DNA damage responses in progeroid syndromes arise from defective maturation of prelamin A

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Summary

The genetic diseases Hutchinson-Gilford progeria syndrome (HGPS) and restrictive dermopathy (RD) arise from accumulation of farnesylated prelamin A because of defects in the lamin A maturation pathway. Both of these diseases exhibit symptoms that can be viewed as accelerated aging. The mechanism by which accumulation of farnesylated prelamin A leads to these accelerated aging phenotypes is not understood. Here we present evidence that in HGPS and RD fibroblasts, DNA damage checkpoints are persistently activated because of the compromise in genomic integrity. Inactivation of checkpoint kinases Ataxia-telangiectasia-mutated (ATM) and ATR (ATM- and Rad3-related) in these patient cells can partially overcome their early replication arrest. Treatment of patient cells with a protein farnesyltransferase inhibitor (FTI) did not result in reduction of DNA double-strand breaks and damage checkpoint signaling, although the treatment significantly reversed the aberrant shape of their nuclei. This suggests that DNA damage accumulation and aberrant nuclear morphology are independent phenotypes arising from prelamin A accumulation in these progeroid syndromes. Since DNA damage accumulation is an important contributor to the symptoms of HGPS, our results call into question the possibility of treatment of HGPS with FTIs alone.

Key words: DNA damage responses, Progeria, Lamin A, Farnesyltransferase inhibitor, DNA double-strand breaks, ATR and ATM checkpoints

Introduction

Hutchinson-Gilford progeria syndrome (HGPS) is a severe childhood disease characterized by accelerated aging, which is caused by a de novo point mutation (1824C→T) in the *LMNA* gene, which encodes lamin A and the splice variant lamin C and germ cell-specific lamin C2 (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). These lamins are intermediate filament proteins composing the nuclear lamina, a scaffold underlying the inner nuclear membrane that structurally supports the nucleus and organizes chromatin (Goldman et al., 2002). The point mutation (1824C→T) of *LMNA* results in defective maturation of lamin A from its precursor prelamin A by causing a deletion of 50 amino acids near the C-terminus of prelamin A, which contains an endoprotease (Zmpste24) cleavage site required for the proteolytic maturation of lamin A (Eriksson et al., 2003). Zmpste24 mutation leads to another progeroid disorder, restrictive dermopathy (RD), which is neonatally lethal (Navarro et al., 2005). Loss of Zmpste24 activity arrests the processing of prelamin A at a stage similar to HGPS, although a unique truncated prelamin A (progerin) is accumulated in HGPS cells. Based on our prior elucidation of the prelamin A processing pathway (Sinensky et al., 1994b), these mutations are predicted to result in accumulation of farnesylated and carboxymethylated prelamin A. These two diseases have been suggested to be manifestations of the same cellular problem to different degrees (Misteli and Scaffidi, 2005). Although the molecular mechanisms by which theses mutations result in premature aging are far from full understanding, Liu et al. recently reported that human HGPS fibroblasts and *Zmpste24*-deficient mouse embryonic fibroblasts (MEFs) showed increased DNA damage and repair defects (Liu et al., 2005a). In addition, Varela et al. showed that *Zmpste24* deficiency in mouse elicits the upregulation of p53 target genes (Varela et al., 2005). These studies suggest that the genomic integrity was compromised in HGPS and RD cells because of the accumulation of progerin and prelamin A, respectively.

Accumulation of DNA damage might activate DNA damage and replication checkpoints, which attenuate cell-cycle progression and arrest replication, thereby preventing DNA lesions from being converted to inheritable mutations (Li and Zou, 2005). Two protein kinases of the phosphoinositide 3-kinase-like kinase (PIKK) family, Ataxia-telangiectasia-mutated (ATM) and ATM- and Rad3-related (ATR), play the central roles in initiating the damage and replication checkpoints (Abraham, 2001; Li and Zou, 2005). ATM is activated primarily in response to DNA double-strand breaks (DSBs) (Shiloh, 2003), whereas ATR is activated by a broad range of DNA damage and replication interference (Abraham, 2001; Li and Zou, 2005). Upon activation, ATM and ATR phosphorylate two major signal-transducing kinases Chk1 and Chk2, which in turn regulate downstream targets, such as Cdc25A, Cdc25C and p53, to control cell-cycle progression and DNA synthesis (Li and Zou, 2005; Sancar et al., 2004). It has been reported that in telomere-initiated senescence, a checkpoint response similar to that in the cells with DNA-

