

ABSTRACT

Hutchinson-Gilford Progeria syndrome is most often caused by a single C to T nucleotide switch that leads to the translation of a defective lamin A protein called progerin. Progeria is a premature aging disease that causes those affected to die from health issues like heart disease and strokes. Progeria cells experience an accumulation of DNA damage that similar to the one caused by ionizing radiation, which is the most common form of exogenous DNA damage. To better understand the effects of progerin accumulation on cells, we examined senescence, DNA damage, growth patterns, and morphology in fibroblasts from Progeria patients and compared them to healthy fibroblast cell lines. We examined the Progeria affected fibroblast cell lines AG11513- 8 y/o female, HGADFN167- 8 y/o male, and AG03199- 10 y/o female against unaffected fibroblast cell lines AG03257- mother and GM08398- 8 y/o male. We hypothesized that the Progeria cell lines would have a higher ratio of senescent cells than the healthy cell lines, and that they would have more DNA damage accumulation. To test this hypothesis, we performed multiple rounds of β -galactosidase staining and γ -H2AX staining to determine the levels of senescence and DNA damage, respectively. To gain even more understanding of how the Progeria cell lines compared to the healthy fibroblasts, the cell lines' proliferation rates and morphology were recorded with every cell passage. Our results showed that the Progeria cell lines not only possessed a higher level of senescence and DNA damage, but that the levels of these also increase with higher cell passages. In contrast, the healthy cells showed much lower levels of senescence and DNA damage and had no dramatic increase with cell passage number. These results further our understanding of the similarities and differences in Progeria phenotypes across different patient cell lines and contribute toward understating the parallels between exogenous DNA damage and the naturally occurring DNA damage in Progeria. These results will contribute to future comparisons of how these different sources of DNA damage affect the 3D genome organization.

INTRODUCTION

One of the most common forms of exogenous DNA damage is caused by ionizing radiation (IR), and the most common form of radiation that affects humans is caused by background levels of radon that can be found in the ground or in building materials.¹ However, advances in medicine and technology have led to the introduction of man-made IR sources. The introduction of low- and high-linear energy transfer (LET) in X-Ray equipment or common mechanical equipment like televisions and computer screens have become common sources of man-made sources of radiation. IR exposure damages DNA with lasting effects, potentially leading to apoptosis. Failure to repair double-stranded breaks (DSBs) caused by IR can result in genomic abnormalities and mutations that can become oncogenic, causing tumors². Cells exposed to IR undergo an adaptive response leading to the activation of DNA damage response (DDR) pathways to prevent long-term effects^{3,4}. Our lab has shown that though chromatin can be repaired after exposure to IR, there are long-lasting effects to the genome that can persist and ultimately result in permanent damage and genome organization changes⁵. However, IR and other exogenous agents are not the only cause of DNA damage; oxidative stress, replication errors, or errors in the activity of topoisomerases can also cause DNA damage to occur⁶. In Progeria specifically, the absence of PCNA at replication centers due to its defective maturation

of prelamin A may lead to stalled replication forks; the collapse of the replication forks may result in double stranded breaks⁷.

One of the known repair pathways responsible for sensing and regulating DNA damage is the *ataxia telangiectasia mutated* (ATM) DNA repair pathway⁵. The detection of DSBs leads to the recruitment of the ATM kinase which phosphorylates the histone variant H2AX on serine 1397^{8,9}. This phosphorylation event recruits repair factors to bind and begin the repair process in both the non-homologous end joining pathway (NHEJ)⁹ and the homologous recombination pathway (HR) pathway¹⁰.

Hutchinson-Gilford Progeria Syndrome (HGPS), or Progeria, is an incapacitating premature aging disease that is seen in 1 in twenty million births worldwide. Progeria was originally described by Jonathan Hutchinson in 1886 and later by Hastings Gilford in 1897^{11,12}. Both Hutchinson and Gilford's studies documented symptoms of premature aging in Progeria patients, but the mechanisms that contribute to Progeria phenotypes have only recently been discovered. Children born with this disease experience symptoms like short stature, skin wrinkling, alopecia, decreased bone density, joint pain, and atherosclerosis, which ultimately leads to their premature death by heart disease or stroke at an average age of 14.6 years. The cause of Progeria has been identified as a mutation in the LMNA gene. As a result of this mutation, the LMNA gene produces progerin, a mutated form of lamin A that results in a dominant negative phenotype that increases in severity during aging due to subsequent rounds of cellular division^{13,14}.

Since progeria is caused by mutations in the LMNA gene, it is classified as a laminopathy, a group of rare genetic disorders caused by mutations in the genes that encode for proteins of nuclear lamina. The most common mutation in HGPS is a *de novo* point mutation at position 1824 which contains a C to T nucleotide replacement. This point mutation activates a cryptic splice site that does not change the encoded amino acid but leads to the translation of a defective lamin A protein: progerin. Progerin carries a deletion of 150 nucleotides that result in 50 amino acids that contain the cleavage site for ZMPSTE24 and in turn results in the preservation of the C-terminus and a permanently farnesylated lamin A^{13,14}. Progerin does not only lead to defects in the structural stability of lamin; it also interacts with regions of the genome and proteins causing abnormal gene expression and damaged proteins that lead to defects that are observed in HGPS patients¹⁵. The phenotype in bone, fat, joint and vascular health of Progeria patients suggests a defect in the mesenchymal stem cell (MSC) lineage which differentiate into osteoblasts, chondrocytes, adipocytes and pericytes as well as to myocytes, and myofibroblasts necessary for wound repair¹⁶.

The effects of progerin expression are not initially observed in children suffering from HGPS and carrying the LMNA mutation; patients are asymptomatic until around the age of two. This is due to the fact that LMNA is only expressed in differentiated cells¹⁷, unlike *type B lamins* which are also expressed in stem cells. The most noticeable defect observed in Progeria cells is a defect in nuclear morphology. Progeria nuclei contain characteristic blebbing, caused by the formation of round protrusions that project from the nucleus, invaginations, and wrinkling which increase as the passage number of the cells increased and with aging in Progeria. Progeria cells have also been shown to experience increased DNA damage, and an increase in γ -H2AX has been observed in nuclei expressing progerin. This suggests that DNA damage is increased in Progeria cells. The accumulation of DNA damage is believed to lead to an impaired DNA damage repair pathway, by overwhelming the normal DNA damage repair methods^{7,18}. Progeria cells, like aged individuals, have decreased levels of tri-methylation of histone 3 lysine 9 (H3K9me3) and tri-methylation of histone 3 lysine 27 (H3K27me3), which are marks associated

with heterochromatic regions of the genome¹⁹. ATM's activation is inhibited in the Progeria cells that have a significant loss of H3K9me3²⁰.

