability of WRN mRNA, as well as truncation of the protein with loss of the nuclear localization signal and all or some enzymatic domains of the protein [19]. Biochemical studies have shown that WRN protein has helicase and exonuclease activities of $3' \rightarrow 5'$ polarity, as well as associated ATPase activity [20,21]. The WRN protein is one of the five members of the human RecQ DNA helicase family, which also includes RecQ1 (also referred as RecQL), BLM, RecQ4, and RecQ5 [22]; mutations in BLM and RecQ4 cause Bloom's syndrome (BS) and Rothmund Thomson syndrome (RTS), respectively [23,24].

Many roles have been suggested for WRN protein with respect to DNA metabolism, including DNA repair and replication, recombination, replication, and telomere metabolism [25].

In particular, information correlating WRN protein to different aspects of DNA replication will be reviewed in this paper (Fig. 1).

WRN interacts with at least 16 proteins, some of which are involved in DNA replication, structure-specific flap endonuclease 1 (FEN-1), DNA polymerase (Polδ), topoisomerase I (TopoI), replication protein A (RPA), and proliferating cell nuclear antigen (PCNA). WRN is also a component of the replisome [26]. Evidence suggests that WRN is involved in processing stalled replication forks to promote replication restart [25]. There are four possible mechanisms by which WRN could do this.

First, WRN could have a role in the resolution of aberrant DNA structures that might impede DNA biosynthesis at stalled replication forks [27].

Second, WRN could have a role in recombination-mediated gap repair after replication-fork stalling [28]. In these models, WRN may associate with the RAD 51 protein and RPA to facilitate strand exchange, Holliday junction resolution and/or branch migration; after recombination, the single strand gap is filled, and repair enzymes can remove the lesion [28].

Third, based on a recent study that strengthened the association between certain *Escherichia coli* proteins in the Rec F pathway and the recovery of DNA replication after UV-induced arrest, it has been proposed that WRN might promote the removal of the lagging strand at stalled replication forks by virtue of its abilities to displace Okazaki fragments and stimulate the flap endonuclease and/or $5'—3'$ exonuclease activities of FEN-1 [25].

Selective degradation of the lagging strand would permit the replication fork to be stabilized within a triple-stranded structure, perhaps involving Rad 51; then replication could be resumed at the site of disruption without strand breakage or recombination, after the repair of the downstream blocking lesion [25].

Fourth, lesions or secondary structures in the DNA cause replication forks to stall and lead to the formation of Holliday junctions where stalled forks collapse upon themselves. It has been proposed that WRN may function at Holliday junctions as an exonuclease degrading the overhangs from the leading strand and as a helicase facilitating branch migration, which would allow replication to proceed [16].

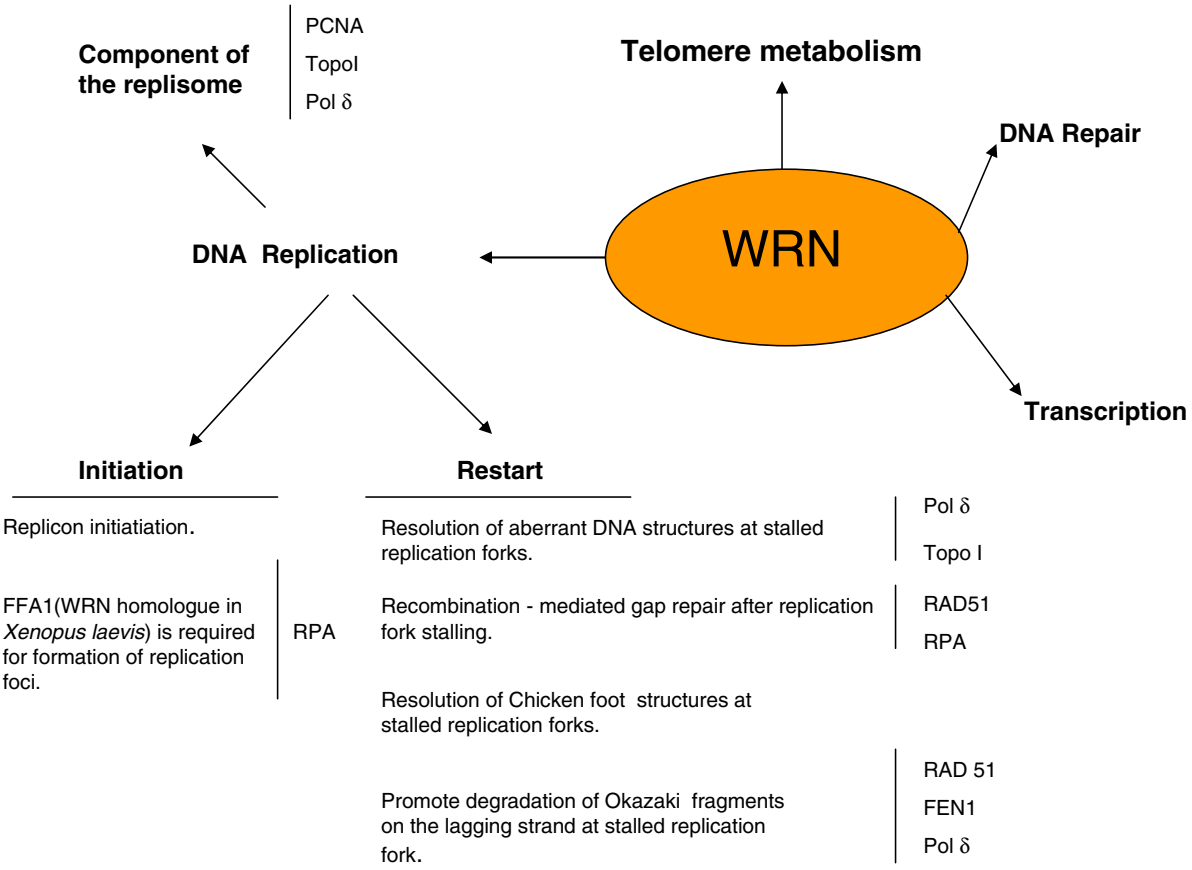

Figure 1 A summary of the DNA metabolic pathways WRN may be involved in and its probable roles in DNA replication. Implicated proteins are indicated.

