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Mutant lamin A links prophase to a p53 independent senescence program

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Expression of oncogenes or short telomeres can trigger an anticancer response known as cellular senescence activating the p53 and RB tumor suppressor pathways. This mechanism is switched off in most tumor cells by mutations in p53 and RB signaling pathways. Surprisingly, p53 disabled tumor cells could be forced into senescence by expression of a mutant allele of the nuclear envelope protein lamin A. The pro-senescence lamin A mutant contains a deletion in the sequence required for processing by the protease ZMPSTE24 leading to accumulation of farnesylated lamin A in the nuclear envelope. In addition, the serine at position 22, a target for CDK1-dependent phosphorylation, was mutated to alanine, preventing CDK1-catalyzed nuclear envelope disassembly. The accumulation of this mutant lamin A compromised prophase to prometaphase transition leading to invaginations of the nuclear lamina, nuclear fragmentation and impaired chromosome condensation. Cells exited this impaired mitosis without cytokinesis and re-replicated their DNA ultimately arresting in interphase as polyploid cells with features of cellular senescence including increased expression of inflammatory gene products and a significant reduction of tumorigenicity *in vivo*.

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Introduction

Cellular senescence is a permanent cell cycle arrest with a dual role in biology. First, senescence is a barrier for tumorigenesis since it prevents the expansion of cells with the potential to form malignant tumors. On the other hand, senescent cells accumulate in old tissues and could be responsible for age-associated diseases.[1] The mechanisms promoting senescence during aging are currently unknown. Senescent cells accumulate in mouse models of premature aging where the underlying defect affects mitosis.[2,3] In humans, the Hutchinson-Gilford Progeria Syndrome (HGPS) mimics some aspects of human aging.[4-6] HGPS cells accumulate a variant of the nuclear envelope protein lamin A, known as progerin, which is responsible for the disease. Progerin can induce cellular senescence although the direct mechanism remains to be identified. Cells expressing progerin have genomic instability,[7] telomere dysfunction,[8] altered epigenetic modifications of histones,[9,10] abnormal chromosome segregation, binucleation[11] and changes in nuclear architecture.[12] The exact molecular mechanism explaining these alterations and their connection to cellular senescence is unknown. It is plausible that a defect in some key function of the nuclear lamina underlies cellular senescence and aging.

The nuclear lamina is mostly composed of several types of lamins and their associated proteins. Lamins of type A and B undergo C-terminal farnesylation, a process that contributes to their localization at the nuclear membrane. However, the C-terminus of lamin A is subsequently cleaved-off by the protease ZMPSTE24/FACE1.[13] Progerin is a lamin A mutant with 50-aa deletion near the C-terminus.[4,5] The deleted fragment contains the cleavage site for the ZMPSTE24 protease, so progerin cannot go through the last endoproteolytic step that cleaves the C-terminal extension of pre-lamin A and stays permanently farnesylated in the nuclear lamina.[14] Inhibitors of farnesyl transferases can rescue some of the defects of cells expressing progerin[12] suggesting that permanently farnesylated lamin A is responsible for the disease. Hutchinson-Gilford Progeria Syndrome is not accompanied by cancer development[15] despite the accelerated aging caused by progerin expression. Although, it is plausible that children with this disease do not live long enough to show an increase in cancer incidence, it is also likely that the ability of progerin to impair cell cycle progression and regulate senescence[8] can halt cancer progression in these patients perhaps pointing to a novel mechanism to treat human cancers.

During every cell cycle the nuclear lamina is disassembled at prophase and then reassembled during cytokinesis.[16] The mitotic CDK/cyclin complexes phosphorylate the N-terminus conserved domain of lamin A at serine 22 (S22) and the C-terminus, at serine 392 (S392), inducing lamina depolymerization.[17,18] Mutations in these phosphorylation sites in lamin A inhibit nuclear lamina disassembly.[19] Here, we report that mutating the serine 22 phosphorylation site of progerin (S22A-progerin) enhances its

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ability to induce cellular senescence characterized by DNA damage, expression of inflammatory cytokines and inhibition of tumor progression in mice. The effects requires farnesylation and the formation of a thick nuclear envelope, is associated with nuclear membrane invaginations, nuclear fragmentation and polyploidy and is independent of p53.

Results

Accumulation of farnesylated prelamin A inhibits tumor cell proliferation

Progerin and L647Rprelamin A are mutant alleles of prelamin A with a disabled ZMPSTE24 cleavage site.[20] Stable expression of these mutant prelamin A genes from retroviral vectors in the human osteosarcoma cell line U-2 OS induced a strong growth inhibition over 6 d of culture while overexpression of wild type prelamin A had a lesser effect (**Fig. 1A and B**). We also expressed wild type prelamin A and its mutants in HeLa cervical cancer cells (containing the papillomavirus proteins E6 and E7 that inactivate p53 and RB), H 1299 lung cancer cells (with mutant p53) and PC-3 prostate cancer cells (with mutant p53). Again, mutant prelamin A alleles inhibited the proliferation of these tumor cells over a 6 day period of culture although the effect was not as striking as in U-2 OS cells (**Fig. S1A-C**).

Since progerin and L647R prelamin A remain both farnesylated we mutated the cysteine in the farnesylation site (the CaaX-motif) into serine to prevent farnesylation.[14] Non-farnesylated progerin (ProgCS) and L647R mutant (L647RCS) had the same proliferation rates as control cells (**Fig. S1D and E**). Farnesylation of prelamin A contributes to targeting prelamin A to the nuclear membrane.[20] In agreement, farnesylated mutant lamins strongly accumulated in the nuclear lamina of interphase cells, while elimination of farnesylation led to different patterns for progerin and L647R staining (**Fig. S1F**). Non-farnesylated progerin (ProgCS) formed intranuclear foci in around 50% of cells and the rest of the cells had low lamin A/C signal localized both at the nuclear envelope and the nucleoplasm. Non-farnesylated L647R did not form intranuclear foci as ProgCS but its accumulation at the nuclear envelope was reduced in comparison with farnesylated L647R. These results are consistent with previous work reporting that non-farnesylated progerin was unable to induce nuclear alterations in cell culture[21] or the progeria phenotype in mice.[22] We conclude that farnesylation is required for the growth inhibition induced by progerin and the L647R mutant prelamin A.

The farnesylated C-terminus of prelamin A is cleaved off by the zinc metallopeptidase ZMPSTE24/FACE-1[23] in the inner nuclear membrane.[24] It is known that siRNAs against ZMPSTE24/FACE-1 lead to accumulation of farnesylated prelamin A and growth inhibition in HeLa cells.[25] Using an shRNA against ZMPSTE24/FACE-1 (shZMPSTE24) (**Fig. 1C**) we confirmed this growth inhibition phenotype in osteosarcoma U-2 OS cells (**Fig. 1D**). Western blot analysis revealed accumulation of uncleaved prelamin A in U-2 OS cells expressing shZMPSTE24 and induction of the p53 target p21 (**Fig. 1E**). In p53 null H 1299 cells, shZMPSTE24 was not as efficient as in U-2 OS cells in leading to accumulation of prelamin A and growth inhibition (**Fig. 1F and G**). Combining expression of wild type lamin A with shZMPSTE24 induced a stronger growth arrest in H 1299 cells (**Fig. 1G**) suggesting that growth inhibition depends on the amount of prelamin A. This accumulation of prelamin A in the nuclear lamina was observed by immunofluorescence using an antibody against lamin A/C in H 1299 cells (**Fig. 1H**).

Inhibition of phosphorylation at serine 22 in progerin increases its ability to inhibit tumor cell proliferation

Phosphorylation of the N-terminus of lamin A is required for disassembly of the nuclear lamina during mitosis.[18,19] Synchronization of U-2 OS cells expressing progerin, or non-farnesylated progerin by nocodazole treatment revealed that progerin could be serine 22-phosphorylated at G2/M (Supplemental **Fig. S2A**). Nevertheless, accumulation of progerin compromised proper nuclear lamina disassembly (**Fig. S2B**) resulting in moderate cell growth inhibition (**Fig. 1B**). We thus reasoned that a progerin mutant unable to undergo phosphorylation at serine 22 should accumulate in the nuclear lamina at higher levels and inhibit further tumor cell proliferation. We expressed S22A-progerin in several tumor cell lines and compared the effects with the same cells expressing progerin or an empty vector control. In all cases, S22A-progerin led to stronger growth arrest as measured in growth curves over 6 d or a colony formation assay over 13 d (**Fig. 2A-E**).