We know that one of the ways the progerin is thought to affect cell phenotypes is through the disruption of chromosome structure¹⁹, and while further investigating the impact of progerin on 3D genome structure our lab identified aspects of change in genome structure in Progeria that matches signatures of 3D genome structure change after X-ray induced DNA damage. Our lab characterized, for the first time, the topologically associating domains (TADs) landscape in Progeria cells. Due to the essential activation of ATM in Progeria cells, the average TAD boundary strength was calculated in early and late passage Progeria fibroblasts. This increase in TAD boundary strength is consistent with the TAD boundary strength changes seen in the IR data (Figure 1A) suggests that the increase in damage observed in Progeria cells may be caused by ATM recruitment and DNA repair within TADs.

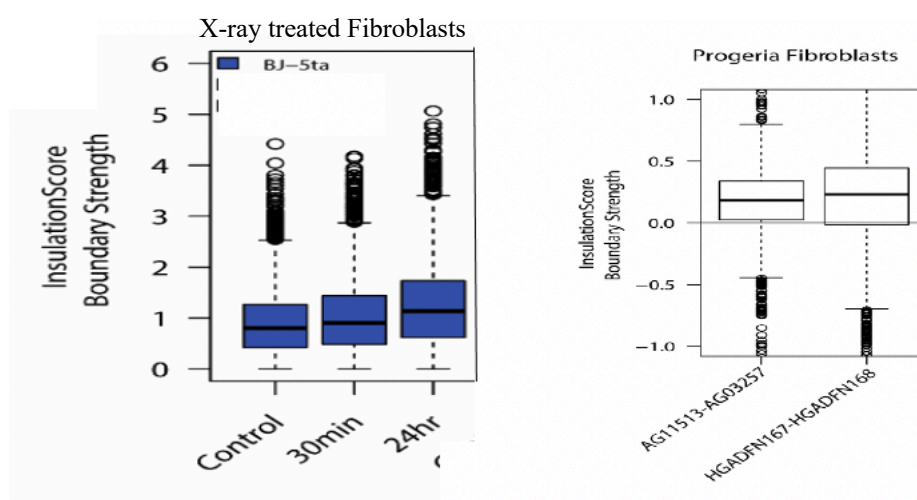


Figure 1: Preliminary data, Jacob Sanders. Box and whisker plot showing the disruption of TAD boundary strengths before and after X-ray in BJ-5ta and in progeria minus parental cell lines.

While it has been shown previously that Progeria cells experience increased DNA damage, in order to compare the X-ray induced and Progeria induced effects we see in genome structure, we decided to quantify the levels of DNA damage occurring in our particular Progeria cells across different ages of cells in culture⁹. The fraction of senescent cells in the population could relate to other aspects of 3D genome structure changes. Finally, there has been little comparison of phenotypes such as DNA damage and growth rate between cells taken from different individual patients. Because of this, we aim to measure DNA damage, senescence, and growth rate of different Progeria cell lines to provide phenotypic data to inform our 3D genome structure measurement interpretation and to assess the diversity of these phenotypes across Progeria cell lines.

RESULTS

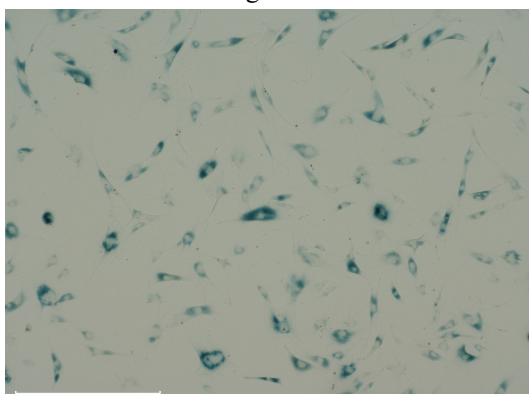
Senescence β -Galactosidase Staining:

To observe senescence in the Progeria and Wild Type fibroblast cell lines, we used a senescence β -galactosidase staining kit from CellSignaling (#9860). Senescence-associated beta-galactosidase (SA- β -gal) is an enzyme that catalyzes the hydrolysis of β -galactosides into monosaccharides, and it is only present in senescent cells, making it a biomarker of cellular senescence. This particular protocol suggests that the adherent cells incubate in the β -galactosidase staining solution for 24 hours, but we found in order to accurately differentiate between stained and unstained cells it was best to let the cells incubate for only 15 hours. Cell lines AG11513, AG03257, GM08398, and HGADFN167 were all imaged with the EVOS FL Auto 2 microscope. Progeria cell lines, AG11513 and HGADFN167 were stained for senescence on two different occasions at two different passage numbers. Since premature cellular senescence contributes to aging of tissues, our measurements aim to quantify whether different proportions of cells become senescent more quickly in Progeria compared to healthy fibroblasts.

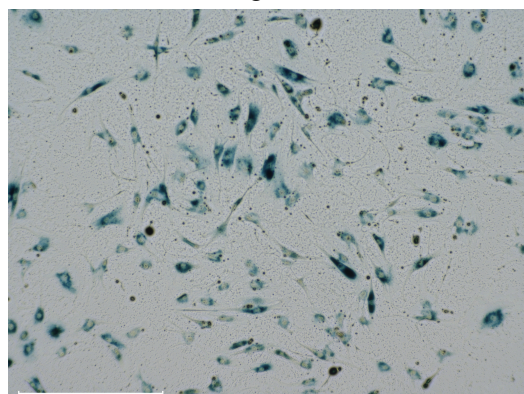
The microscopic images simple visual inspection of the resulting images is not sufficient because it is hard to quantify what images have a higher brightness of blue by eye (Fig. 2A). Using ImageJ, we were able to set a color brightness threshold of less than 105 in order to select for every cell in each image and a threshold of less than 95 to select only for the cells that have a certain amount of blue staining. (Lower threshold values capture darker staining.) By dividing the blue cells by the total cells, we were able to fairly compare the amount of senescence staining in each cell line. It can be seen when Progeria cell lines AG11513- 8 y/o female and HGADFN167- 8 y/o male were stained at a higher passage the level of senescence staining also increased. This trend continued across multiple replicates, but the increased level of senescence is the most dramatic in the AG11513 cell line (Fig. 2B). The two healthy fibroblast cell lines, AG03257- AG11513 mother and GM08398- 8 y/o male, were at passage 6 and 16 respectively and have almost equal average levels of senescence staining at 0.0999 and 0.0988, so even at drastically different passage numbers the ratio of blue to total cells did not change (Fig. 2C).