Replication foci and origins in Werner syndrome

Before the WS gene was cloned, a large amount of experimental work had been carried out to correlate the cell phenotype of WS fibroblasts with DNA replication defects.

It was first determined that WS fibroblasts in culture exhibit a retarded rate of DNA replication [29], a normal rate of chain elongation, and a prolonged S phase and cell cycle [17].

Next, fiber autoradiographic studies correlated the defect with a decrease in the frequency of replication initiation as measured by the center to center distance (CCD) in WS cells [14,30]. However, these studies failed to demonstrate either altered chain elongation or a CCD change in low PD fibroblasts obtained from three normal donors of different ages, though the normal skin fibroblasts in late passages were not tested for increased CCD as in WS [14]. The distance between replicon initiation (CCD) sites is also decreased in SV40-infected WS cells, whereas no change is observed in the rate of chain elongation; this again suggests that the cause could be the decrease in the amount of protein that is required for the initiation of replicons [31].

Given that a decreased frequency of initiation of adjacent replicons (increase in CCD) was observed, this was the most probable cause of the decrease in DNA synthesis and prolonged S phase. Thus, a model was proposed in which WS cells misfire or delay the initiation of at least one origin in the replication foci, which may cause the imbalance of initiations that was noted [32]. The resultant replicon fusions in foci are eventually delayed or arrested until synthesis proceeds beyond the termini or backup synthesis occurs [32].

However, a recent study comparing one WS cell line with the MRC-5 embryonic lung fibroblast cell line, which used bromodeoxyuridine (BrdU) labeling and visualization after immunostaining in spread DNA, did not find a CCD increase in WS cells [16].

Surprisingly, the WRN orthologue in *Xenopus*, focus-forming activity1 (FFA1), has been shown to be

essential for the establishment of replication foci in the very early stages of DNA replication [15,33] and could also have a function in stalled replication forks [34]. A comparison of a cell line from a WS patient and the embryonic lung fibroblast cell line MRC-5 found no apparent defect in replication focus usage in the WS cell line [16].

In summary, WS cells have an increased S phase length probably due to a replicon initiation failure, or foci formation, since FFA1 is the orthologue of WRN, and/or because of a failure or delayed resolution of intermediates at stalled replication forks. In my opinion, it is plausible that WRN could have both functions (replication initiation and restart), which would reflect the heterogeneity that is seen in patients with the syndrome, although more work is necessary to elucidate this point.

Replication foci and origins

According to the Jesuit model [35], metazoan chromosomes carry many potential origin sequences, but only a subset of them is actually activated for usage.

The existence of replication foci (or centers, factories) was first suggested by fiber autoradiographic studies [36]. Replication foci were later visualized directly by labeling somatic cells with BrdU or biotin-UTP and subsequently by immunofluorescent antibody [36].

The number of origins of a domain can be as high as 300/focus. However, it appears that some foci contain just one replicon, though an average of five is estimated [36]. Thus, the number of sites (foci) varies considerably. Many reports estimate 100—350 sites in the early S phase. However, more recently, some researchers have concluded that there are 1000 individual replication foci [36]. In the very early S phase, the number of foci is smaller than in the later part of the early S phase; the number of foci in the mid and late S phase is smaller than in the early S phase, though the sizes of the mid and the late S phase foci are larger [36].

Moreover, the FFA1 protein (the orthologue of the WRN protein), which is essential for the binding of RPA to these foci, binds to chromatin before RPA does [37].

One model for foci-DNA interactions during DNA replication involves association of the origins to the foci proteins at the time that FFA1 generates loop replicons. The replication complexes remain tethered to the foci until completion of replication, and the newly replicated DNA remains attached to foci [37].

The role of methylation in replication origin activation

In *E. coli*, DNA methylation regulates both origin usage and the time required to reassemble prereplication complexes at replication origins [38]. In mammals, at least three replication origins are associated with a high density cluster of methylated CpG dinucleotides (mCpG) [39].

In the dihydrofolate reductase (*DHFR*) locus of Chinese hamster ovary (CHO) cells, replication can initiate at a large number of sites in a 55 kb region downstream of the *DHFR* gene with two preferred subregions, ori-beta, and ori-gamma [39].

In one study, ori-beta was found to contain a cluster of methylated CpGs, and this was also true for another origin at the 5′-end of the *RPS14* gene in the cells that were studied [39].

Moreover, when these cells were treated with the demethylating agent 5-azacytidine, initiation was no longer present at ori-beta [40].Therefore, at some loci, DNA methylation promotes the initiation process.

However, this cluster of mCpG should be considered to be different from the CpG islands, based on such criteria as G+C richness, lack of methylation, and promoter association.

CpG islands are assumed to be completely unmethylated. In agreement with this observation, replication origins located within the CpG islands, such as human c-*myc*, have been found to be unmethylated [40], and human lamin B2 is only partially methylated [41].

It has also been suggested that most of the unmethylated CpG islands contain replication origins [42], but this is not always true, as, for example, the CpG island at the 5′-end of the *DHFR* lacks origin activity [39,40].

It has been proposed that origins that lie proximal to promoters may utilize their transcription factors to facilitate transferring the origin recognition complex (ORC) to a specific site [39,43]. Origins that do not lie near transcription binding sites may utilize clusters of mCpGs to do this, since mCpG bind specific proteins, such as MeCP2, that may associate with members of the pre replication complex (pre-RC) [39,43].

Some origins, such as human beta-globin, do not have an mCpG cluster but have sparse CpGs that are methylated [41].