For visualization of S22A-progerin and progerin we performed immunofluorescence and found that S22A-progerin formed a thicker nuclear lamina than progerin (**Fig. 2F** see also **Fig. 4**). Intriguingly, the majority of the S22A-progerin expressing cells displayed multiple nuclei and a very large nuclear and cell size (**Fig. 2G**). To investigate whether farnesylation was also required for growth inhibition by S22A-progerin we created a mutant in which cysteine in the CaaX-motif was replaced by serine preventing farnesylation.[14] S22A-progerinCS, like progerinCS did not affect cell growth in U-2 OS cells (**Fig. S3A and B**) and did not accumulate in the nuclear envelope (**Fig. S3C**). Importantly, the level of expression of S22A-progerinCS was higher than S22A-progerin indicating that interfering with nuclear envelope functions rather than an overexpression artifact is the cause of the cell cycle arrest. Together, these results suggest that a stable lamin A cage around the nucleus impairs cell proliferation.

S22A-progerin induces nuclear envelope invaginations and polyploidy

We next characterized in detail the phenotype of large multinucleated cells induced by S22A-progerin. DNA content analysis revealed that a large portion of S22A-progerin-expressing cells had abnormally high levels of DNA peaking at 4 times the normal diploid genome (**Fig. 3A**). The low expression of cyclin B1 and cyclin A (**Fig. 3B**) suggests that the S22A-progerin cell population contained a significant fraction of cells arrested at G1, since these 2 proteins mainly

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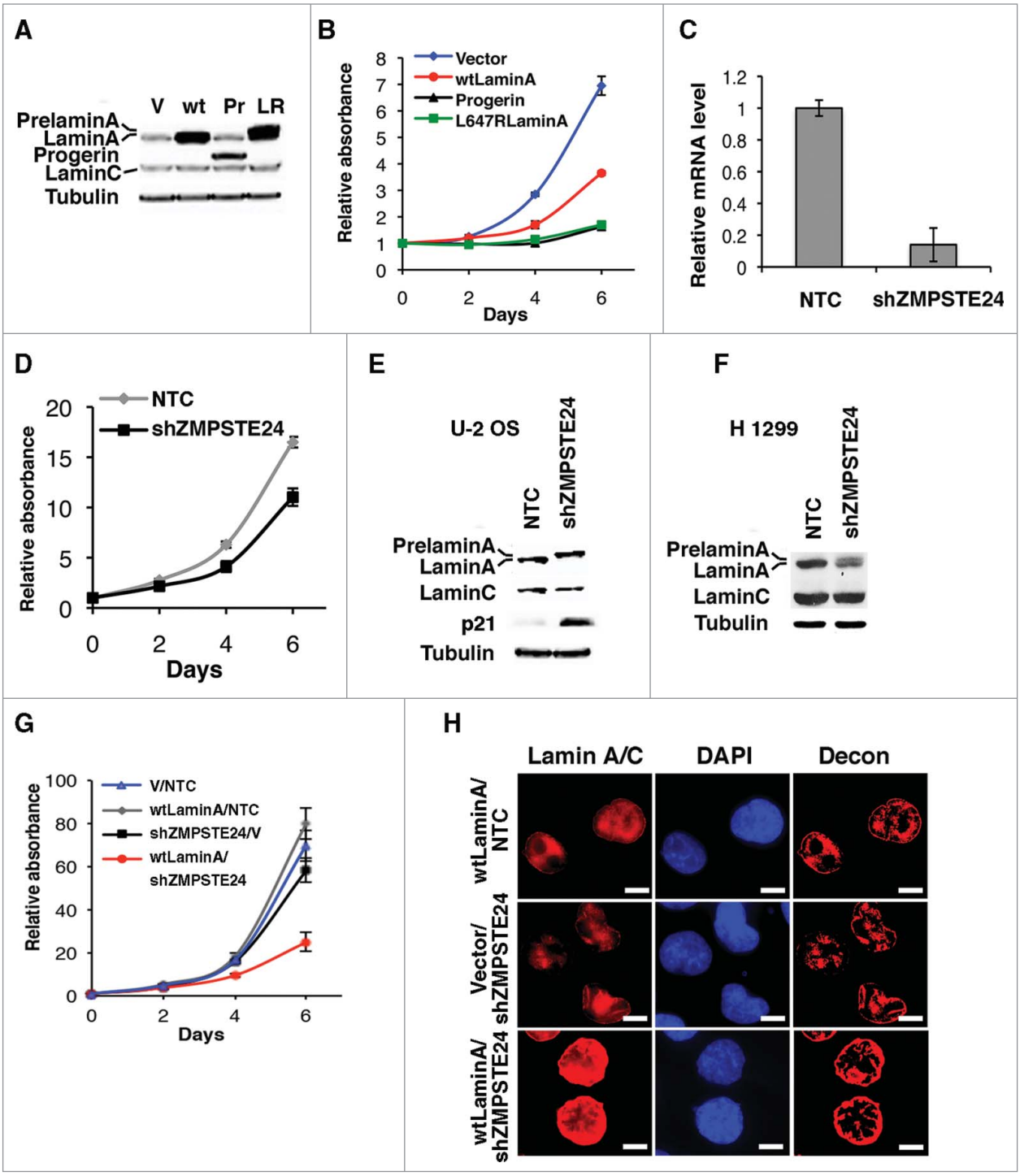

Figure 1. Accumulation of lamin (**A**) in the nuclear envelope inhibits cancer cell growth. (**A**) Immunoblot for lamin A/C of U-2 OS cells expressing vector (V), wild type lamin A (wt), progerin (Pr) or L647R lamin A (LR). (**B**) Growth curves of U-2 OS cells expressing vector, progerin, L647R lamin A or wild type lamin A (wtLaminA). Cells were plated in triplicate 4 d after infection with a retroviral vector expressing wtLamin A, progerin, or L647R lamin A mutant. Relative cell numbers were estimated from a crystal violet staining assay. (**C**) qPCR showing *ZMPSTE24* mRNA levels of U-2 OS cells with a control shRNA (NTC) or shZMPSTE24. (**D**) Growth curves of U-2 OS cells expressing shZMPSTE24 or NTC. (**E**) Immunoblots for the indicated proteins of U-2 OS cells and (**F**) H 1299 with a control shRNA (NTC) or shZMPSTE24. (**G**) Growth curves of H 1299 cells expressing wild type lamin A and NTC (wtLaminA/NTC), shZMPSTE24 and vector (shZMPSTE24/V), or wild type lamin A and shZMPSTE24 (wtLaminA/shZMPSTE24). (**H**) Immunofluorescence images of H 1299 cells expressing the vectors indicated in (**G**). Magnification = 10 μm.