damage stress was activated involving ATM, ATR and downstream kinases Chk1 and Chk2 (d'Adda di Fagagna et al., 2003; von Zglinicki et al., 2005). Kinase inactivation experiments showed that this signaling pathway has to be maintained in order to keep cells in a senescent state (d'Adda di Fagagna et al., 2003; von Zglinicki et al., 2005). DNA damage accumulation and responses resulting from repair defects might lead to phenotypes associated with premature aging and might have causal roles in normal aging (Lombard et al., 2005). Furthermore, evidence has been presented that progerin expression occurs during the normal aging process (Scaffidi and Misteli, 2006). Given the similarities between these progeroid syndromes and normal aging, we speculated that the same signaling pathway of DNA damage response is activated in HGPS and RD cells as in telomere-initiated senescence.

Several recent studies have shown that inhibition of prelamin A farnesylation by protein farnesyltransferase inhibitors (FTIs) reversed the aberrant nuclear morphology of progeroid cells (Capell et al., 2005; Mallampalli et al., 2005; Toth et al., 2005). However, the important question as to whether treatment with FTIs concurrently restores the genomic integrity in these cells remains to be addressed.

In this study, we report that DNA damage checkpoints were constantly activated in HGPS and RD cells because of DNA defects. Strikingly, inactivation of ATR and ATM by a specific kinase inhibitor or RNA interference (RNAi) partially restored DNA replication in HGPS cells. Also importantly, treatment of the patient cells with a protein FTI was found to have no effect on DNA damage in these cells.

Results

Early replication arrest of RD cells and HGPS cells

Results from our studies (see below) and others (Liu et al., 2005a; Varela et al., 2005) showed that a considerable amount of phosphorylated histone H2AX (γ-H2AX), a molecular marker for DNA DSBs (Sedelnikova et al., 2002), formed in HGPS and RD cells. This indicates that DNA damage accumulates in patient cells. To determine the status of DNA replication in these cells, a DNA replication assay with replicative incorporation of [methyl-^{3}H] thymidine was performed. As shown in Fig. 1, DNA synthesis in the HGPS and RD cells of passage 11 occurred at rates more than 2-fold slower than that in BJ cells, a non-transformed human diploid fibroblast cell line. Moreover, replicative capacity was lost at passage 15 for RD cells and at passage 21 for HGPS cells (Fig. 1), in contrast to the more typical replicative behavior of BJ cells which exhibit many more passages before undergoing replicative senescence (Steinert et al., 2000). This premature replicative senescence of the patient cells is consistent with the previous reports that the percentage of S-phase cells in *Zmpste24*-deficient MEFs was lower than that in normal MEFs (Liu et al., 2005a; Varela et al., 2005).

Activation of ATM and ATR in RD cells and HGPS cells

The premature replicative senescence of HGPS and RD cells suggested that G1-S and/or intra-S-phase checkpoints were probably activated. To test this notion, we assessed the activation of ATM and ATR, two central initiators of DNA damage checkpoints (McGowan and Russell, 2004), in HGPS and RD cells using the method of immunofluorescence microscopy. In

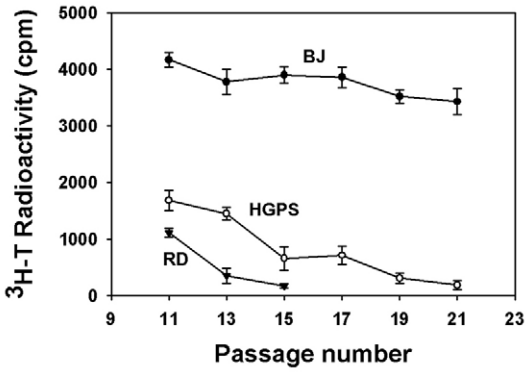

Fig. 1. Early replication arrest of HGPS and RD cells. The replication assay was performed with [methyl-^{3}H] thymidine labeling as described in Materials and Methods. Symbols represent radioactivity values as follows: ●, BJ cells; ○, HGPS cells; ▼, RD cells. The values were calculated from three independent experiments. Error bars represent standard deviations.