The indirect function of DNA methylation in DNA replication could involve the regulation of the expression of genes whose products determine the distribution of initiation sites [39].

Moreover, DNA methyltransferase was found to be associated with foci [44]; the inhibition of methyltransferase also inhibits DNA replication [45].

In summary, mCpG clusters associated with origins can promote the initiation process, though mCpG clusters are not components of all origins. Methylation may also regulate the expression of genes whose products are important for the initiation of DNA replication.

Replication origins inactivation and strand specific mutagenesis

Replicational origins impose additional control on mutagenesis through replication errors [46,47]. As the synthesis of the two antiparallel strands of duplex DNA occurs in the 5′ → 3′ direction, they can be copied by different proteins and processes. This asymmetry creates the potential for different error rates between the leading-strand and lagging-strand replication machinery. This would predict that strand-specific mutation rates may vary as a function of active replication origins found within eukaryotic chromosomes [46]. The switching off of nearby replication origins could change the mutability of gene blocks [47] and could modulate cellular aging.

By regulating origin activation, methylation and other epigenetic parameters could be involved in the origin regulation that is involved in aging.

Methylation and replication in aging mammalian cells

Having considered the importance of methylation in the regulation of replication origins in mammals, the way that replication capacity and methylation vary during cellular aging will now be discussed.

It is known that the total methylation level declines during aging [48—53]. This is true for cells cultured in vitro [48,49] and in vivo [50]. Evidence indicates that DNA methylation declines in mice in vivo at a greater rate than in vivo DNA methylation in humans; this suggests that the rate of decline is related to longevity [48].

In immortal cell lines, there is a major increase in de novo methylation, which is probably due to the increased activity of the de novo Dnmts (3a and 3b) [54]. A single treatment of human diploid fibroblasts with either 5-azacytidine or 5-aza 2′-deoxycytidine, which causes an irreversible decrease in DNA methylation in these cells, has been found to shorten their proliferative lifespan [49].

Under normal circumstances, CpG islands are devoid of methylation and coincide with active promoters. In cancer, it is well known that there is suppression of promoter activity by aberrant hypermethylation of tumor suppressor genes [55]. In the aging process it has been shown that some gene promoters are also inactivated by promoter hypermethylation or by partial methylation of the island that diminishes their expression [56,57].The reduced genome-wide methylation that occurs in aging cells may be attributed to attenuated Dnmt1 expression; on the other hand, CpG island promoter hypermethylation or partial methylation may be linked to an increase in Dnmt3b expression [54,58].

These results indicate that the limited number of cell divisions of normal cells that is seen with normal cells could be related to the progressive demethylation of DNA and hypermethylation or the partial methylation of some CpG islands associated with promoter activity.

Another parameter that declines during aging is DNA replication. DNA replication also depends on two parameters: the number of active replicons and the rate of chain elongation [59]. DNA fiber autoradiography studies indicate that in MRC-5 cells, the rate of DNA chain growth is slower in late passage cultures than in early passage cultures [11]. In MRC-5 fibroblasts and WI-38, the reduction in the rate of DNA synthesis was not partly due to a reduced rate in DNA chain growth, but could have been due to a decrease in the number of active replicons, or more likely due to a smooth decline in replicon clusters (foci) inactivation [60—62]. These results are in agreement with previous work showing that X-ray exposition may temporarily inactivate replication foci, but not individual origins [63—65].

Studies in the IMR-90 cell line of human diploid fibroblasts using alkaline sucrose density gradient centrifugation have also suggested that the change in DNA synthesis that is seen in aging cells is not due to a low chain elongation rate [66]. However, contradictory results using the same cell line (IMR-90) have also been presented by the same authors who reported that they did not find a decline in replication capacity during cellular aging [67].

One reason that conflicting results have been reported with respect to the evaluation of DNA replication capacity (rate of DNA chain elongation and number of active replicons) during in vitro cellular aging is that researchers used cell lines derived

from fetal fibroblasts in the experiments. If replication origins in mammals are regulated by methylation and epigenetic parameters [39,40], and the patterns of methylation in cell lines derived from fetal fibroblasts are in some way different from cell lines derived from adult fibroblasts [68], then this may explain the conflicting results.

Other studies have also shown that there is a correlation between the decline of DNA replication and aging in cells [69,70]. A study of hepatocyte DNA synthesis in rats of different ages found that the potential for DNA replication markedly diminished with age [8]. It has been shown that there is a slowing down of DNA replication in the old rat liver [71], the regenerating old rat liver [72], and in the old rat spleen and kidney [7].

To conclude, evidence indicates that a decline in global methylation, regional or gene localized hypermethylation, and DNA synthesis reduction occurs during cell aging. This reduction is most probably due to a decrease in the number of active replicons or slight inactivation of replication foci.

A molecular model of cellular aging

Although WRN protein participates in many metabolic pathways that are essential for cellular life, evidence suggests that one of the major roles of WRN protein is in DNA replication, since WS cells have a reduced life span, extended S phase, and a reduced frequency of replication initiation sites [14,17]. In particular, since WRN protein has an extensive homology with *Xenopus laevis* focus-forming activity 1 (FFA1) [15], WRN protein could be very important in resolving aberrant intermediates, such as those resulting from collapsed replication forks [16], as well as in replication initiation by establishing replication foci.

As WRN is not essential for cell survival it has been suggested that a redundant protein function exists and that other RecQ helicases have been proposed as important candidates [33]. Thus, it is not a remote possibility that different RecQ helicases with tissue specificity could be involved in the activation of particular kinds of replication foci. According to the tissue-specific hypothesis of genome instability, the distinct clinical symptoms of each RecQ disease result from genomic instability due to defective helicases that are present in specific tissues in which a high expression of particular helicases is needed for normal function [73].