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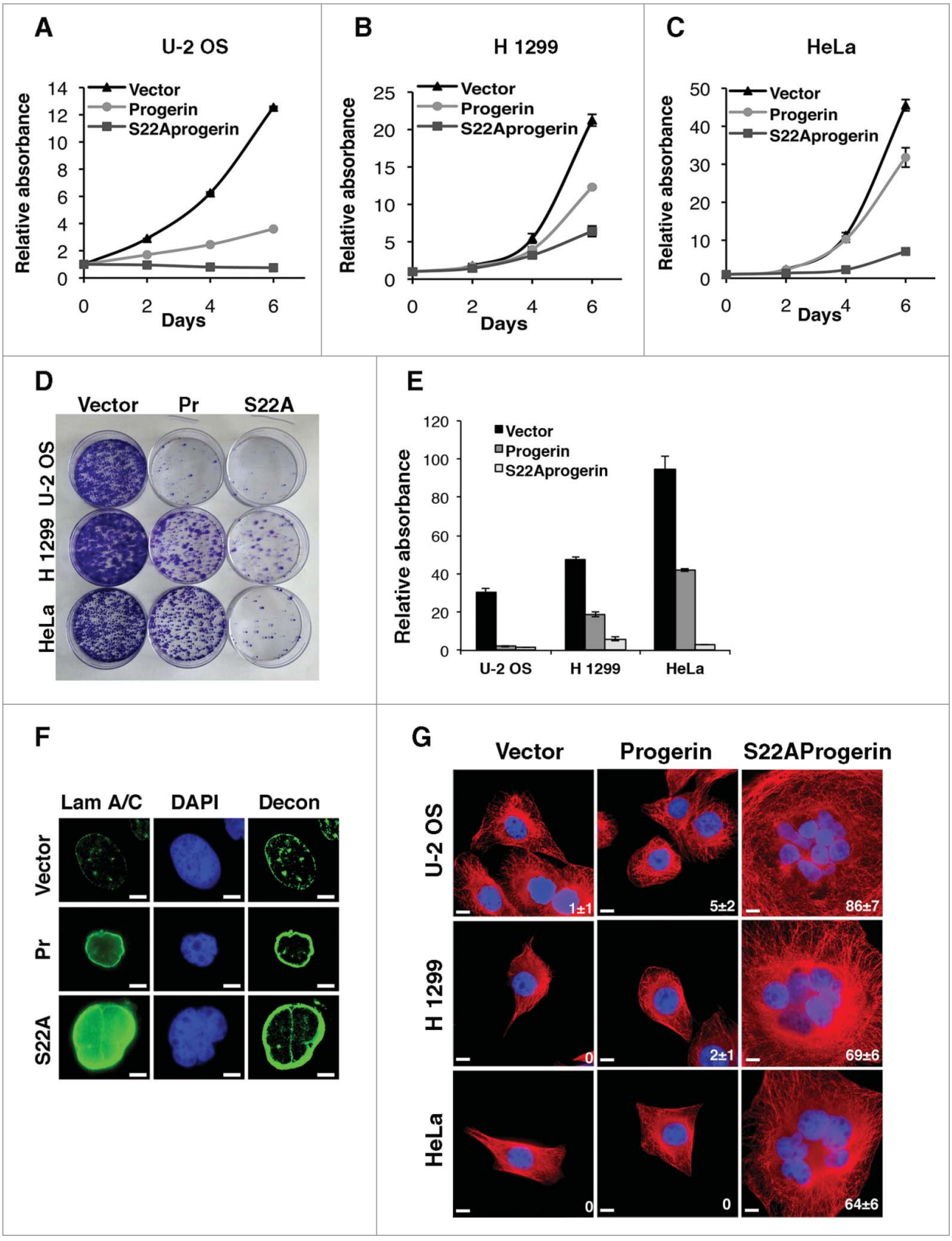

Figure 2. S22A-progerin triggers a cell proliferation arrest and a multinucleated phenotype in tumor cells. (**A-C**) Growth curves of different cancer cell lines expressing the indicated vectors. (**D**) Colony formation assay of cell lines as in (**A-C**). (**E**) Quantitation of cell growth in the colony formation assay using crystal violet staining. (**F**) Immunofluorescence for lamin A/C of U-2 OS cells expressing a vector control, progerin (Pr) or S22A-progerin (S22A). Decon means deconvolution option of Metamorph software. (**G**) Immunofluorescence showing the cell nucleus (blue-DAPI staining) and the cytoskeleton (red-Tubulin) in the indicated cell lines expressing vector, progerin or S22A-progerin. The percent and S.D of multinuclear cells are indicated at the bottom right of each panel. Magnification = 10 μm.

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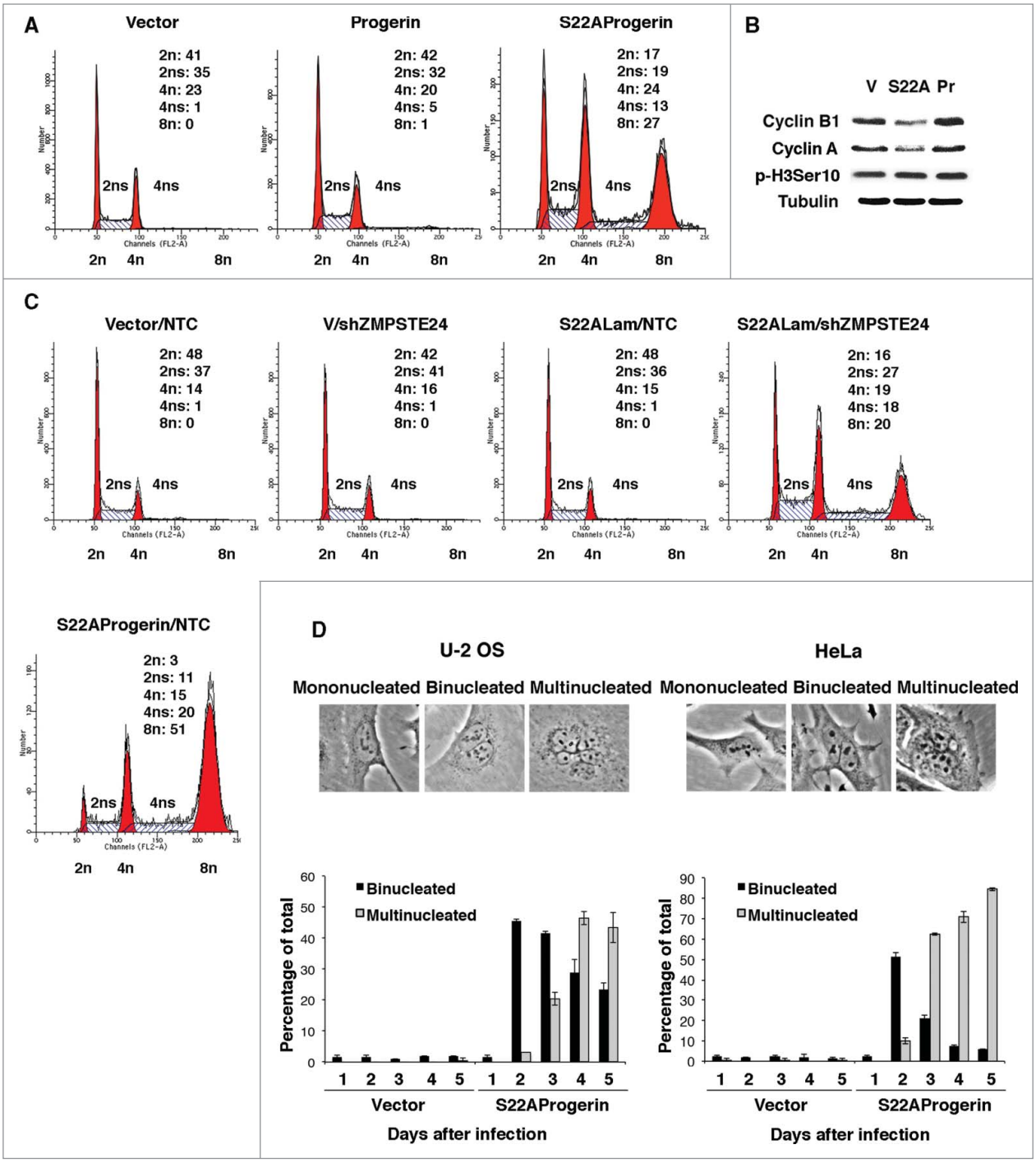

Figure 3. DNA content analysis reveals the polyploidy of the S22A-progerin-expressing cells. (**A**) DNA content analysis was performed by fluorescence activated cell sorting (FACS) of U-2 OS cells expressing vector, progerin, or S22A-progerin. Quantification of the DNA content analysis (%) is shown in each panel. (**B**) Immunoblots for the indicated proteins of U-2 OS cells expressing the vectors as in (**A**). (**C**) FACS of U-2 OS cells expressing vector and a control shRNA (NTC), vector (V) and shZMPSTE24, S22A-wild type lamin A (S22ALam) and NTC, S22ALam and shZMPSTE24, or S22A-progerin and NTC. Quantification of DNA content analysis (%) of FACS is shown in each panel. (D) Quantification of cells with 2 or more nuclei in U-2 OS or HeLa cells after infections with a retroviral vector expressing S22A-progerin or an empty vector control.

peak at G2/M. However, the reduction of levels of cyclin A and B is moderate and we can not discard that some cells are also arrested in G2. Simultaneous expression of S22A-wild type-lamin A (S22ALam) with shZMPSTE24 also induced polyploidy in U-2 OS cells, although in smaller fraction of cells compared with S22A-progerin (**Fig. 3C**). Careful observations of nuclei in U-2 OS or HeLa cells shortly after expressing S22A-progerin indicates first an accumulation of

binucleated cells that later become multinucleated without overall increase in cell numbers (**Fig. 3D**).