BJ cells, the majority of ATM was homogenously distributed in the nucleus (Fig. 2A). The treatment of BJ cells with camptothecin (CPT), a radiomimetic agent widely used to induce DSBs and activate ATM in cells (Shiloh, 2003), caused ATM focus formation in the nuclei (Fig. 2A). Interestingly, a very similar pattern of ATM nuclear focus formation was observed in HGPS and RD cells even without treatment with CPT, suggesting that ATM was activated in these cells (Fig. 2A). By contrast, a different pattern of activation was observed for ATR in these patient cells. In unstressed BJ cells, ATR was mainly localized in cytoplasm, with little or no nuclear staining (Fig. 2B). After UV irradiation, a known DNA damaging stress that induces ATR activation (Abraham, 2001), ATR translocated from cytoplasm into the nucleus. Interestingly, whereas the majority of ATR was in the nuclei of untreated RD cells, only part of ATR was distributed in the nuclei of HGPS cells and formed large foci or aggregates (Fig. 2B). The nuclear distribution of ATR in RD and HGPS cells suggests its activation in these cells, which is confirmed by the phosphorylation of its primary substrate Chk1 (see below). To verify that nuclear translocation of checkpoint kinases in the patient cells arises from expression of prelamin A, HeLa cells were transfected with a plasmid encoding progerin (LAΔ50) for immunofluorescence analysis. As shown in Fig. 2C, the majority of ATR was in cytoplasm in HeLa cells transfected with an empty parent vector. By contrast, ATR was mainly located in nuclei, forming large foci in the HeLa cells transfected with the LAΔ50-expression plasmid. This indicated that the nuclear translocation of checkpoint kinases was indeed induced by the presence of progerin. Thus, DNA damage in HGPS and RD cells, arising from prelamin A accumulation, results in nuclear distribution of ATR and ATM, consistent with activation of cell cycle checkpoints.

To confirm the presence of checkpoint response pathways in HGPS and RD cells, we next examined the activation of downstream signal-transducers Chk1 and Chk2, and the effector p53, by assessing their phosphorylation status at specific sites (Helt, 2005). As shown in Fig. 3, besides phosphorylation of H2AX, phosphorylation of Chk1 (Ser-345), Chk2 (Thr-68) and p53 (Ser-15) were all readily detected in

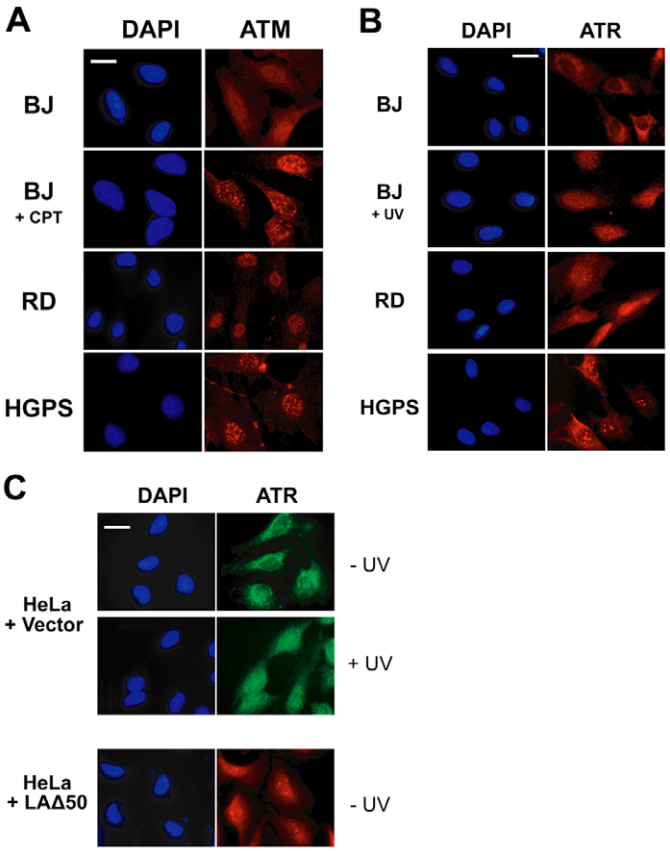

Fig. 2. Activation of ATM and ATR in HGPS and RD cells. (A) CPT treatment was done by incubating the cells with 4 μM camptothecin for 1 hour. Cells with or without treatment were fixed with cold methanol (–20°C) followed by immunofluorescence microscopy with ATM antibody staining. Blue, DAPI; red, ATM. (B) UV treatment was performed by irradiating the cells with 20 J/m^2 UV. Two hours after the treatment, the cells with or without treatment were fixed and stained with ATR antibody for immunofluorescence microscopy. Blue, DAPI; red, ATR. (C) HeLa cells grown on coverslips were transfected with the plasmid for expressing progerin (LAΔ50) or empty parent vector. Twenty-four hours after transfection, the cells were irradiated with 20 J/m^2 UV or mock treated. After additional 2-hour culture, the cells were processed for immunofluorescence microscopy. Blue, DAPI; green or red, ATR. Photomicrographs were taken at 63× magnification. Bar, 50 μm.