Furthermore, in atypical Werner syndrome, which has a mutation in the *LMNA* gene [74], a defect in DNA replication may occur, since it is well known that lack of lamin A/C could disrupt lamin organization, replication foci, and continuation of DNA synthesis, but not the initiation of DNA synthesis [75]. It has been reported that normal primary fibroblasts grown in culture start to develop a small number of foci that coincide with intranuclear lamin A/C structures [76].

If one of the consequences of WS is an alteration in replication foci formation, leading to difficulty in replication origin activation and resolution of DNA junctions at stalled replication forks, then the regulation of replication origins during cell aging has fundamental importance (Fig. 2).

In the model presented here (Fig. 3), the decrease of global methylation due to a reduction in Dnmt1 expression and specific hypermethylation due to an increase in Dnmt3b expression observed in cell aging [58] could lead to the progressive inactivation of replication origins, resulting in a

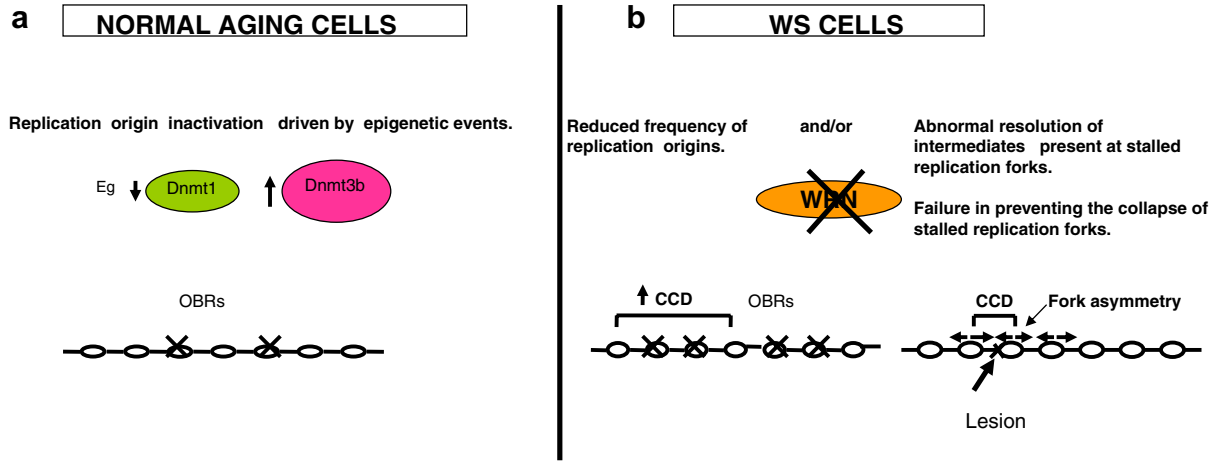

Figure 2 Proposed DNA replication defect in aging cells compared with that in WS cells. OBR, origin of bidirectional replication; CCD, center to center distance, which is the distance between initiation sites along DNA.

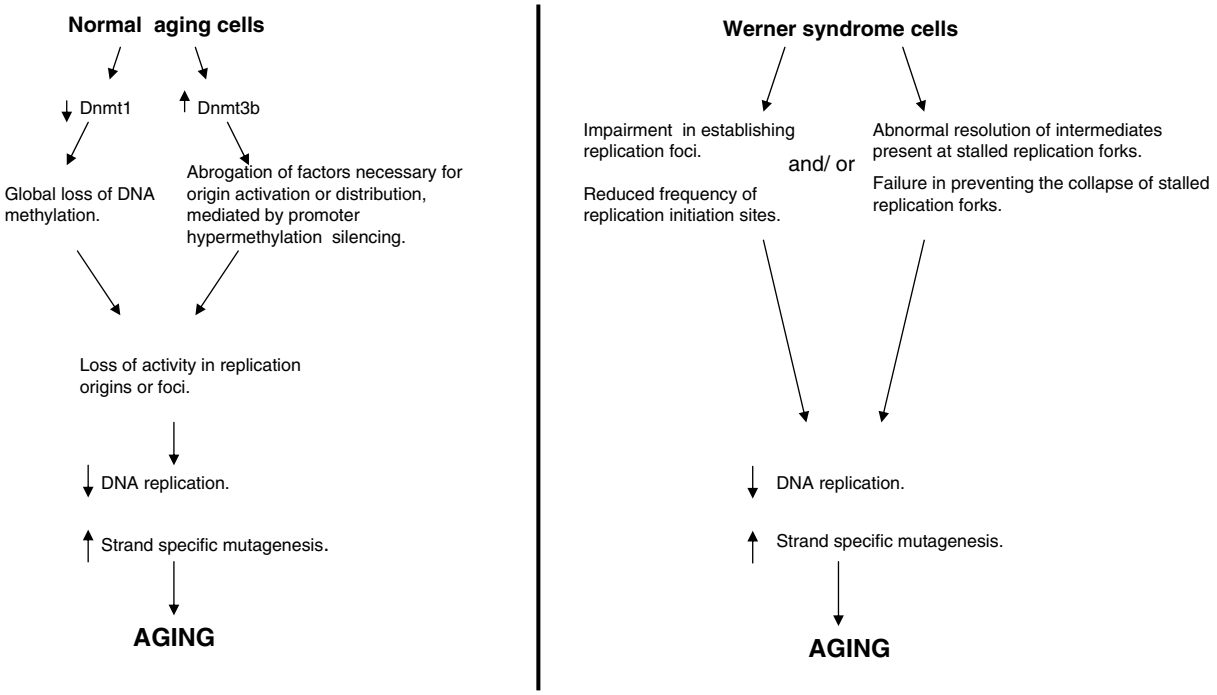

Figure 3 Model of cellular aging.

decline in DNA synthesis, as has been observed, [6] and to a change in the strand-specific mutation rates [46,47].