Multinucleation could arise by normal mitotic progression with a failure in cytokinesis[26] or by nuclear fragmentation as seen in cancer cells treated with chemotherapeutic drugs.[27] Visualization of progerin at early times after their expression, when cells are not yet multinucleated revealed an invagination of progerin and presumably its attached nuclear membrane in cells that express S22A-progerin consistent with a nuclear fragmentation model for multinucleation (**Fig. 4A**). Electron microscopy clearly revealed invaginations of the nuclear envelope that occasionally fragmented the nucleus (**Fig. 4B** and **Fig. S4**). Treatment of S22A- progerin expressing cells with nocodozale did not allow visualizing condensed chromosomes as seen in progerin expressing cells or in control cells with an empty vector (**Fig. 4C**). This result suggests that S22A-progerin-expressing cells are compromised at the transition between prophase and prometaphase, the stage at which chromosomes condense and the nuclear envelope dismantles.

We noticed that the proportion of multinucleated cells was higher in the p53 disabled cell line HeLa when compared with the p53 wild type U 2-OS (**Fig. 3D**). To investigate whether p53 limits multinucleation in cells expressing S22A-progerin we inactivated p53 expression in U 2-OS using a validated shRNA. We found that expression of S22A progerin in U 2-OS cells expressing a shRNA against p53 expression increased the number of cells with a polyploid DNA content (**Fig. 5A**) and multiple nuclei (**Fig. 5B**). Immunoblots confirmed that the shRNA decreased p53 expression and its target p21 that were both induced by S22A progerin. In contrast, p16INK4a expression was not affected by expression of S22A progerin (**Fig. 5C**). S22A progerin increased DNA damage signals as measured with anti-γH2AX antibody and inactivation of p53 further amplified this signal (**Fig. 5C**). Likewise, S22A progerin decreased colony formation and inactivation of p53 also magnified this effect (**Fig. 5D, E**). Although most cells expressing S22A progerin remained viable, p53 inhibition increased the proportion of cell death as well (**Fig. 5F**). These results indicate that p53 is required for a checkpoint response that limits DNA replication, DNA damage and multinucleation in cells with altered nuclear envelope but is not required for growth arrest in response to S22A-progerin expression. Further proof that checkpoint functions of p53 limit multinucleation was obtained by using resveratrol, a drug that can prevent cell cycle progression at S phase.[28] Treatment of S22A progerin expressing cells with resveratrol induced accumulation of cells in S phase (**Fig. S5**) and reduced the number of multinucleated cells (**Fig. 5G**).

S22A-progerin induces tumor cell senescence

Drugs that interfere with mitosis usually kill cells by apoptosis in a process that requires the spindle checkpoint.[29] However, most cells expressing S22A-progerin remained viable. Since normal fibroblasts expressing progerin prematurely senesce[8] we next investigated the presence of senescence markers in tumor cells after introduction of progerin or S22A-progerin. We first used the senescence associated β-galactosidase marker and found that H 1299 and PC-3 cells expressing S22A-progerin but not progerin were positive for this marker (**Fig. 6A**). We also found DNA damage foci labeled with anti-γH2AX antibody in H1299 and PC3 cells expressing S22A progerin (**Fig. 6B**). We could not stain U-2 OS for senescence associated β-galactosidase because this cell line may have defects in the expression of this enzyme. However, many other senescence markers were positive in U-2 OS cells expressing S22A-progerin but not progerin. These markers included PML bodies (**Fig. 6C**) and DNA damage foci (**Fig. 6D**). Intriguingly, DNA damage foci in S22A progerin expressing were in association to the nuclear envelope decorating the nuclear periphery or the lamin A invaginations described above (**Fig. 6D** and **E**). S22A progerin induced senescence was also characterized by low expression of the proliferation antigen Ki-67 (**Fig. 6F**) and the E2F target MCM6 (**Fig. 6G**) and high expression of the CDK inhibitor p21 (**Fig. 6H**). We also compared the gene expression pattern of U-2 OS cells expressing S22A-progerin with the cells expressing wild type lamin A. Data from 3 independent experiments were analyzed with Flexarray 1.6.1.1 using robust multiarray average for normalization. Data is available at the GEO repository (GSE51349). Gene expression changes showing a fold of 2 or more were used for pathway analysis using the platform Babelomics.[30] The main functional category of upregulated genes involved cytokine and extracellular factors (**Fig. S6A, B** and **Table 1**) consistent with the concept that senescent cells exhibit a senescence associated secretory phenotype or SASP.[31] The expression of some of these extracellular factors was then confirmed by qPCR (**Fig. S6C**). Gene Set Enrichment Analysis (GSEA) revealed an overlap with gene sets related to senescence and the NF-κB transcription factor (Supplemental tables I and II). Immunoblots show phosphorylation of RelA at serine 536 and the p38 MAPK at the activation loop Thr180/Tyr182 both in progerin and S22A-progerin expressing cells indicating activation of these pathways by both mutant lamins (**Fig. 6I**). This result suggests that additional mechanisms may explain the increased expression of cytokines genes in S22A-progerin expressing cells. Both progerin and S22A progerin induced accumulation of the DNA damage marker γ-H2AX but the effect was much higher in S22A progerin expressing cells. Taken together, the results indicate that S22A-progerin, unlike progerin can force tumor cells to senesce. Progerin can induce senescence in normal human cells and this event is associated with telomere dysfunction.[32] The failure of progerin to induce senescence in a significant number of tumor cells could be due to better telomere maintenance in tumor cells or higher CDK activity due to defects in p16INK4a and other CDK inhibitors. In contrast, S22A-progerin creates a thicker lamin A cage around the nucleus (**Fig. 2F and 4A**) activating a senescence program in tumor cells with different genetic backgrounds and telomere maintenance.

To investigate whether the growth inhibition and senescence phenotype induced by S22A-progerin in tumor cells is sufficient to inhibit tumor formation in mice we inoculated 10^6 H 1299 cells expressing S22A-progerin or a vector control into nude mice. Most injections with H 1299 cells (9 out of 10) developed tumors that were detected 10 d after

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inoculation and grew aggressively 2 weeks after. In contrast, only 4 out of 10 injections with S22A-progerin formed tumors that appeared much later (25 d after inoculation) and were considerably smaller (**Fig. 7A–C**).

It is likely that the few and late growing tumors observed in mice injected with S22A-progerin expressing cells are the result of cells that express lower levels of lamin A. To investigate this issue we isolated tumors from mice inoculated with H 1299 cells expressing a vector control of S22A-progerin. Then we fixed the tumors and stained sections with an anti-lamin A antibody. We found a similar level of lamin A staining around the nucleus when compared tumors with empty vectors or S22A-progerin. These levels were not comparable to the high levels of lamin A around the nucleus in H 1299 cells expressing S22A-progerin before inoculation (**Fig. 7D**), suggesting that tumorigenesis selected against cells with a S22A-progerin nuclear cage. Intriguingly, qPCR analysis of S22A-progerin gene expression showed that tumors expressing this gene had high levels of the mRNA comparable to the cells before inoculation (**Fig. 7E**) but the protein was not detectable (**Fig. 7F**). The events leading to loss of S22A-progerin protein remain to be investigated. It has been reported that senescent cells can generate proliferating cells by a special kind of asymmetric cell division involving budding.[33] It is plausible that this mechanism may generate the tumor cells expressing low levels of S22A-progerin.