HGPS and RD cells, confirming the activation and signaling of checkpoint pathways in these cells.

Restoration of replication activity by inactivation of ATM and ATR

DNA damage checkpoint responses are complex signaling pathways orchestrated by the PIKK family including ATM and ATR (Abraham, 2001). Cells with deficient ATM and/or ATR are defective in initiating DNA damage-induced cell-cycle arrest (Shiloh, 2003). To test whether inactivation of ATM and ATR could abolish the premature senescence observed in the patient cells, we treated cells with 5 mM caffeine, an ATM and ATR inhibitor (Sarkaria et al., 1999), and measured their DNA synthesis by [methyl-^{3}H] thymidine labeling. As shown in Fig. 4A, both ATM and ATR were efficiently knocked down in BJ and HGPS cells transfected with ATR and ATM small

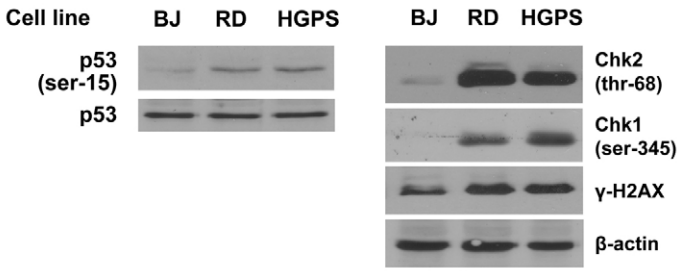

Fig. 3. Phosphorylation of Chk1, Chk2 and p53 in HGPS and RD cells. Western blotting was performed as described in Materials and Methods. The phosphorylation status of p53 was determined with p53 Ser-15 phosphorylation-specific antibody. The total p53 was probed as the loading control to ensure that the same amounts of p53 were loaded for BJ, RD and HGPS cells. In the right panel, β-actin was probed to ensure that similar amounts of proteins were loaded for the three cell lines.

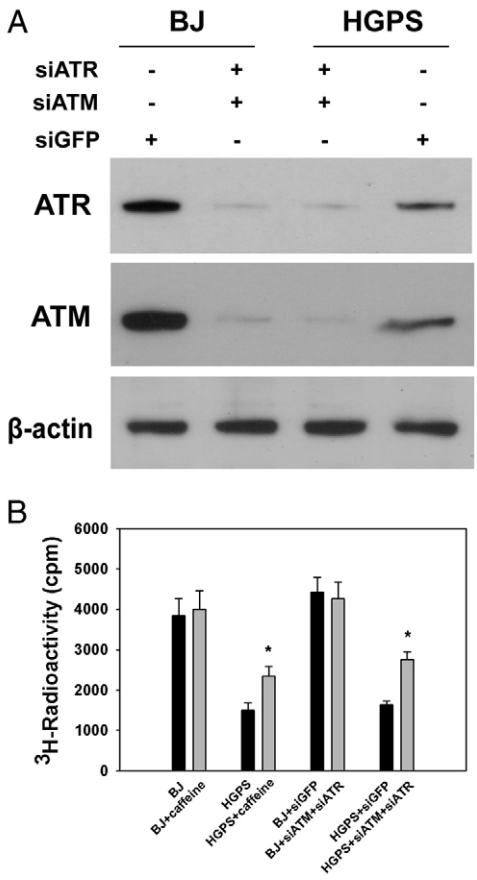

Fig. 4. Restoration of replication activity in HGPS cells by inactivation of ATM and ATR. (A) Western blotting shows the knockdown of ATR and ATM in BJ and HGPS cells by RNAi. β-actin was used as sample loading control. (B) The replication assay was performed with [methyl-^{3}H]-thymidine labeling as described in Materials and Methods. BJ cells and HGPS cells used were at passage 12. The asterisk indicates a significant difference, P<0.05.

interfering RNAs (siRNAs). The observation of lower cellular levels of ATM and ATR in green fluorescent protein (GFP) siRNA-transfected HGPS cells than in corresponding BJ cells could be because of the tight chromatin association of ATM