A

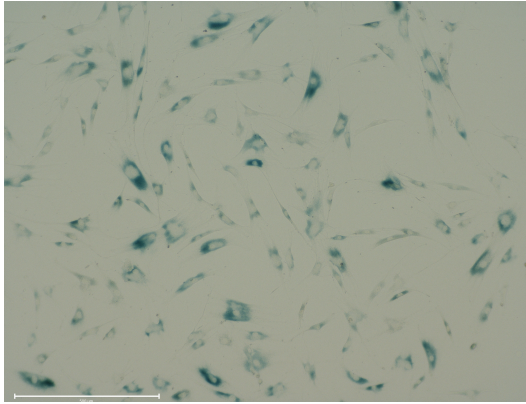
AG11513- Progeria Female P12



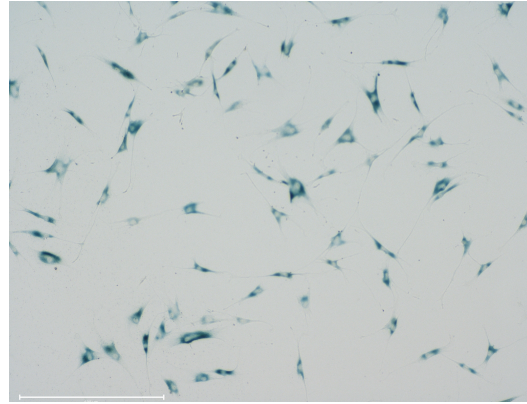
AG11513- Progeria Female P17



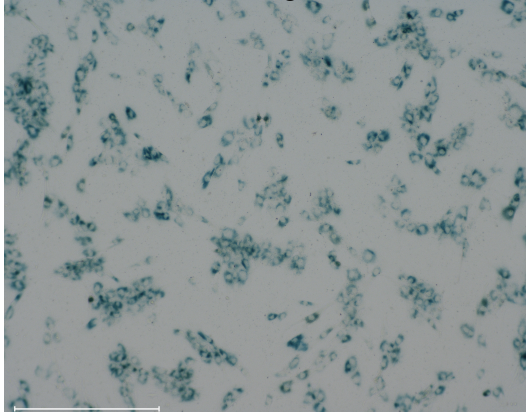
AG03257- Wild Type Mother P6



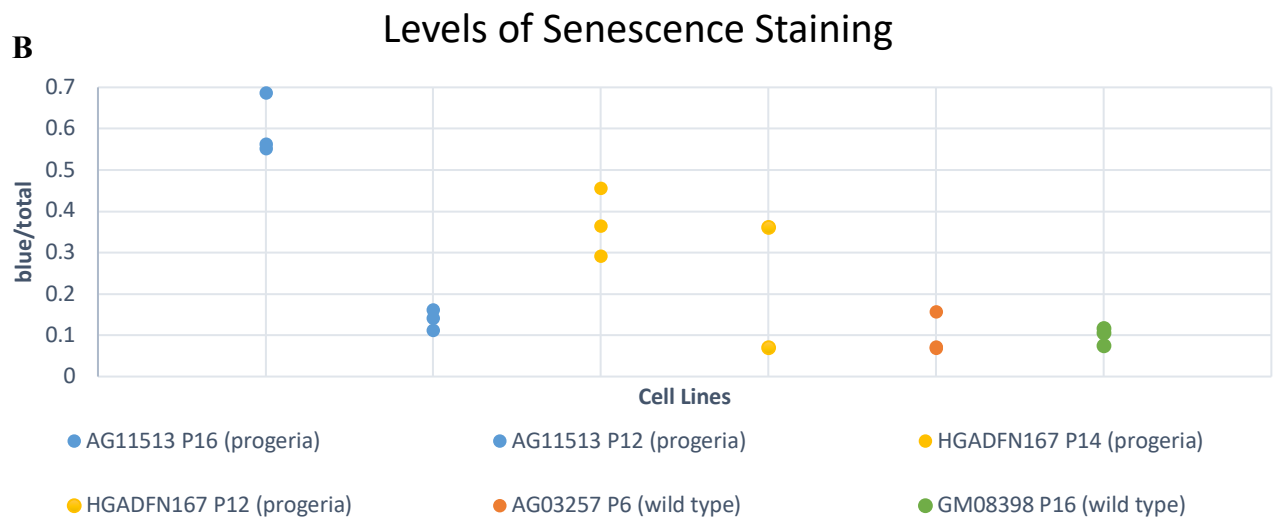
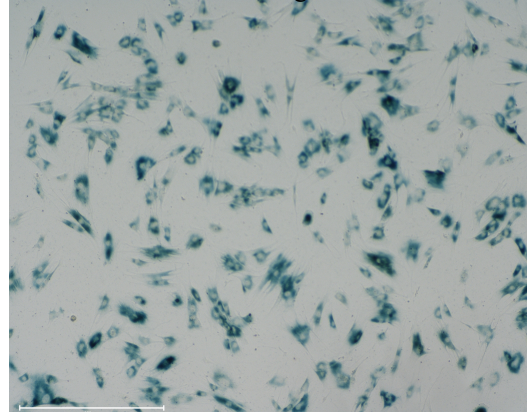
GM08398- Wild Type Male P16