Some origins are coincident with mCpG clusters [40] that are not located near promoters as CpG islands. According to the model, these origins could be inactivated during the aging process by the diminished expression and activity of Dnmt1 that causes global demethylation [58] (Fig. 4a). In fact, following this line of thought, it has been found that hamster cells treated with the demethylating agent 5-azacytidine had a 50% reduction in methylation and no longer activated ori-beta [40].

CpG islands are devoid of DNA methylation and are situated near promoters [42]. It has been shown that some, but not all, origins are located in such islands [42].

The origins located within CpG islands are active when the island is in an unmethylated state because it has been suggested that they probably use promoter transcription factors to facilitate the binding of ORC to a specific site [40] (Fig. 4b).

According to the model presented here, the increased expression and activity of Dnmt3b in aging [58] could induce hypermethylation of some CpG islands where origins are located. Subsequently, promoter expression, as well as, possibly, origin function, would be lost (Fig. 4b). However, one recent study compared the mouse and human inactive X chromosome, where most CpG are methylated, to the active X chromosome and found that replication initiation was comparable at both alleles, but replication origins (ORIs) at nonmethylated CpG islands replicated earlier [77].

On the other hand, this model postulates that promoter silencing due to hypermethylation of CpG islands (a very well known fact in cancer cells) [55] by the Dnmt3b increases with aging [58] and could induce the loss of certain factors that are necessary for origin activation, for example, transcription factors that can facilitate the loading of ORC to the origin [40] (Fig. 4b).

Dnmt3b can also regulate the expression of genes whose products could determine the distribution of initiation sites [40] and/or can resolve intermediates at stalled replication forks, similar to the *WRN* gene itself.

To test this hypothesis, it would be interesting to first evaluate the status of methylation of the mCpG cluster in ori-beta in different PD in vitro for hamster cells using the bisulphite technique and correlate initiation assessed by the ''nascent strand abundance assay'' and the level of global methylation.

Second, it would be necessary to evaluate the methylation status of the CpG islands for the origins that are within the islands located at different PD and to determine whether there is hypermethylation of the islands and if hypermethylation is correlated with origin inactivation or late origin activation.

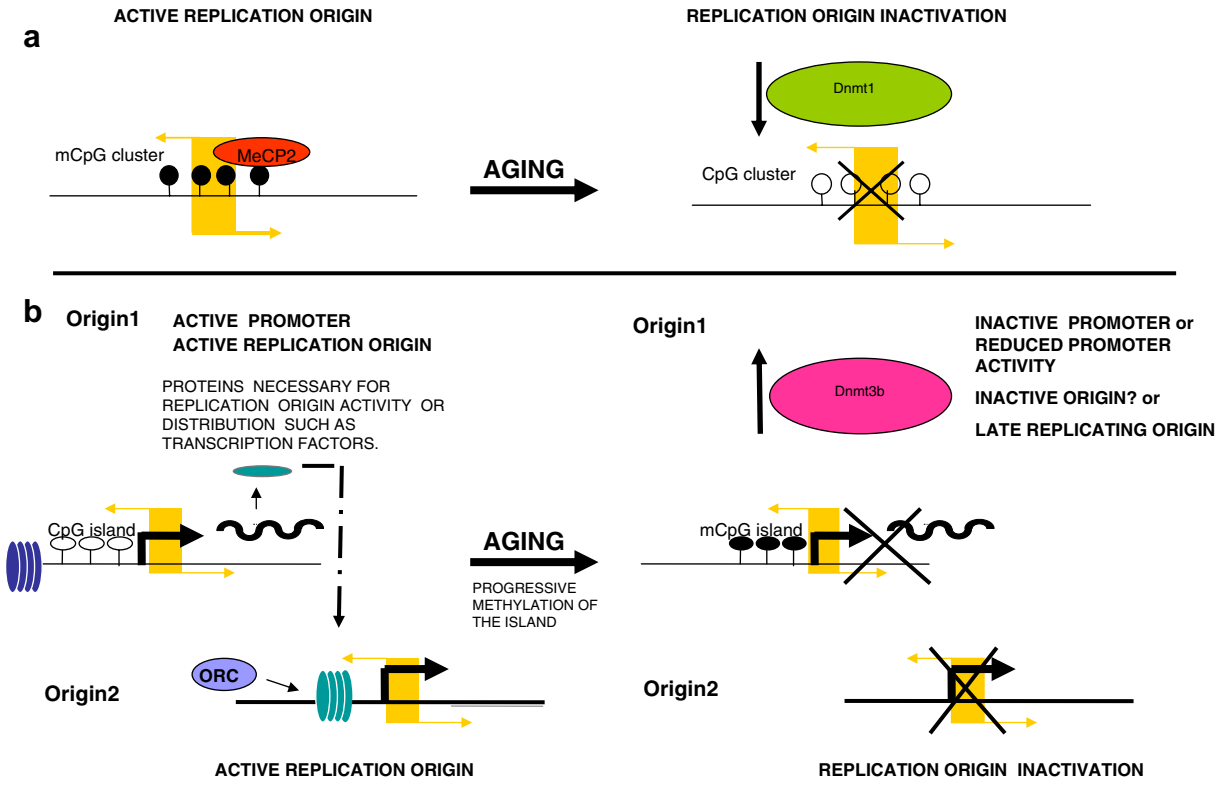

Figure 4 Author's proposed models for origin inactivation according to the Dnmts changes in aging. (a) The transcriptional downregulation of Dnmt1 and the reduced protein production and activity causes global hypomethylation and origin of bi-directional replication (OBR) inactivation; origins like the hamster (ori-beta), in which the mCpG cluster may bind to MeCP2 and associate with members of the pre-RC, could be inactivated by this mechanism. (b) CpG island promoter hypermethylation caused by transcriptional upregulation of Dnmt3b and the increased protein production and activity with aging could ablate the transcription of genes (promoter coincident with origin1 in figure) whose protein products are necessary for origin activation (origin2 in figure), such as transcription factors, as they may facilitate binding of ORC to a specific site. Yellow arrows indicate the direction of the leading strand DNA synthesis. White and black lollipops represent nonmethylated and methylated CpG, respectively. Black arrow is the transcription origin. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As well, the methylation status of the gene promoters for genes whose products are involved in origin activity or distribution and the coordination of origins in high PD cultured fibroblasts would need to be established.