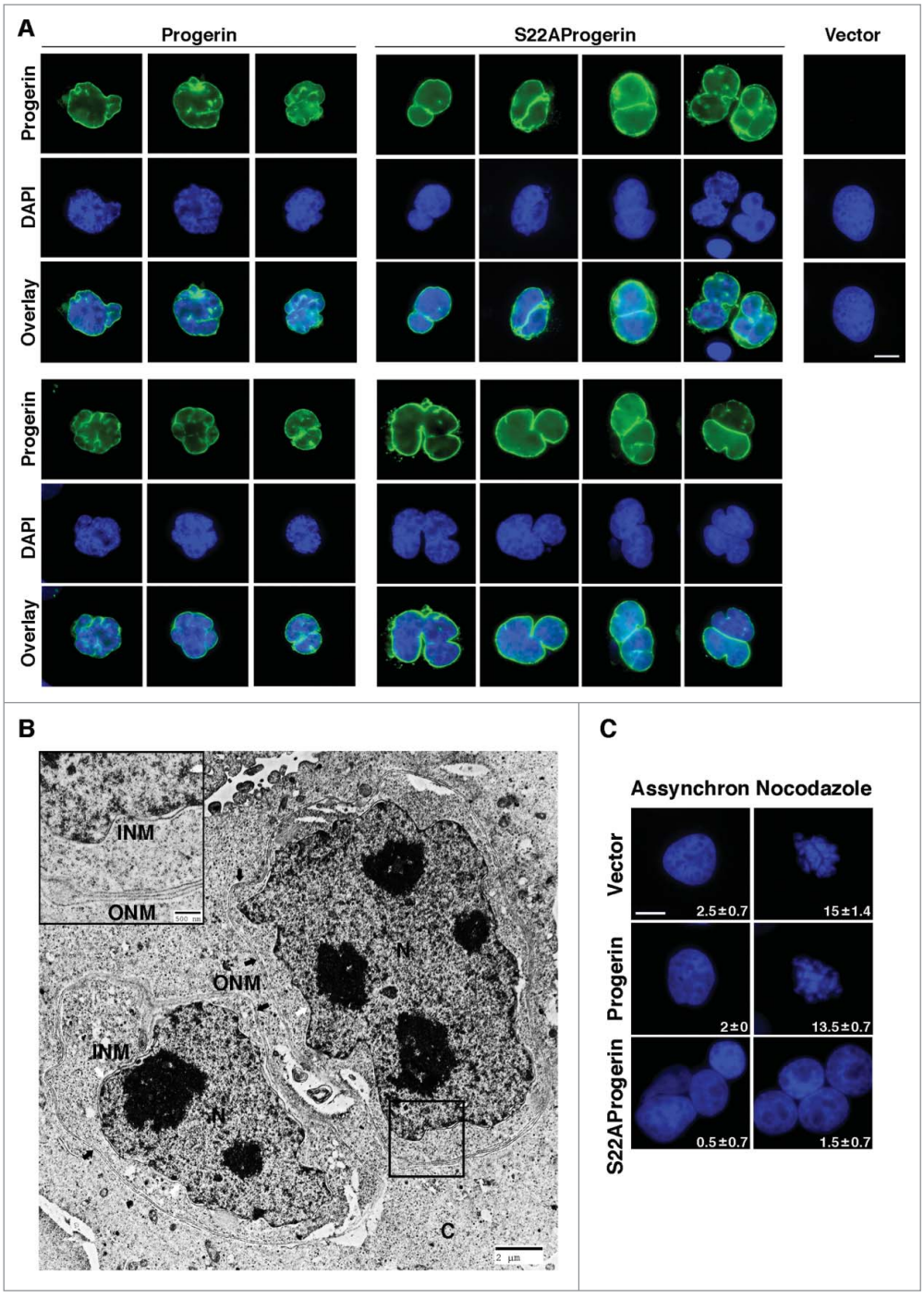

Figure 4. S22A-progerin expressing cells display invaginations of the nuclear envelope, nuclear fragmentation and a failure in chromosome condensation. (**A**) Immunofluorescence with a progerin specific antibody in U-2 OS cells expressing vector, progerin, or S22A-progerin. Magnification = 10 μm. (**B**) Electron microscopy showing a cell where a nuclear envelope invagination divides the nuclei in 2 fragments. The black arrows indicate the outer nuclear membrane (ONM) and the white arrows the inner nuclear membrane (INM). (**C**) Dapi staining of U-2 OS cells expressing vector, progerin, or S22A-progerin. The percent of cells with condensed chromosomes is indicated at the bottom right of each panel. Magnification = 10 μm.

Discussion

We show here that tumor cells can be forced into a senescent cell cycle arrest by expression of a mutated form of lamin A with 2 molcular defects. First, our lamin A mutant cannot be processed by the protease ZMPSTE24/FACE1 and stays farnesylated in the nuclear lamina. Second, we mutated the phosphorylation site at serine 22, implicated in nuclear envelope disassembly

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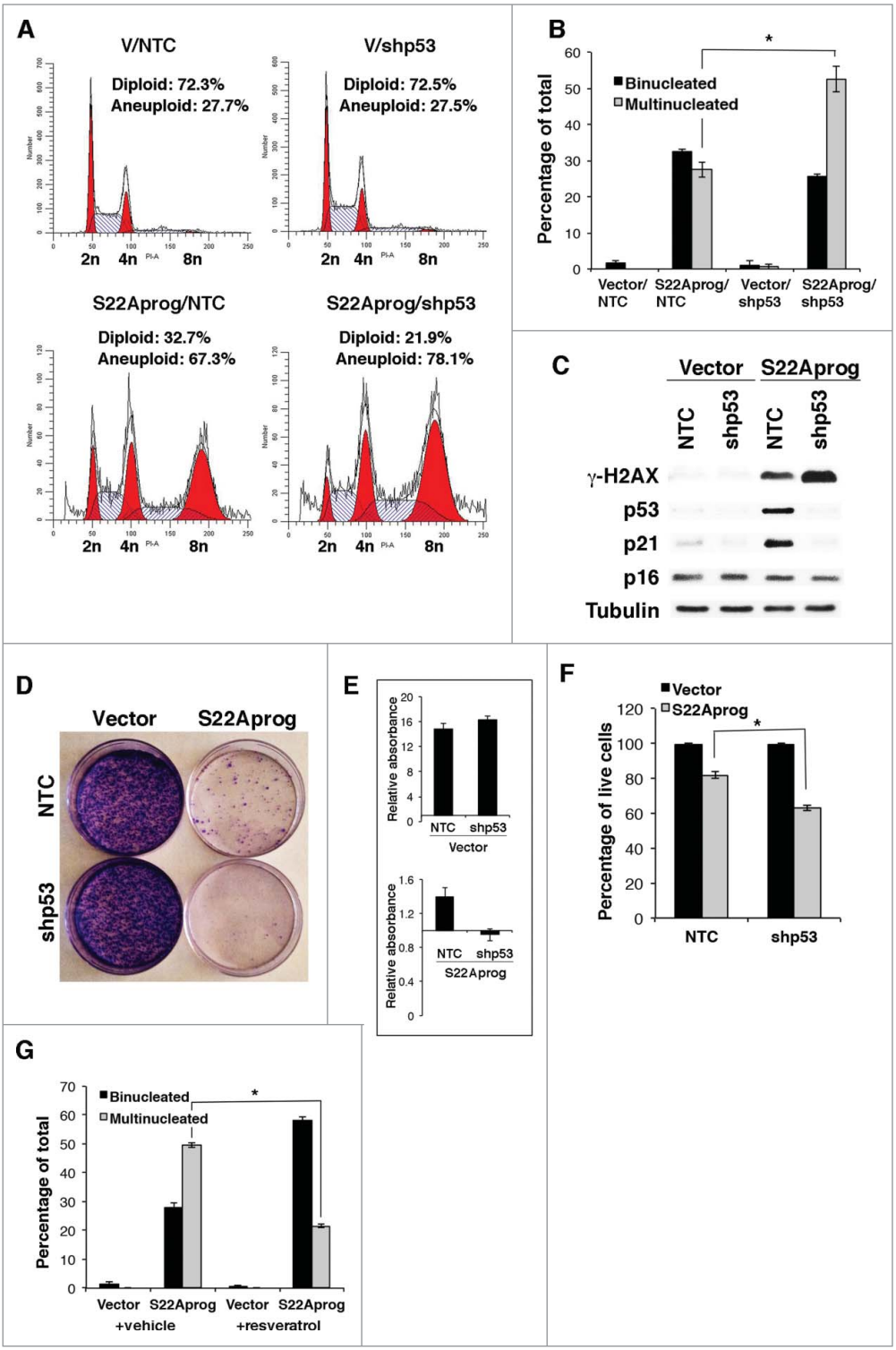

Figure 5. Role of p53 in S22A-progerin expressing cells. (**A**) DNA content analysis of U-2 OS cells expressing vector or S22A-progerin with shRNA control (NTC) or shRNA against p53. Quantification of the DNA content analysis (%) is shown in each panel. (**B**) Quantification of cells with 2 or more nuclei in U-2 OS as in (**A**). (**C**) Immunoblots for the indicated proteins using extracts from cells as in (**A**). (**D**) Colony formation assay of cell lines as in (**A**). (**E**) Quantitation of cell growth in the colony formation assay using crystal violet staining. (**F**) Cell viability in the indicated cell lines using a trypan blue exclusion assay. (**G**) Quantitation of cells with 2 or more nuclei in U-2 OS expressing the indicated vectors and treated with 40 μM resveratrol or vehicle for 48 hours.

during mitosis.[19] As a result, cells expressing S22A-progerin arrest as multinucleated senescent cells, expressing senescence biomarkers including the senescence associated secretory phenotype.