HGADFN167- Progeria Male P12



HGADFN167- Progeria Male P14



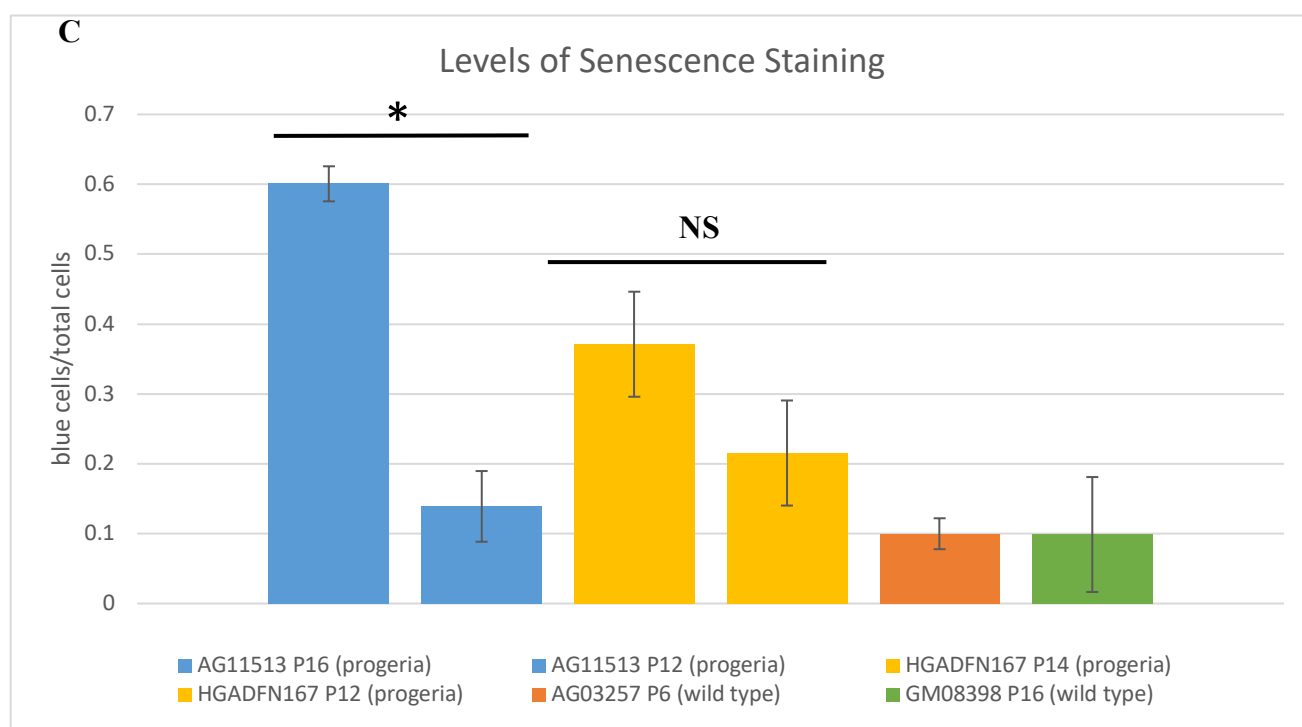


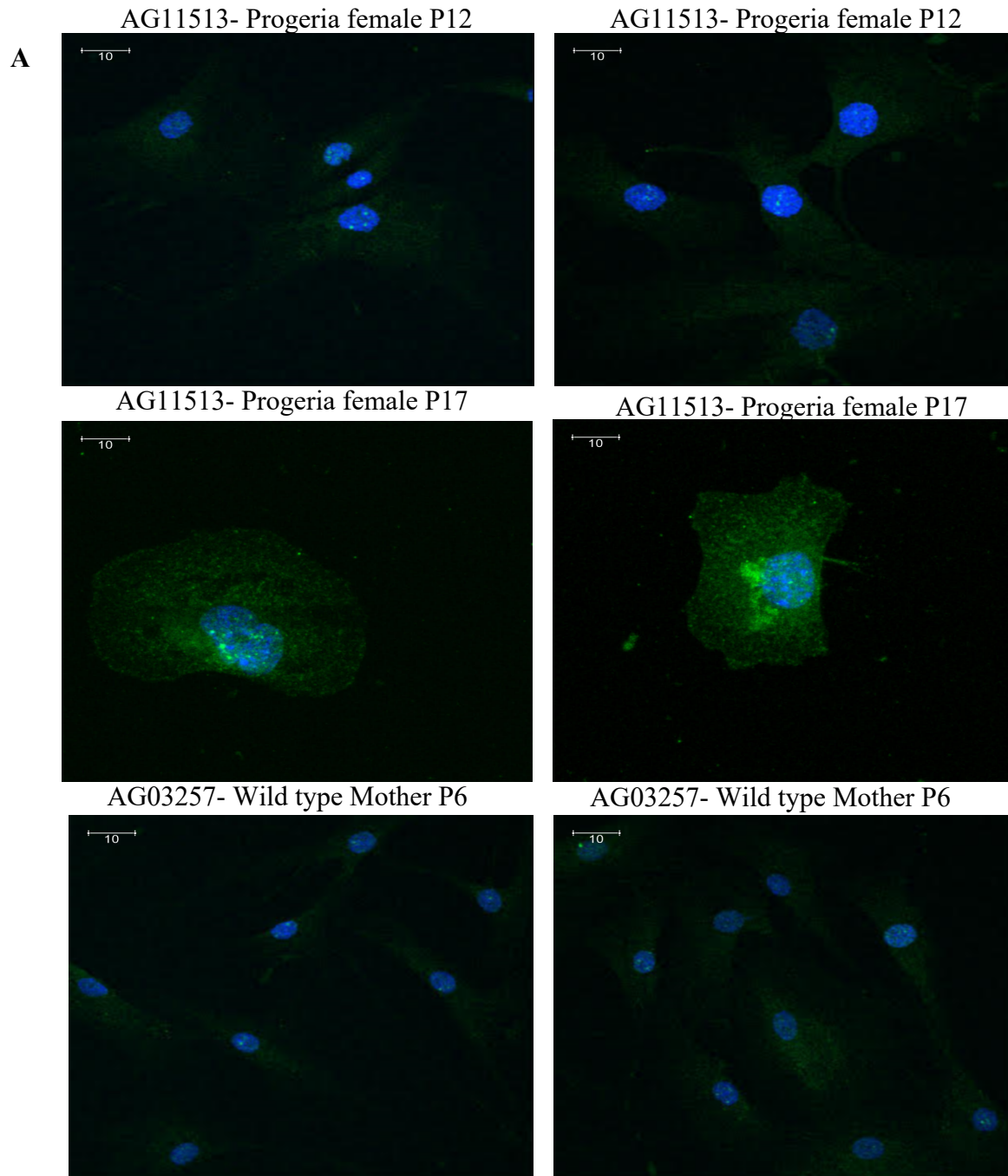
Figure 2: Senescence staining results. (A) Senescence β -Galactosidase Staining results imaged with EVOS FL Auto 2. (B) . Each replicate's ratio of blue to total cells shows an overall pattern across the different cell lines. (C) Average amounts of senescence in each image were calculated using the number of blue cells divided by the total number of cells. The average level of senescence staining is higher for the Progeria cell lines when compared to the healthy cell lines and higher at later passages for Progeria cells. A ttest was done between the groups with a solid line above them. Comparing AG11513 P16 and P12 resulted in a p value of 0.00477, so it is marked with an Asterisk to show significance. When HGADFN167 P14 and P12 were compared it resulted in a p value of 0.465 so it is not significant (NS).