Recently, it was determined that WRN and lamin A/C expression was abrogated in cancer cells by aberrant promoter hypermethylation [78,79]. Therefore, it would be interesting to test if this is also true for cultured primary fibroblasts in high PD.

Other epigenetic mechanisms, such as histone modification, show no clear correlation with origin activation [80]. However, a fascinating mechanism that has been proposed involves nucleotide pools determining origin choice and the efficiency of activation [81]. In fact, changes in nucleotide pools have been observed in aging lymphocytes [82].

According to the proposed model, another level of regulation in cellular aging could exist, since the pattern of activation of replication foci has a temporal and spatial organization in the S phase [36].

The evolving patterns of origin epigenetic changes, just as methylation of individual foci, could lead to their slight inactivation; foci inactivation rather than individual origin inactivation may be more important in explaining the decline of DNA replication capacity in aging cells. The temporal and spatial organization of foci in the S phase could become altered in successive cellular passages leading to a decrease in the capacity of DNA replication and, subsequently, to cellular aging.

In WS patient cells, the inability of the cells to properly form at least some replication foci could lead to a faster and uncoordinated decline in replication origin activation; an accelerated reduction

of DNA replication capacity would occur, leading to cellular aging and to a related change in the pattern of strand-specific mutagenesis.

Conclusion

A molecular model based on WS cells that explains normal cellular aging has been proposed. This hypothesis could be tested experimentally as has been suggested. Since cellular replicative senescence is related to organismal aging, this model could help elucidate the mechanism behind human aging.

Acknowledgments

The author would like to thank all of the members of the Centro de Documentacion e Informacion en Cancer (Comision Honoraria de Lucha contra el Cancer, Uruguay) for their kind assistance during the writing of this manuscript; colleagues Dr. Fernando Setien, Dr. Lionel Gresh, Dr. Jesus Espada, and Professor Carlos Scotti (Departamento de Laboratorio Clinico-Facultad de Medicina-Universidad de la Republica, Uruguay) for their support and encouragement, and Dr. Manel Esteller (Director of the Cancer Epigenetics Laboratory — Spanish National Cancer Research Centre, Madrid, Spain) for introducing me to the study of cancer epigenetics.

References

[1] Cristofalo JV, Sharf BB. Cellular senescence and DNA synthesis Thymidine incorporation as a measure of population age in diploid cells. Exp Cell Res 1973;76: 419—27.
[2] Maciera-Coelho A, Ponten J, Philipson J. The division cycle and RNA synthesis in diploid human cells at different passage in vitro. Exp Cell Res 1966;42:673—84.
[3] Kapp LV, Kleveczz RR. The cell cycle of low passage and high passage human diploid fibroblasts. Exp Cell Res 1976;101: 154—8.
[4] Hayflick L. The longevity of cultured human cells. J Am Geriatr Soc 1974;22:1—12.
[5] Schneider EL, Mitsui Y. The relationship between in vitro cellular aging and in vivo human age. Proc Nat Acad Sci 1976;73:3584—8.
[6] Petes TD, Farber RA, Tarrant GM, Holliday R. Altered rate of DNA replication in ageing human fibroblasts cultures. Nature 1974;251:434—6.
[7] Levitsky EL. Age-dependent changes of DNA replication in rat spleen and kidney. Gerontology 1980;26:321—6.
[8] Sawada N, Ishikawa T. Reduction of potential for replicative but not unscheduled DNA synthesis in hepatocytes isolated from aged as compared to young rats. Cancer Res 1988;48:1618—22.
[9] Goto M. Hierarchical deterioration of body systems in Werner's syndrome: implications for normal ageing. Mech Aging Dev 1997;98:239—54.
[10] Kyng KJ, May A, Kolvraa S, Bohr VA. Gene expression profiling in Werner syndrome closely resembles that of normal aging. Proc Natl Acad Sci USA 2003;100:12259—64.
[11] Martin GM, Sprague CA, Epstein CJ. Replicative life-span of cultivated human cells. Lab Invest 1990;23:86—92.
[12] Salk D, Au K, Hoehn H, Martin GM. Cytogenetics of Werner's syndrome cultured skin fibroblast: variegated translocation mosaicism. Cytogenet Cell Genet 1981;30:92—107.
[13] Fokuchi K, Martin GM, Monnat JR. Mutator phenotype of Werner syndrome is characterized by extensive deletions. Proc Natl Acad Sci USA 1989;86:5893—7.
[14] Takeuchi F, Hanaoka F, Goto M, et al. Altered frequency of initiation sites of DNA replication in Werner's syndrome cells. Hum Genet 1982;60:365—8.
[15] Yan H, Chen CY, Kobayashi R, Newport J. Replication focus forming activity 1 and the Werner syndrome gene product. Nat Genet 1998;19:375—8.
[16] Rodriguez-Lopez AM, Jackson DA, Iborra F, Cox LS. Asymmetry of DNA replication fork progression in Werner's syndrome. Aging Cell 2002;1:30—9.
[17] Takeuchi F, Hanaoka F, Goto M, Yamada M-A, Miyamoto T. Prolongation of S phase and whole cycle in Werner's syndrome fibroblasts. Exp Geront 1982;17:473—80.
[18] Yu CE, Oshima J, Fu YH, et al. Positional cloning of the Werner's syndrome gene. Science 1996;272:258—62.
[19] Huang S, Lee L, Hanson NB, et al. The spectrum of WRN mutations in Werner syndrome patients. Hum Mutat 2006;6:558—67.
[20] Shen J-C, Gray MD, Oshima J, Loeb LA. Characterization of Werner syndrome protein DNA helicase activity: directionally, substrate dependence and stimulation by replication protein A. Nucleic Acids Res 1998;26:2879—85.
[21] Kamath-Loeb AS, Shen J-C, Loeb LA, Fry M. Werner syndrome protein. II. Characterization of the integral 3′—5′ DNA exonuclease. J Biol Chem 1998;273:34145—50.
[22] Hisama FM, Bohr VA, Oshima J. WRN' tenth anniversary. Sci Aging Knowledge Environ 2006;10:18.
[23] Ellis NA, Groden J, Ye TZ, et al. The Bloom's syndrome gene product is homologous to RecQ helicases. Cell 1995;83:655—66.
[24] Kitao S, Shimamoto A, Goto M, et al. Mutations in RecQL4 cause a subset of cases of Rothmund Thomson syndrome. Nat Genet 1999;22:82—4.
[25] Opresko PL, Harrigan JA, Cheng WH, Brosh Jr RM, Bohr VA. Proposed biological functions for the Werner syndrome protein in DNA metabolism. In: Lebel M, editor. Molecular mechanisms of Werner's syndrome. NY: Kluwer Academic/ Plenum Publishers; 2003. p. 123—32.
[26] Lebel M, Spillare EA, Harris CC, Leder P. The Werner's syndrome gene product co-purifies with the DNA replication complex and interacts with PCNA and topoisomerase I. J Biol Chem 1999;274:37795—9.
[27] Kamath-Loeb AS, Loeb LA, Johansson E, Burgers Peter MJ, Fry M. Interactions between the Werner syndrome helicase and DNA polymerase specifically facilitate copying the tetraplex and hairpin structures of the d (CGG) n trinucleotide repeat sequence. J Biol Chem 2001;276:16439—46.
[28] Shen C-J, Loeb LA. The Werner syndrome gene the molecular basis of RecQ helicase deficiency diseases. Trends Genet 2000;16:213—20.
[29] Fujiwara Y, Higashikawa T, Tatsumi M. A retarded rate of DNA replication and normal level of DNA repair in Werner's syndrome fibroblasts in culture. Cell Physiol 1977;92: 365—74.