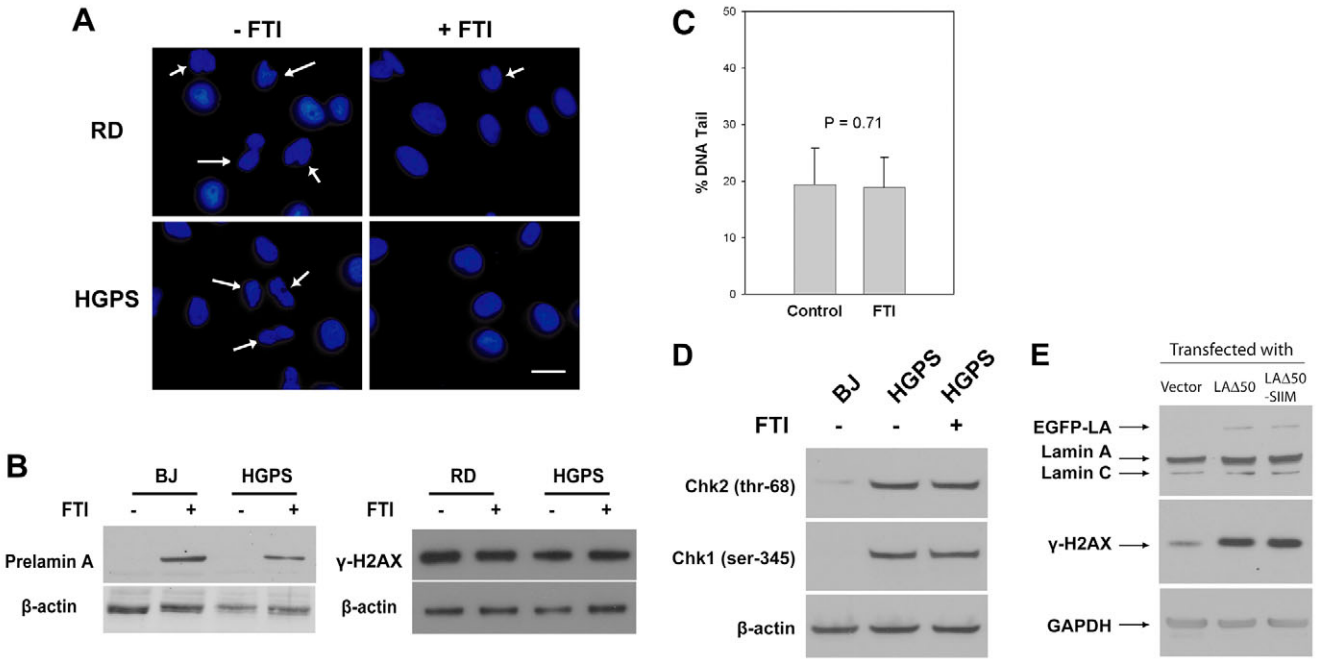

Fig. 5. FTI treatment of HGPS and RD cells. (A) Improvement of nuclear shapes of HGPS and RD cells by FTI treatment. The patient cells were treated with FTI and prepared for immunofluorescence microscopy as described in Materials and Methods. The nuclei were stained with DAPI. Misshapen nuclei are indicated by arrows. Photomicrographs were taken at 63× magnification. Bar, 50 μm. (B) FTI treatment blocked farnesylation of prelamin A, but did not change the production of γ-H2AX, a molecular marker for DSBs. In the left panel, BJ and HGPS cells were lysed in 2× SDS gel loading buffer and probed with the antibody directly against prelamin A. In the right panel, HGPS and RD cells were lysed and probed with the antibody for γ-H2AX. β-actin was loaded to ensure similar amounts of samples were used for each pair of experimental groups. (C) Comet assays were performed with HGPS cells in the presence and absence of FTI as described in Materials and Methods. DSBs of 50 randomly chosen cells were quantitated as the percentage of DNA in tail. (D) Phosphorylation of Chk1 and Chk2 in HGPS cells with or without FTI treatment. The FTI treatment of BJ and HGPS and western blotting were performed as described in Materials and Methods. The phosphorylation statuses of Chk1 at Ser-345 and Chk2 at Thr-68 were determined with corresponding antibodies. β-actin was probed as a loading control. (E) Accumulation of γ-H2AX in cells expressing prelamin A mutants. HeLa cells were transfected with plasmid pEGFP (empty parent vector), pEGFP-LAΔ50 or pEGFP-LAΔ50-SSIM, followed by western blot analysis. GAPDH was probed as a loading control.

and ATR in the checkpoint-activated HGPS cells. These proteins could be partially resistant to extraction for western blot analysis. However, regardless of the basis for the lowered levels of ATM and ATR in the controls, a relative knockdown by the siRNAs was observed. As shown in Fig. 4B, the knockdown significantly increased DNA synthesis in HGPS cells, while having no obvious effect on that of the control BJ cells. Similar data were produced by treating the cells with caffeine (Fig. 4B). These results confirm that DNA damage checkpoints were activated in the patient cells, and demonstrate that the replicative senescence of these patient cells can be reversed by inactivation of checkpoint kinases.