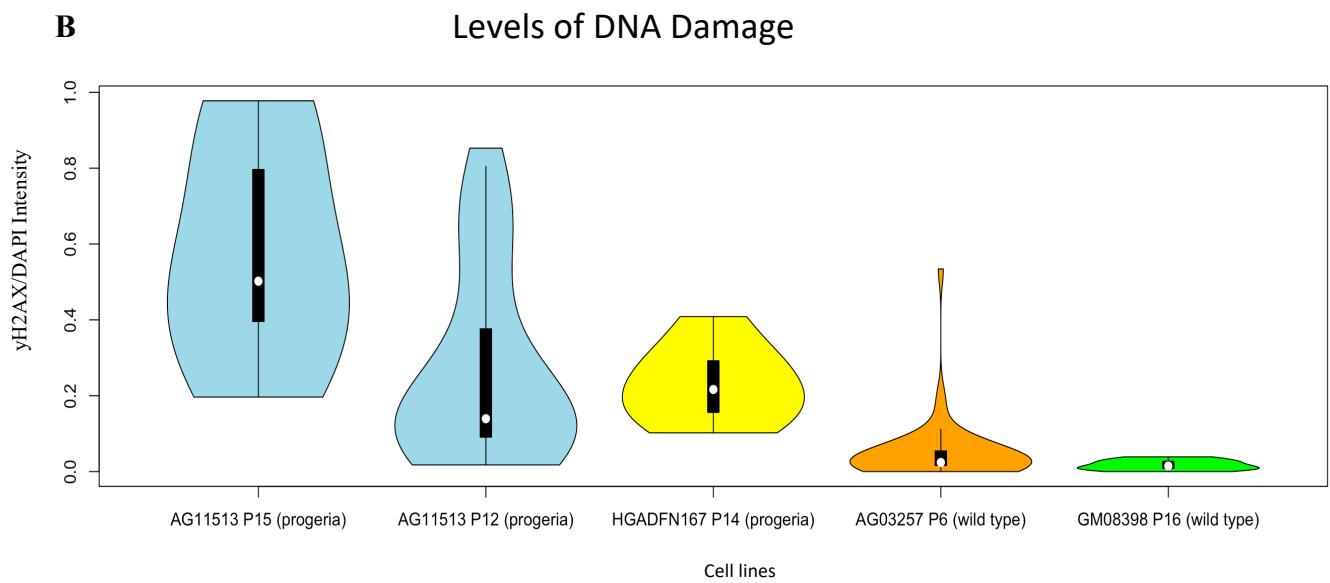
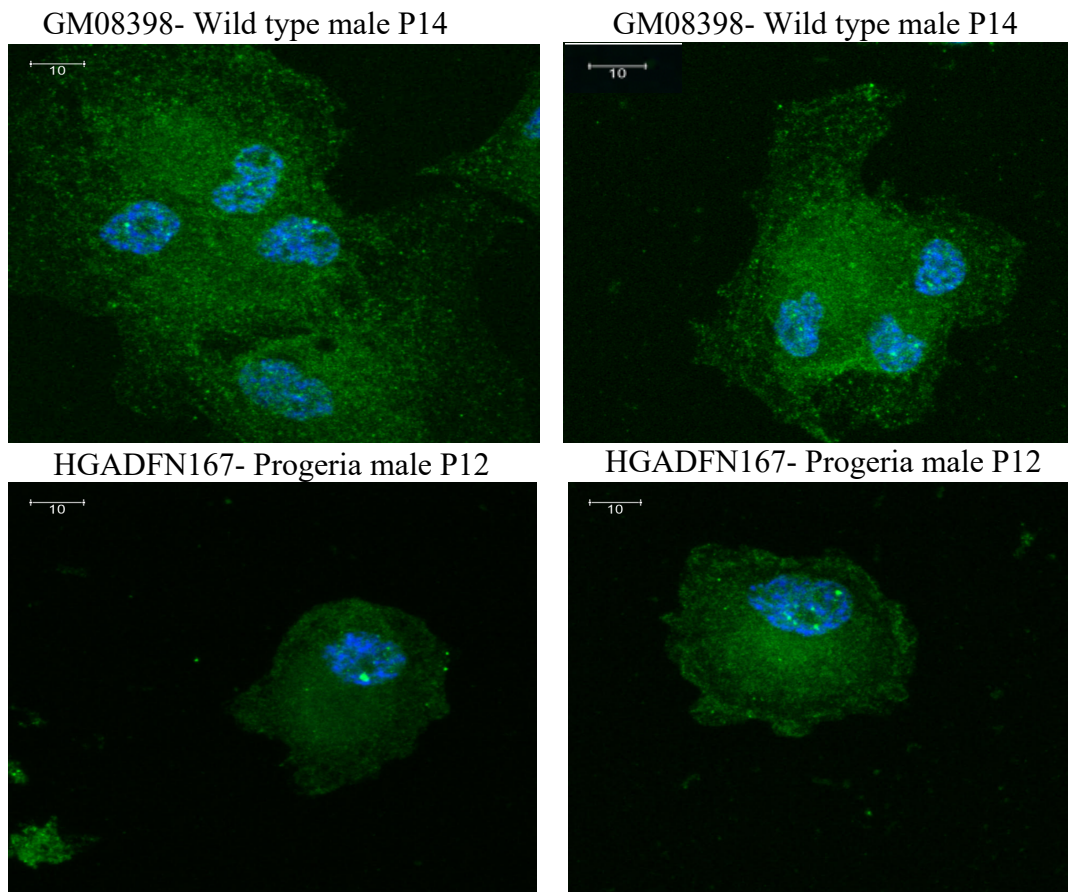
γ -H2AX DNA Damage Staining:

To observe the DNA damage in the various cell lines we seeded the cells on poly-D-lysine coverslips and treated with the γ -H2AX primary antibody, Alexa-Fluor-488 goat anti-mouse secondary antibody (Invitrogen, # A32723), and mountant with DAPI according to the manufacturer's instructions. The images obtained are all maximum projections of Zstacks created with the Leica SP8 White Light Laser Confocal System. Like the senescence staining, the Progeria cell line AG11513 was stained for DNA damage at two different passages in order to see if DNA damage increased with passage number.

When looking at the max projections, the amount of DNA damage appears to be slightly evaluated by eye due to the green γ -H2AX foci in the nucleus, a sign of DNA damage. In order to truly quantify the amount of DNA damage in each cell line we separated the blue and green channels in ImageJ, so we were able to record the intensity of each individual color and normalize the intensity of the green (γ -H2AX) by the intensity of the blue (DAPI). This ensured that the amount of DNA damage relative to the DNA content of the nucleus could be evaluated. The cell line with the highest ratio of γ -H2AX to DAPI was both passage 12 and passage 15 of the AG11513 cell line, followed by the other Progeria cell line, HGADFN167 (Fig. 3B). The

higher the ratio of γ -H2AX and DAPI intensity, the more DNA damage is present. It can be seen in Figure 3C that the average DNA damage is much higher in the Progeria cell lines when compared to the healthy fibroblast cells. Just like the senescence staining, the higher the cell passage number for Progeria fibroblasts, the more DNA damage is present, but both of the healthy fibroblast cell lines show far less DNA damage (Fig. 3C).