Senescence induction by S22A-progerin involves the ability of this protein to form a thick stable nuclear lamina that interferes with mitotic progression and promotes nuclear fragmentation. Consistent with this model, overexpression of S22A progerin with a mutation in its farnesylation signal prevented its localization to the nuclear membrane and totally abolished its ability to cause growth arrest or senescence. We propose that a lamin A cage physically interferes with nuclear mechanics during prophase inhibiting the nuclear envelope breakdown required for karyokinesis.[34] A failure to disassemble the nuclear envelope can inhibit the transition from prophase to prometaphase and eventually chromosome disjunction leading to aberrant mitosis, also called mitotic catastrophe.[35] Morphologically, mitotic catastrophe is associated with multinucleated cells, which ultimately undergo cell death or senescence.[35] Aberrant mitosis of nontransformed cells commonly leads to accumulation of G1-arrested tetraploid binucleated cells[36] exhibiting markers of senescence.[37] In normal human cells, this G1 arrest is dependent on p53 and the p38 MAPK.[38] In contrast, transformed cells after tetraploidization continue DNA synthesis leading to polyploidy,[36,39] multinucleation[11] and death,[39] the latter associated to the spindle checkpoint.[29] The fact that most S22A progerin-expressing cells remain alive but senescent suggests that they do not activate the spindle

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checkpoint-dependent apoptosis pathway but a novel pathway linking altered prophase to senescence.

S22A-progerin induced senescence is p53-independent since it occurs in cell lines that do not express p53 (PC3 and H1299 cells) or in U 2-OS cells where p53 was knocked down by RNA interference. In fact, p53 restricted polyploidy in S22A-progerin-expressing cells. Since hyperploid can trigger senescence,[40] p53 null cells could be more sensitive to treatments capable of activating the S22A-progerin senescence pathway. Consistent with this interpretation, p53 can prevent cellular senescence by decreasing TOR activity and forcing cells into quiescence.[41] S22A-progerin induced senescence was associated to inhibition of chromosome condensation, invaginations of the nuclear membrane and DNA damage. Intriguingly, DNA damage foci in S22A-progerin expressing cells were associated to the nuclear envelope suggesting a direct effect of progerin on DNA damage. Lamins are known to bind DNA, mainly at sequences that bind the nuclear matrix called matrix attachment regions or MARs.[42] It is plausible that tension promoted by S22A-progerin and to a lesser extent progerin can damage DNA at these sites. Chromosome condensation requires the CDK1/cyclin B complexes and is inhibited by calcium.[43] It is intriguing that nuclear membrane invaginations which involve the nuclear membrane[44] can lead to nuclear fragmentation but also connect the calcium rich endoplasmic reticulum to the nucleus[45] providing a potential for calcium mediated signals to regulate mitotic progression.[43] These invaginations of the nuclear envelope were previously noticed in cells expressing progerin[46] or overexpressing wild type lamin A [44] and we report here that mutation of serine 22 in progerin magnifies this phenotype. The mechanism leading to nuclear invaginations remain to be investigated but as proposed above to explain DNA

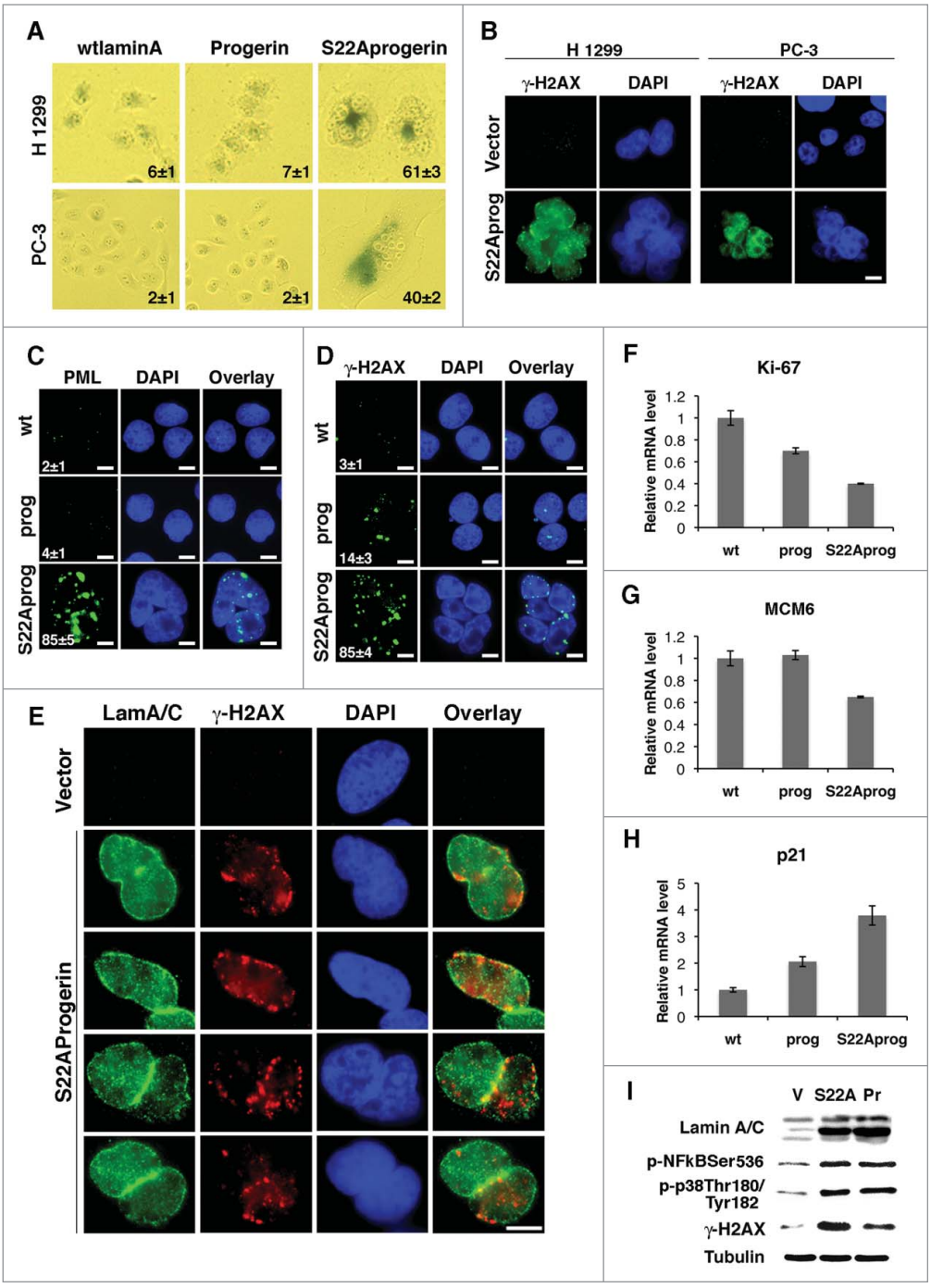

Figure 6. S22A-progerin induces tumor cell senescence. (**A**) Senescence associated β-galactosidase staining in the indicated cell lines expressing wild type lamin A (wtlamin A), progerin or S22A-progerin. (**B**) Immunofluorescence for γ-H2AX in the indicated cell lines. (**C-D**) Immunofluorescence for PML bodies (**C**) or γ-H2AX (**D**) of U-2 OS cells expressing wild type lamin A (wt), progerin (prog) or S22A-progerin (S22Aprog). The percent and S.D labeled cells are indicated at the bottom right of each panel. Magnification = 10 μm. (**E**) Immunofloures-cence of cells co-stained for lamin A and γ-H2AX (F-H) qPCR for genes whose expression change with senescence in U-2 OS cells. (**I**) Immunoblots for the indicated proteins of U-2 OS cells with empty vector (V), S22A-progerin (S22A) or progerin (Pr). All markers in A-I were measured at day 5 post-infection.