FTI treatment, a potential therapy for HGPS and RD?

Recent studies showed that FTI treatment can correct aberrant nuclear morphology in HGPS fibroblasts (Capell et al., 2005; Mallampalli et al., 2005; Toth et al., 2005) and RD fibroblasts (Toth et al., 2005). FTI treatment also ameliorates disease phenotypes in *Zmpste24*-deficient mice (Fong et al., 2006). Since DNA damage accumulation is believed to be one of the major causes of accelerated aging, cellular senescence and normal aging (d'Adda di Fagagna et al., 2003; Gorbunova and Seluanov, 2005; Kirkwood, 2005; Lees-Miller, 2005; Lombard et al., 2005; Misteli and Scaffidi, 2005; von Zglinicki et al., 2005), it is of great interest to test whether FTIs can also reduce the accumulated DNA damage in these cells. As shown in Fig. 5A, treatment of HGPS and RD fibroblasts of passage 15 with L-744832, a potent FTI (Capell et al., 2005), significantly reduced the percentage of cells with misshapen nuclei (from 47% to 11% for RD cells, $P<0.005$; from 33% to 6% for HGPS cells, $P<0.001$). The misshapen nuclei were defined as nuclei with blebs, folds or gross irregularities in shape (Toth et al., 2005), and the counting was performed by two observers who randomly chose 200 cells from each experimental group. This result confirms that FTI treatment can normalize the nuclear morphology of the patient cells. Consistently, FTI treatment of BJ and HGPS fibroblasts caused accumulation of prelamin A in these cells as analyzed by western blotting (Fig. 5B) with prelamin A-specific antibody (Sinensky et al., 1994a), demonstrating the efficacy of the FTI in blocking farnesylation in cells. By contrast, however, no substantial reduction of DSBs was detected in HGPS and RD cells after FTI treatment, as evidenced by the amount of γ-H2AX analyzed by western blotting (Fig. 5B). The same HGPS cells were also subjected to single cell gel (SCG) electrophoresis or comet assays that directly measured the DSBs in cells. As shown in Fig. 5C, there was no substantial difference in the amount of DNA damage generated in the cells with and without FTI treatments. In

addition, ATM and ATR damage checkpoint signaling was also examined. As shown in Fig. 5D, both the checkpoint substrates, Chk1 and Chk2, of ATR and ATM, were well phosphorylated in FTI treated and untreated HGPS cells, indicating their activation. Importantly, the phosphorylation was equally efficient in the cells with or without FTI treatment. These observations indicated that FTI treatment was unable to reduce the accumulated DNA damage in these cells despite its capacity to improve the nuclear morphology.

To further confirm the results, pEGFP-LAΔ50 and pEGFP-LAΔ50-SIIM plasmid constructs, respectively, were transfected into HeLa cells. pEGFP-LAΔ50-SIIM is a construct for expression of the LAΔ50 with a mutation at its farnesylation site, making the progerin prenylation-incompetent (Capell et al., 2005). Unlike FTI treatment, which might not be able to completely abolish the farnesylation of progerin, expression of pEGFP-LAΔ50-SIIM produces only the unfarnesylated LAΔ50. As shown in Fig. 5E, the expression of LAΔ50 and prenylation-incompetent LAΔ50-SSIM in HeLa cells induced similar levels of γ-H2AX accumulation, indicating that farnesylation had no substantial effect on the cellular DNA damage accumulation induced by progerin. This is consistent with the above results obtained with HGPS cells. Our results also suggested that DNA damage accumulation and misshapen nuclei are perhaps two independent phenotypes produced by prelamin A accumulation in HGPS and RD.