C

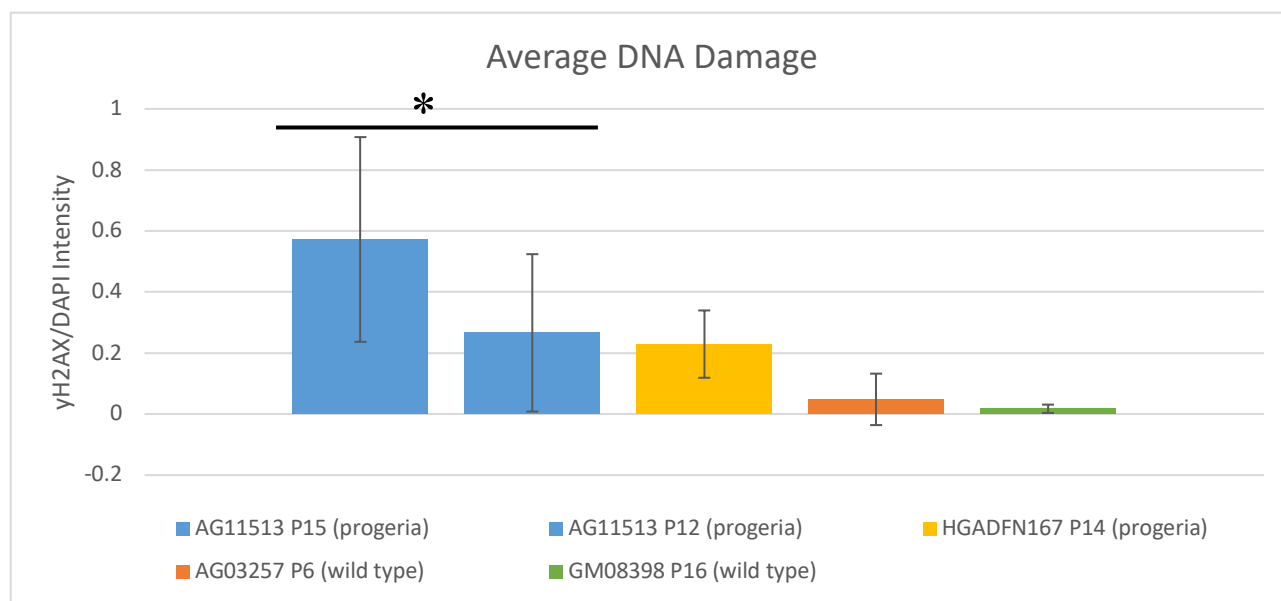


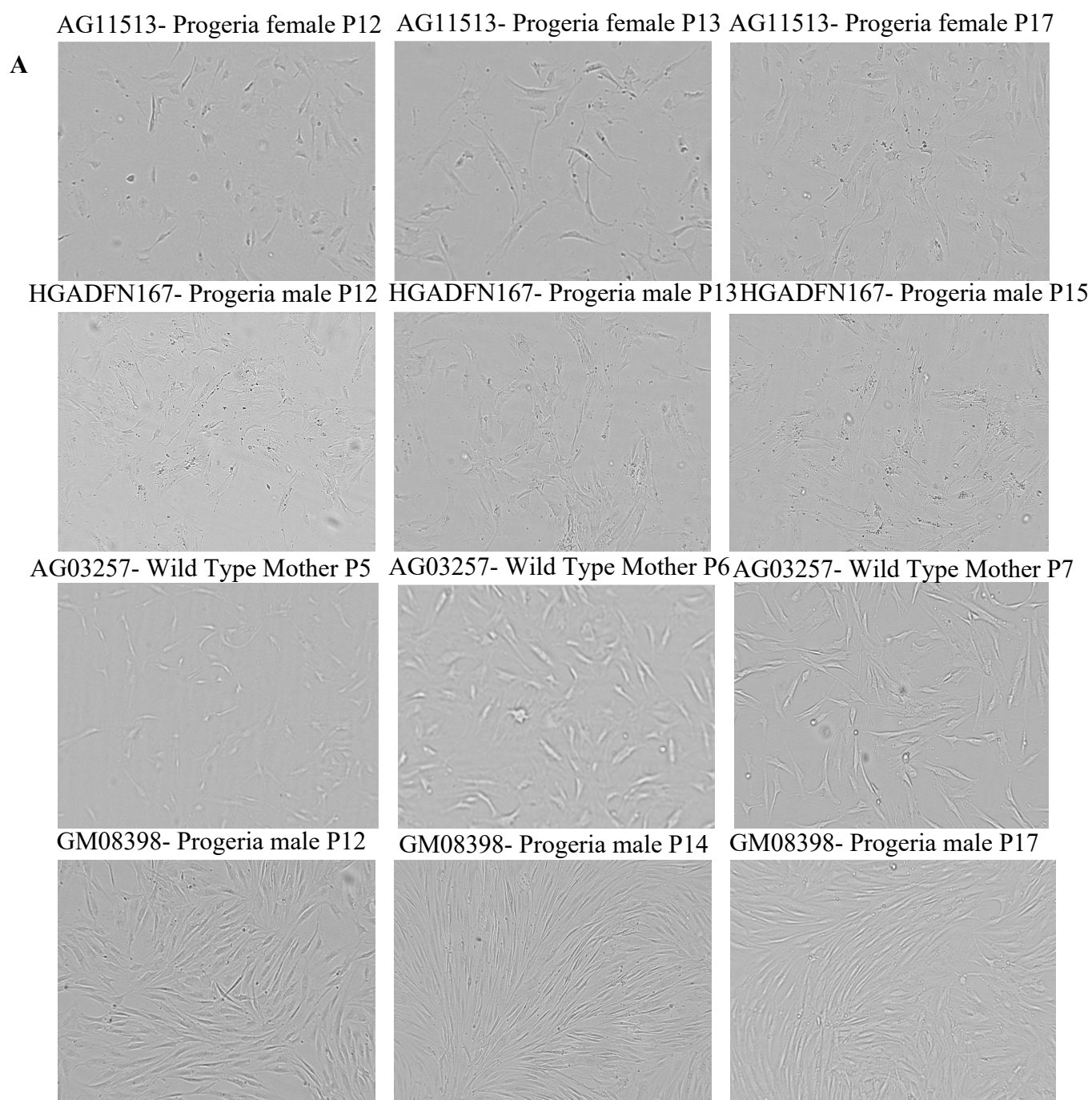
Figure 3: y-H2AX DNA damage staining results. (A) y-H2AX and DAPI max projection Zstacks imaged with Leica SP8 confocal microscope. (B) Violin plot of y-H2AX/DAPI density of multiple nuclei for every cell type quantified using ImageJ. (C) Average DNA damage for each cell line quantified using ImageJ. On average, the Progeria cell lines have more DNA damage than healthy fibroblast cell lines. A Ttest between AG11513 P12 and P16 revealed a p value of 0.045, so the Asterisk shows significance difference between the two groups.