Table 1. The secretome of S22 A-progerin induced senescence in U-2 OS cells

Gene	Fold	FDR p value	Gene	Fold	FDR p value
HTN3	21.99	0.002	IL36B	3.39	0.007
IL1A	18.98	0.003	TNFSF4	3.16	0.024
HTN1	13.74	0.007	PLAU	2.62	0.010
IL8	11.45	0.003	NOV	2.61	0.008
GDF15	11.44	0.0008	MMP9	2.58	0.004
MMP3	10.17	0.002	FGF5	2.56	0.025
CCL2	7.62	0.003	SERPINB4	2.43	0.093
AREG	5.74	0.002	VIP	2.43	0.012
CCL20	5.61	0.002	SERPINB2	2.38	0.023
IL24	5.46	0.002	SERPINB7	2.29	0.009
miR-146*	5.02	0.002	SPP1	2.27	0.008
SAA2	4.89	0.009	GMFG	2.26	0.034
SAA1	4.67	0.00002	FAS	2.25	0.002
CSF2	4.36	0.003	CCL26	2.06	0.021
PI3	4.21	0.00001	IL7	2.06	0.006
MMP1	4.0	0.002	BMP2	2.06	0.005
TNFSF15	3.81	0.006			

*this micro RNA is linked to the SASP and modulates the process.[59]

damage they could result from increased tension created at the nuclear envelope but higher levels of lamin A. Progerin is highly expressed in HGPS cells but is also present in old cells at lower concentrations.[47] CDK inhibitors such as p16INK4a increase their expression with age[48] suggesting an endogenous pathway that inhibits CDKs and could prevent phosphorylation of progerin at serine 22. p16INK4a or any other factor that inhibits CDK-dependent phosphorylation of progerin can in principle potentiate its ability to induce senescence and aging.

Our results thus provide evidence for a new pathway to induce senescence effective in p53 disabled tumor cells, which is based on mechanical obstacles to karyokinesis. This pathway involves an alternative route in cell cycle progression where cells experiencing a block in prophase sidestep the rest of mitosis entering G1 and S, increasing their DNA content. A similar cell cycle variation termed mitotic slippage was described in cells where the mitotic spindle was compromised with drugs. In this case some cells manage to gradually exit mitosis entering G1 and progressing to the rest of the cell cycle.[49] Senescence may then act as a barrier for the expansion of cells that undergo mitotic slippage and a mechanism for tumor suppression in response to drugs targeting mitosis. It is not entirely clear whether S22A-progerin-induced senescence requires the p16/RB pathway. S22A-progerin induced senescence in HeLa cells that express the RB inhibitor E7, suggesting that RB is not essential. On the other hand in GSEA of our microarray data obtained form U-2 OS cells, there is a clear signature of E2F genes downregulation that matched data published for Ras-induced senescence (Supplemental **table II**). This suggests that E2Fs genes are repressed, a task normally associated to RB pathway activation. It has been proposed that the p53 pathway is sufficient to trigger senescence in the absence of RB and that both p53 and RB should be disabled to bypass ras-induced senescence.[50–53] The CDK inhibitor p21 can mediate RB-independent E2F repression and senescence in cells where lamin A is disabled.[53] However, U2-OS cells expressing a shRNA against p53 express little p21 and senesced in response to S22A-progerin. A senescence pathway acting independently of both RB and p53 is very interesting for cancer therapies because these tumor suppressors are often inactivated in advanced tumors. Further mechanistic understanding on how a lamin A nuclear cage and altered prophase force cells into senescence may help to identify druggable targets to induce senescence in advanced tumors with mutations in the p53 and RB tumor suppressor pathways.

Senescence is a promising endpoint for anticancer therapies not only due to its capability to halt cell growth but also for its ability to engage the immune system.[54,55] However, senescence can have both pro-tumor and anti-tumor effects and more work is needed to find treatments that avoid the pro-tumor activity of senescent cells. Otherwise, the good anticancer activity of a progerin nuclear cage may anticipate novel cancer therapies acting by providing mechanical barriers for cell cycling. Such physical barriers might work despite the mutations present in cancer cells, and therefore could be effective for advanced tumors.

Materials and Methods

Cell culture and reagents

Human non-small cells lung carcinoma H 1299 (CRL-5803, ATCC), human osteosarcoma U-2 OS cells (HTB-96, ATCC), human cervix carcinoma HeLa cells (CCL^{-}2, ATCC) were cultured in Dulbecco's modified Eagle's medium (Wisent, St-Bruno, QC) supplemented with 10% fetal bovine serum (FBS) (Wisent). Human prostate carcinoma PC-3 cells (CRL-1435, ATCC) were cultured in RPMI-1640 medium (Wisent) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% L-glutamine (Wisent). Crystal violet retention assay used for cell proliferation analysis and senescence associated β-galactosidase assay were described before.[51] For cell proliferation the data is expressed as relative absorbance of crystal violet extracted from the cells and diluted in 10% acetic acid.

Retroviral vectors and retroviral-mediated gene transfer

Retroviral vectors pLPC and MLP are from S.W. Lowe. pRetroSuper-shp53 and pRetroSuper-shGFP were described by Voorhoeve and Agami.[56] pLPC wtlaminA, pLPC L647RlaminA and pLPC progerin were constructed by subcloning wild type or mutated laminA from pMXIH-hygro vectors kindly provided by Dr. B. Kennedy. pLPC L647RlaminACS, pLPC S22AlaminACS and pLPC progerinCS were generated by substitution of cysteine in CaaX-motif by serine (TGC→TCC) using PCR. S22A point mutation was introduced into progerin by site-directed mutagenesis using forward primer 5′-AGCTCCACTCCGCTGGCACC-CACCCGCATCACC-3′ and reverse primer 5′-GGTGATGCGGGTGGGTGCCAGCGGAGTGGAGCT-3′. Hairpin ZMPSTE24/FACE1 (shZMPSTE24) was designed against the sequence 5′-GATCATGGATTCTGAAACATT-3′ of the human ZMPSTE24 gene. Retroviral-mediated gene transfers were done as in.[57]

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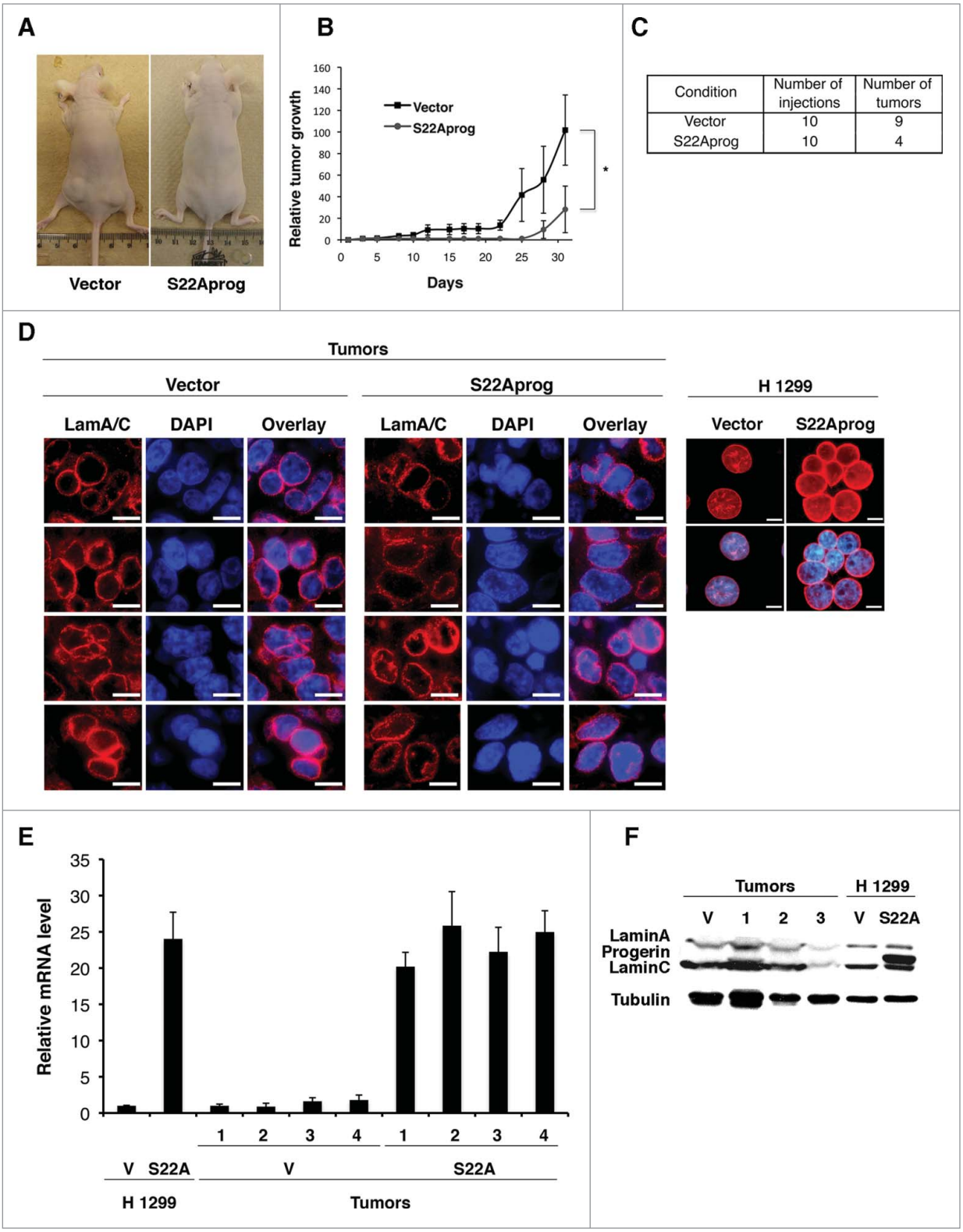