Discussion

DNA damage is believed to contribute to both aging and cellular senescence (Lombard et al., 2005) which has been regarded as a permanently maintained DNA damage response state (von Zglinicki et al., 2005). Defects in several DNA repair proteins lead to DNA damage accumulation and damage responses, which cause phenotypes reminiscent of premature aging (Lombard et al., 2005). As there is no evidence for any mutations in DNA repair genes in HGPS and RD cells, we, and others, hypothesize that prelamin A accumulation affects DNA repair in these syndromes. In this study, we present direct evidence that DNA damage checkpoints were constantly activated in HGPS and RD cells because of accumulated DNA damage. We also demonstrated that the subcellular distribution of checkpoint kinases ATM and ATR might be used as an indicator for their activation in vivo. Inactive ATM is homogenously distributed in the nucleus, whereas nuclear focus formation of ATM indicates its activation. In unstressed cells, ATR is mainly localized in cytosol, but translocates into the nucleus upon DNA-damaging stress such as UV irradiation. Our finding is contradictory to that of Manju et al., who recently reported that ATR was normally localized in the nucleus (Manju et al., 2006). The explanation for this discrepancy might lie in the fact that Flag-ATR was ectopically expressed in cells in the Manju et al. study, whereas we used normal cells and stained endogenous ATR directly. The expression of exogenous proteins probably induces stress to the cells, which might cause the translocation of ATR from cytosol to the nucleus. In the present study, activation of ATR and ATM in the patient cells as evidenced by immunofluorescence determination was confirmed by the phosphorylation of their downstream substrates Chk1, Chk2 and p53 as analyzed by western blotting.

Interestingly, inactivation of ATM and ATR in HGPS cells partially restored cell-cycle progression into S-phase. This confirms that a form of prelamin A (progerin) activated DNA damage responses, leading to replicative senescence. Importantly, this senescence can be suppressed by inactivating DNA damage response pathways in HGPS cells. That only partial restoration of S-phase progression is observed is probably because of the incomplete repression of the DNA damage responses, as even a residual kinase activity could be sufficient to enforce a DNA damage checkpoint (Cortez et al., 2001). Varela et al. showed that p53 knockout completely recovered the proliferative capacity of Zmpste24$^{-/-}$ mouse cells (Varela et al., 2005). We expect that a highly efficient checkpoint inhibition would permit a significant recovery of cell division and alleviate many other senescence-associated phenotypes in progeroid cells.

Aberrant nuclear morphology is the most obvious phenotype caused by prelamin A accumulation in HGPS and RD fibroblasts (Goldman et al., 2004; Toth et al., 2005). Recent studies showed that FTI treatment could correct the nuclear morphology defects of progeroid cells (Capell et al., 2005; Glynn and Glover, 2005; Mallampalli et al., 2005; Toth et al., 2005). However, we found that FTI treatment could not reduce the accumulated DSBs in both HGPS and RD cells. This suggests that DNA damage accumulation and misshapen nuclei are probably two unrelated phenotypes caused by prelamin A accumulation in HGPS and RD. Consistent with this notion, p53 knockout can restore proliferative capacity of Zmpste24$^{-/-}$ mouse cells, but only partially reverse other disease phenotypes (Varela et al., 2005), suggesting that independent pathological pathways exist and cooperate with each other in the generation of progeroid phenotypes. Thus, strategies for treatment of HGPS need to combine elimination of DNA damage accumulation as well as normalization of nuclear morphology.

Materials and Methods

Cell culture and drug treatments

Fibroblasts from a HGPS patient with the point mutation 1824C→T were obtained from the Coriell Cell Repository (no. AG11513A). Human RD fibroblasts were a gift from J. H. Miner (Washington University School of Medicine, St Louis, MO). BJ cells and HeLa cells were purchased from American Type Culture Collection (ATCC, numbers CRL-2522 and CCL-2, respectively). All cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) (for RD cells and HeLa cells) or Eagle's minimal essential medium (EMEM) (for HGPS cells and BJ cells) supplemented with 10% fetal bovine serum (FBS) and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) at 37°C under an atmosphere containing 5% CO_2. For FTI treatment, cells were cultured to 70% confluence, and treated with 5 μM FTI L-744832 (Biomol, Plymouth Meeting, PA) daily for 72 hours before harvest. For the inactivation of ATR and ATM, cells were treated with caffeine at a final concentration of 5 mM for at least 2 hours before further analysis.

Immunofluorescence microscopy

Cells were grown on coverslips to 70% confluence, washed twice with PBS, and then fixed with cold methanol (–20°C) or with 1% formaldehyde followed by permeabilization with 0.5% Triton X-100. The fixed cells were blocked with 15% FBS, and then incubated with a primary antibody against ATR (rabbit or mouse; GeneTex, San Antonio, TX), ATM (mouse; GeneTex), GFP (rabbit; Abcam, Cambridge, UK) or γ-H2AX (mouse; StressGen Biotechnologies Group, Victoria, Canada). After three washes with PBS-1% Tween-20, the cells were incubated with a secondary antibody, Alexa Fluor 488-conjugated donkey anti-rabbit immunoglobulin G (IgG) or Alexa Fluor 568-conjugated goat anti-mouse IgG (Molecular Probes). Nuclei were counterstained with DAPI. Cells were visualized by using a Zeiss Axioscope microscope.