[30] Hanaoka F, Yamada M, Takeuchi F, Goto M, Miyamoto T, Hori T. Autorradiographic studies of DNA replication in Werner's syndrome cells. Adv Exp Med Biol 1985;190: 439–57.
[31] Hanaoka F, Takeuchi F, Matsumura T, et al. Decrease in the average size of replicons in a Werner syndrome cell line by simian virus SV 40 infection. Exp Cell Res 1983;144: 464–67.
[32] Fujiwara Y, Kano Y, Ichihashi M, Nakao Y, Matsumura T. Abnormal fibroblast aging and DNA replication in the Werner syndrome. Adv Exp Med Biol 1985;190:459–77.
[33] Chen CY, Graham J, Yan H. Evidence for a replication function of FFA-1, the Xenopus orthologue of Werner syndrome protein. J Cell Biol 2001;152:985–96.
[34] Sasakawa N, Fukui T, Waga S. Accumulation of FFA-1, the Xenopus homolog of Werner Helicase,and DNA polymerase {delta} on chromatin in response to replication fork arrest. J Biochem (Tokyo) 2006;40:95–103.
[35] De Pamphilis ML. Origins of DNA replication in metazoan chromosomes. J Biol Chem 1993;268:1–4.
[36] Berezney R, Dubey DD, Huberman JA. Heterogeneity of eukaryotic replicons, replicon clusters and replication foci. Chromosoma 2000;108:471–84.
[37] Newport J, Yan H. Organization of DNA into foci during replication. Curr Opin Cell Biol 1996;8:365–8.
[38] Kimura T, Asai T, Imai M, Takanami M. Methylation strongly enhances DNA bending in the replication origin region of the *Escherichia coli* chromosome. Mol Gen Genet 1989;219:69–74.
[39] Rein T, Zorbas H, De Pamphilis ML. Active mammalian replication origins are associated with a high density cluster of mCpG dinucleotides. Mol Cell Biol 1997;17: 416–23.
[40] Rein T, Kobayashi T, Malott M, Leffak M, De Pamphilis ML. DNA methylation at mammalian replication origins. J Biol Chem 1999;274:25792–800.
[41] Araujo FD, Knox JD, Szyf M, Price GB, Hadjopoulos Z. Concurrent replication and methylation at mammalian origins of replication. Mol Cell Biol 1998;18:3475–82.
[42] Delgado S, Gomez M, Bird A, Antequera F. Initiation of DNA replication at CpG islands in mammalian chromosomes. EMBO J 1998;17:2426–35.
[43] De Pamphilis ML. How transcription factors regulate origins of DNA replication in eukaryotic cells. Trends Cell Biol 1993;3:161–7.
[44] Liu Y, Oakeley EJ, Sun L, Jost JP. Multiple domains are involved in the targeting of the mouse methyltransferase to the DNA replication foci. Nucleic Acids Res 1998;26: 1038–45.
[45] Knox JD, Araujo FD, Bigey P, et al. Inhibition of DNA methyltransferase inhibits DNA replication. J Biol Chem 2000;24:17986–90.
[46] Radman M. DNA replication: One strand may be more equal. Proc Natl Acad Sci USA 1998;95:9718–9.
[47] Pavlov YI, Newton CS, Kunkel TA. Yeast origins establish strand bias for replicational mutagenesis. Mol Cell 2002;10: 207–13.
[48] Wilson VL, Jones PA. DNA methylation decreases in aging but not in immortal cells. Science 1983;220:1055–7.
[49] Fairweather S, Fox M, Margison GP. The in vitro life span of MRC-5 cells is shortened by 5 azacytidine induced demethylation. Exp Cell Res 1987;168:153–9.
[50] Wilson VL, Smith RA, Ma S, Cutler RG. Genomic 5-methyl deoxycytidine decreases with age. J Biol Chem 1987;262: 9948–51.
[51] Wilson VL, Jones PA. DNA methylation decreases with age. J Biol Chem 1983;262:9948–51.
[52] Cooney CA. Are somatic cells inherently deficient in methylation metabolism A proposed mechanism for DNA methylation loss, senescence and aging. Growth Dev Aging 1993;57:261–73.
[53] Catania J, Fairweather FS. DNA methylation and cellular aging. Mutation Res 1991;256:283–93.
[54] Lopatina N, Haskell JF, Andrews LG, Poole JC, Saldanha S, Tollefsbol T. Differential maintenance and de novo methylating activity by three DNA methyltransferases in aging and immortalized fibroblasts. J Cell Biochem 2002;84:324–34.
[55] Esteller M. Aberrant DNA methylation as a cancer-inducing mechanism. Annu Rev Pharmacol Toxicol 2005;45:629–56.
[56] Issa JP, Ottaviano YL, Celano P, Hamilton SR, Davidson NE, Baylin SB. Methylation of the oestrogen receptor CpG island links ageing and neoplasia in human colon. Nat Genet 1994;7:536–40.
[57] Ahuja N, Li Q, Mohan AL, Baylin SB, Issa JP. Aging and DNA methylation in colorectal mucosa and cancer. Cancer Res 1998;58:5489–94.
[58] Casillas Jr MA, Lopatina N, Andrews LG, Tollefsbol TO. Transcriptional control of the DNA methyltransferases is altered in aging and neoplastically transformed human fibroblasts. Mol Cell Biochem 2003;252:33–43.
[59] Edemberg HJ, Huberman JA. Eukaryotic chromosome replication. Annu Rev Genet 1975;9:245–8.
[60] Griffiths TD, Carpenter JG. DNA synthesis in permeabilized WI138 and MRC-5 cells. Exp Cell Res 1980;130:470–3.
[61] Griffifths TD, Carpenter JG. Effects of UV on DNA synthesis in permeabilized mammalian cells. Radiat Res 1981;87: 430–31.
[62] Griffiths TD, Carpenter JG, Ling SY. DNA chain growth as a function of age in intact and permeabilized WI 38 and MRC-5 cells. Mech Aging Dev 1983;21:15–25.
[63] Painter RB, Young BR. X-Ray induced inhibition of DNA synthesis in Chinese hamster ovary human HeLa and mouse L cells. Radiat Res 1975;64:648–56.
[64] Painter RB, Young BR. Formation of nascent DNA molecules during inhibition of replicon inhibition in mammalian cells. Biochem Biophys Acta 1976;418:146–53.
[65] Dohle DB, Griffiths TD, Carpenter JG. Subchromosomal DNA synthesis in X irradiated U-79 cells. Radiat Res 1979;78: 542–49.
[66] Hasegawa N, Hanaoka F, Hori T, Yamada M. Reevaluation of DNA chain elongation rate in human diploid fibroblasts. Exp Cell Res 1982;140:443–7.
[67] Hasegawa N, Hanaoka F, Yamada H. Does capacity of replication change during in vitro ageing? Exp Cell Res 1985;156:478–86.
[68] Halle JP, Schmidt C, Adam G. Changes of the methylation pattern of the c-myc gene during invitro aging of IMR-90 human embryonic fibroblasts. Mut Res 1995;316:157–71.
[69] Levitsky EL. DNA replication in adult and aged rats. Biokhimiia 1982;47:1601–7.
[70] Levitsky EL. Mechanisms of age specific characteristics of nuclear DNA replication. Ukr Biokhim Zh 1984;56:460–72.
[71] Levitsky EL. Age and features of DNA replication in intact white rat liver. Buill Eksp Biol Med 1980;89:733–5.
[72] Obolenskaya MY, Levitsky EL. Age characteristics of DNA replication in regenerating rat liver tissue. Vop Med Khim 1978;24:345–7.
[73] Furuichi Y. Premature aging and predisposition to cancers caused by mutations in RecQ family helicases. Annals NYAS 2001;928:121–31.
[74] Chen L, Lee L, Kudlow BA, et al. LMNA mutations in atypical Werner's syndrome. Lancet 2003;362:440–5.