Figure 7. S22A-progerin blocks tumor progression in mice. (**A**) Tumor formation in BALB/c nude mice injected with 10^6 H 1299 cells expressing vector or S22A-progerin (S22Aprog). (**B**) Relative tumor growth in BALB/c nude mice injected with H 1299 cells expressing a vector or S22A-progerin. * = p value <0.05. Error bars indicate SEM. (**C**) Summary of number of tumors developed in BALB/c nude mice. (**D**) Immunofluorescence for lamin A in tumor sections from mice inoculated with H 1299 cells expressing vector or S22A-progerin. Right panel shows the same staining in H 1299 cells before inoculation in mice. Magnification = 10 μm. (**E**) qPCR for *LMNA* (isoform A-delta50) in H 1299 cells expressing vector (V) of S22A-progerin (S22A) or from tumors as in (**A**). (**F**) Immunoblots for lamin A/C in tumors and H 1299 cells as in (A). Numbers indicate different tumors.

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Mice

For xenografts, BALB/c 5-wk-old nude mice were injected subcutaneously in both flanks with 10^6 H 1299 cells expressing pLPC-Vector or S22A-progerin. Cells were resuspended in 50 μL of PBS mixed with 50 μL of Matrigel (BD Biosciences) at 4°C. Tumor formation was evaluated 3 times per week over a period of 31 d after which the mice were euthanized.

Immunoblotting

To prepare total protein lysates, cells were collected by trypsinization, washed with phosphate-buffered saline (PBS), lysed in sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol), and boiled for 5 min. For Western blotting, 25 μg of total protein were separated on SDS-polyacrylamide gel electrophoresis gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). We used the following primary antibodies: anti-Cyclin B1 (sc-245, 1:200, Santa Cruz Biotechnology), anti-Cyclin A (sc-596, 1:200, Santa Cruz Biotechnology), anti-gamma H2AX (phospho S139) (ab11174, 1:1000, Abcam), anti-LaminA/C (636, sc-7292, 1:1000, Santa Cruz Biotechnology), anti-Lamin A/C (2032, 1:1000, Cell Signaling), anti-phospho-Ser22Lamin A (2026, 1:1000, Cell Signaling), anti-phospho-Ser10Histone H3 (06–570, 1:1000, Millipore), anti-phospho-Thr180/Tyr182p38 MAPK (4511, 1:1000, Cell Signaling), anti-phospho-Ser536NFkB p65 (3033, 1:1000, Cell Signaling), anti-p21 (sc-397, 1:500, Santa Cruz Biotechnology), anti-p53 (DO-1) (sc-126, 1:1000, Santa Cruz Biotechnology), anti-p21 (556431, 1:1000, BD PharMingen), anti-p16 (F-12) (sc-1661, 1:500, Santa Cruz Biotechnology), anti-Tubulin (T5168, 1:5,000, Sigma). Signals were revealed after incubation with anti-mouse or anti-rabbit secondary antibodies coupled to peroxidase (Amersham Pharmacia, Amersham, United Kingdom) by using enhanced chemiluminescence (Amersham Pharmacia).

Fluorescence microscopy

Cells were plated on coverslips at least 24 h prior to fixation with 4% paraformaldehyde in PBS for 15 minutes at room temperature. After washing with PBS, cells were permeabilized using 0.2% Triton X-100 in PBS–3% bovine serum albumin (BSA) solution for 5 min. The cells were then washed 3 times with PBS–3% BSA and incubated for 1–2 h at room temperature with the primary antibody: anti-lamin A/C (636) (sc-7292, 1:500, Santa Cruz Biotechnology), anti-Tubulin (T5168, 1:1000, Sigma), anti-phospho-H2AX (JBW301, 1:200, Upstate Biotechnologies), anti-PML (A301–167A, 1:200, Bethyl), anti-progerin (ab66587, 1:100, Abcam), anti-phospho-Ser22LaminA (2026, 1:200, Cell Signaling) diluted in PBS/BSA. Next, cells were washed 3 times in PBS/BSA and incubated with the appropriate secondary antibody (1:2000, AlexaFluor 488 goat anti-mouse, AlexaFluor 568 goat anti-mouse, or AlexaFluor 488 goat anti-rabbit, Molecular Probes-Invitrogen) for 1h at room temperature. Finally, cells were rinsed 3 times with PBS alone and once with PBS containing 300 nM DAPI for 10 minutes. Images from independent fields were captured with a fluorescence microscope and processed with Metamorph.

Immunofluorescence from paraffin embedding samples

Tumor sections from xenografts were deparaffinised and rehydrated. Antigen epitope retrieval was performed by heating for 25 min at 95°C in Sodium-Citrate Buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0). Samples were permeabilized with Triton 0.5%-PBS solution for 15 min and blocked with 3% BSA, 5% Goat Serum, 0.1% Tween20-PBS solution for 1 h. Then, they were incubated with mouse monoclonal anti-Lamin A/C (1:200, sc-7292, Santa Cruz Biotechnology) at 4°C overnight. The next day, samples were washed in PBS and incubated with goat anti-mouse AlexaFluor 568 (1:1000, A-11031, Molecular Probes, Invitrogen). For mounting, we used ProLong Gold Antifade Reagent with DAPI (P36931, Invitrogen).

Transmission electron microscopy

Cells were fixed in 1% glutaraldehyde for 30 min at room temperature (RT) and washed 2 times with PBS. Then, the cells were incubated with 1% osmium tetroxide solution for 1h at RT, followed by extensive washing with PBS, dehydration with ethanol, and embedding in LR White resin. 70nm sections were cut, stained with 4% uranyl acetate for 2min, and examined in a FEI Tecnai 12 electron microscope (FEI, Eindhoven, The Netherlands), operating at 80 kV.

Real-Time PCR, microarray and bioinformatic analysis of microarray data

Real-Time PCR and Microarray analysis were done as previously described.[58] Briefly, RNA was extracted with Trizol 4 d after infection of U-2 OS cells with wild type lamin A or S22A-progerin. Total RNA was sent to Genome Quebec (McGill University) for cRNA amplification and subsequent hybridization on GeneChIP Human Gene 2.1 ST Array Affymetrix DNA Chip. Data were analyzed using the Affymetrix Softwares Expression Console and Transcriptome Analysis Console, http://www.affymetrix.com. Data is available at GEO (GSE51349).

Statistics

Statistical analysis was performed using SPSS software 16.0 (SPSS, Inc.). The nonparametric Kruskal-Wallis test was used to show significant differences for tumor growth in mice.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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