Growth and Morphology:

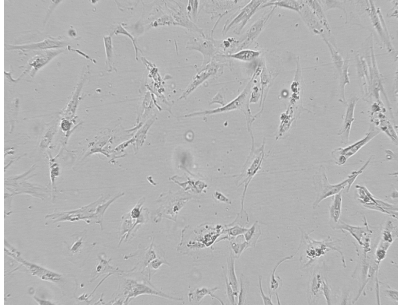
Cellular senescence of fibroblasts in culture is also known to associate with changes in morphology, from elongated to broad and flattened²¹. Therefore, we also wanted to measure how the Progeria cell lines grow in culture, how their morphology differs from healthy fibroblasts, and how it changes as passage number increases. To achieve this, images were captured of each cell line before each passage using a cytoSMART cell counter (Fig. 4A). Each passage also involved recording a live cell count (number of cells/mL) also using the cytoSMART cell counter and trypan blue exclusion. Finally, in an attempt to quantify the cell line's morphology, Image J was used to obtain an area and a major axis measurement, from an ellipse fit, of the cells in these images. The higher the ratio of area to major axis, the more rounded instead of elongated the cell line is by nature.

When looking into the cell growth it can be seen that the cell lines AG03257- healthy Mother and GM08398- healthy male experienced the most exponential growth, which was to be expected. Cell lines AG11513- Progeria male and HGADFN167- Progeria female were relatively easy to grow and maintain and also experienced consistent growth, but at a much slower rate than the unaffected cell lines (Fig 4B). AG03199 has only been through two passages that are reflected here, but we know from past projects that they are a difficult cell line to grow in culture. When comparing the average cell morphology, area/major axes, it is no surprise that Progeria cell lines AGADFN167 and AG03199 experience the roundest morphology. Interestingly, as shown in Figure 3B and 3C is that the AG11513 and AG03257 cell lines experience similar growth patterns and morphology traits. It is surprising that the AG11513 cell

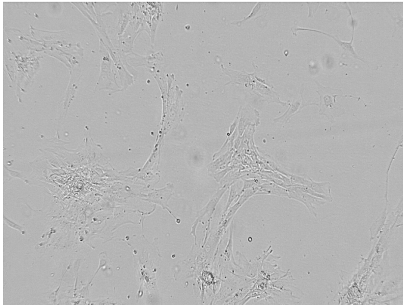
line does not have a different morphology than the healthy fibroblast cell lines, given their higher levels of senescence staining and DNA damage measured above (Fig. 4C).



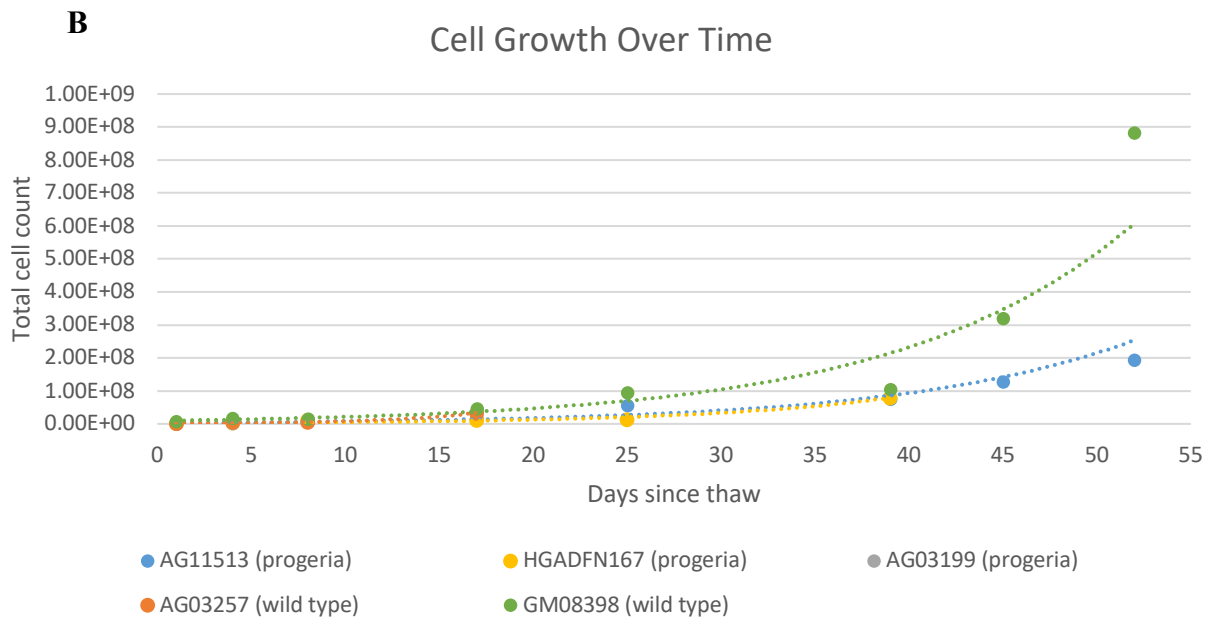
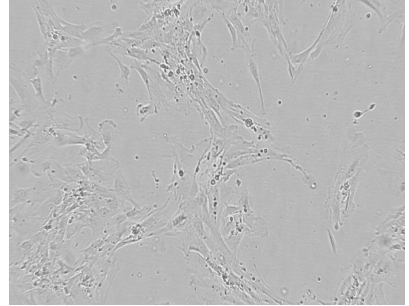
AG03199- Progeria female P10