Transfection of plasmids and siRNA

HeLa cells grown on coverslips were transiently transfected with pEGFP-LAΔ50 and pEGFP-LAΔ50-SSIM plasmid constructs, respectively (both were gifts from Francis Collins, NIH), control plasmid pEGFP or empty parent vector using

GeneJammer transfection reagent (Stratagene) following manufacturer's instructions. Twenty-four hours post-transfection, the cells were processed differently for the following experiments. For examining the activation of ATM and ATR, the cells were irradiated with 20 J/m^2 UV or mock treated. Two hours post-treatment, the cells were processed for immunofluorescence microscopy as described above. To measure the amount of γ-H2AX, the cells were harvested and lysed for western blotting as described below. For detecting the formation of γ-H2AX foci, the cells were fixed with 1% formaldehyde and processed for immunofluorescence.

For the knockdown of ATR and ATM by RNAi, the cells were transfected with ATR siRNA and ATM siRNA (Wu et al., 2006), or GFP siRNA as a control using TransIT-TKO transfection reagent (Mirus, Madison, WI) following manufacturer's instructions. Further analyses were performed 72 hours after transfection.

DNA synthesis assay

DNA synthesis was assayed by the method of thymidine incorporation modified from Shao et al. (Shao et al., 1997). Briefly, 2×10^5 cells were seeded in a 35-mm dish 24 hours before pulse-labeling with 0.5 μCi/ml [methyl-^{3}H] thymidine (Amersham Biosciences) for 30 minutes. The cells were then rinsed with PBS three times and harvested by lysis with 5% trichloroacetic acid (TCA) at 4°C for 1 hour. Cell lysates were subjected to filtering using Whatman glass microfibre filters and a vacuum manifold. The filters were washed twice with 5 ml of 5% TCA, once with 70% ethanol and then dried. The radioactivity of each sample was counted by liquid scintillation.

Western blotting

Cells cultured in 100-mm dishes were grown to 70% confluence and then trypsinized. Cell number was counted by using a hemacytometer. The cells were centrifuged at 377 ***g*** for 5 minutes and washed twice with PBS. Cell pellet was lysed in 2× SDS gel loading buffer and volumes corresponding to 5×10^6 cells were subjected to SDS-PAGE. Immunoblotting was performed as previously described (Liu et al., 2005b) with primary antibodies directed against p53 (Santa Cruz), p53 (ser-15) (Cell Signaling Technology, Beverly, MA), Chk2 (thr-68) (Cell Signaling Technology), Chk1 (ser-345) (Santa Cruz), γ-H2AX (Bethyl Laboratories, Montgomery, TX), GAPDH (Santa Cruz), LaminA/C (Santa Cruz) and β-actin (Santa Cruz). The rabbit anti-mouse prelamin A antiserum used was generated specifically against the carboxyl-terminal prelamin A and cannot bind mature lamin A or lamin C (Sinensky et al., 1994a).

Comet assay

The neutral comet assay was performed to assess DNA strand breaks in cells. The first layer of agarose on microscope slides was prepared by dipping the slides into 1% normal melting agarose (NMA) followed by drying. An amount (85 μl) of 0.5% low melting agarose (LMA) containing 4×10^5 cells was made by mixing 10 μl cell suspension with 75 μl LMA, and then poured onto the pre-coated slides. Slides were immersed in freshly prepared ice-cold buffer (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-HCl, 1% Triton X-100, pH 10) to lyse the cells for at least 1 hour at 4°C in the dark. The slides were then placed in the alkaline buffer (0.3 M NaOH, 1 mM EDTA, pH>13) for 30 minutes for DNA unwinding. The slides were equilibrated in Tris-borate-EDTA (TBE) buffer for 5 minutes twice, followed by electrophoresis at 1 volt/cm in TBE buffer for 10 minutes. The slides were then dipped in 70% ethanol for 5 minutes and dried at room temperature for 1 hour. An amount (50 μl) of 600 μM DAPI was used for staining. All steps described above were performed under dimmed light to prevent additional DNA damage. The quantification of the comets was performed for randomly chosen 50 cells, and DNA damage was expressed as the percentage of DNA in tail.

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