[75] DePamphilis ML. Review: nuclear structure and DNA replication. J Struct Biol 2000;129:186—97.
[76] Barbie DA, Kudlow BA, Frock R, et al. Nuclear reorganization of mammalian DNA synthesis prior to cell cycle exit. Mol Cell Biol 2004;24:595—607.
[77] Gomez M, Brockdorff N. Heterochromatin on the inactive X chromosome delays replication timing without affecting origin usage. Proc Natl Acad Sci USA 2004;101:6923—8.
[78] Agrelo R, Cheng WH, Setien F, et al. Epigenetic inactivation of the premature aging Werner syndrome gene in human cancer. Proc Natl Acad Sci USA 2006;103:8822—7.
[79] Agrelo R, Setien F, Espada J, et al. Inactivation of the lamin A/C gene by CpG island promoter hypermethylation in hematologic malignancies, and its association with poor survival in nodal diffuse large B-cell lymphoma. J Clin Oncol 2005;23:3940—7.
[80] Dazy S, Gandrillon O, Hyrien O, Prioleau M-N. Broadening of DNA replication origin usage during metazoan cell differentiation. EMBO Rep 2005;7:806—11.
[81] Anglana M, Apiou F, Bensimon A, Debatisse M. Dynamics of DNA replication in mammals somatic cells: nucleotide pool modulates origin choice and interorigin spacing. Cell 2003;114:385—94.
[82] Borzi RM, Dal Monte P, Uguccioni M, Meliconi R, Facchini A. Intracellular nucleotides of lymphocytes and granulocytes from normal ageing subjects. Mech Aging Dev 1992;64:385—94.