AG03199- Progeria female P11



AG03199- Progeria female P12



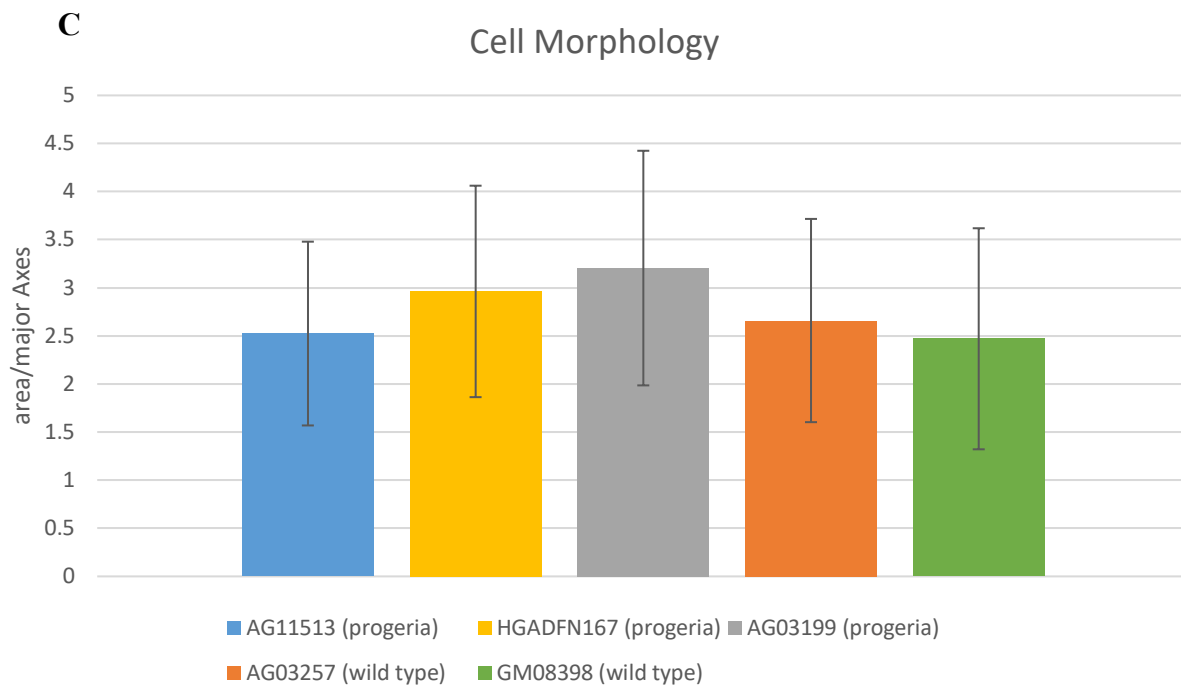


Figure 4: Cell morphology results. (A) Screen captures from cytoSMART cell counter at multiple passages to show cell morphology. (B) Plot of each cell line's growth with exponential trendline. Each point was recorded with the cytoSMART cell counter at each passage. Each point is calculated from the number of cells per milliliter then multiplied by the total volume of cells. (C) Bar graph expressing the cell morphology of each cell line, quantified by ImageJ; Progeria cell lines AG03199 and HGADFN167 are rounder in shape when compared to other cell lines.

DISCUSSION

In this study, we have attempted to better understand how the disease Progeria affects phenotypes at the cellular level through senescence staining, γ -H2AX staining, and by paying close attention to cell growth patterns and morphology. We began by deciding what experimental techniques we wanted to use in order to understand how cells affected with Progeria differ from cells that are normal, healthy, fibroblasts. We chose to complete senescence staining because when a cell is senescent it means that it is in cell-cycle arrest and cannot replicate but is not dead. Senescence is also a marker for normal aging, so we were curious if the rapid aging disease, Progeria, would express senescence at a higher level than the healthy cells. We confirmed this hypothesis, since the Progeria cell lines AG11513 and HGADFN167 both have a higher level of senescent cells when compared to the healthy fibroblasts cell lines AG03257 and GM08398. Something that is also supported in our data is that the ratio of senescence cells to total cells increase at a higher passage number. In both AG11513 and HGADFN167 the higher passage replicate shows a higher level of senescence staining.

In order to compare the DNA damage caused by X-rays, which I studied previously in my undergraduate research career, we decided to stain all cell lines for DNA damage using the exact same protocol and antibodies as was done for the cells exposed to X-rays, meaning direct

comparisons could be drawn between the levels of DNA damage of progeria cells and cells affected by IR. By staining with γ -H2AX and DAPI we were able to calculate their individual intensities and put them into a ratio that fairly and consistently quantified each cell line's amount of DNA damage. Even though in the X-ray project it could be seen that the cells were repairing their DNA, there was still long-lasting damage that occurred. This sort of long-lasting damage occurs naturally in the Progeria cell lines and never presents itself as being fully repaired; even if the DNA is being constantly repaired more damage is constantly being generated. Our data supports this by showing that cell lines, AG11513 and HGADFN167 had more DNA damage than the healthy fibroblast lines. Like the senescence staining, our data also supports the amount of DNA damage increases with the cell passage number. This occurs with both cell lines but is more dramatic in the AG11513 cells.

It was important to gain a better understanding of how these Progeria cell lines grow and how they look. Interestingly, the senescence and DNA damage is more dramatic in the AG11513 cell line, so it would make sense that this Progeria cell line would have the most dramatic cell growth pattern and morphology as well, but this is not the case. Our data supports that the other Progeria cell lines AG03199 and HGADFN167 actually have the roundest morphology and are the most different morphologically when compared to the healthy cell lines. So far, we do not have a complete understanding of why this is, but both cell lines' cells had the tendency to grow in clumps rather than right next to each other in the directional monolayer observed in the healthy GM08398 cell line. Through this study, we have shown that there is a patient-to-patient variability in Progeria cell lines and reaching an overarching conclusion cannot be done by observing just one. For example, AG11513- Progeria affected male, was the only cell line to show a significant increase in DNA damage with a higher passage number but had the least abnormal morphology compared to the other Progeria cell lines.

In summary there is still much left to uncover and understand about the random genetic mutation that causes Progeria and how the disease affects the functionality of the cells. This project provides insight on how the DNA damage seen in normal fibroblasts due to ionization radiation is very similar to how it is seen in these Progeria cells naturally. Moving forward we would like to better understand and compare how this premature aging and DNA damage affects the 3D structure of the genome in progeria patients.

METHODS

Cell culture:

HGADFN167, a Progeria affected fibroblast from an 8-year-old boy, was purchased from the Progeria Research Foundation and AG11513, a Progeria affected fibroblast from an 8-year-old female, AG03257, an unaffected fibroblast from 35-year-old parent to AG11513, AG03199, an affected fibroblast from a 10-year-old female, and GM08398, an unaffected fibroblast from an 8-year-old boy, were all purchased from Coriell. Cells were grown in DMEM supplemented with 15% FBS, 1% Pen-strep, and 1% L-glutamine. Cells were always passed 1:2 at a confluency under 80%.

Senescence Staining and Analysis:

SA- β -gal activity was quantified using reagents from a CellSignal Senescence β -Galactosidase Staining Kit. Cells were seeded straight onto 6-well plate without glass coverslips. The adherent cells were washed with 1X PBS and incubated in 1 mL of fixative solution for 15 minutes. Cells were washed with 1X PBS again and were incubated in 1 mL 1X β -gal staining solution for 15 hours in 37°C in absence of CO₂ and light. After 15 hours, the 1X β -gal staining solution was replaced with 1X PBS and cells were imaged using EVOS FL Auto 2 microscope. All images were quantified using Image J using a scale of 5.5 pixels per micron. Once an image was loaded into ImageJ a color brightness threshold was applied that highlighted all the cells in the image. The highlighted cells were analyzed in order to get a total cell count. This process was repeated with a higher brightness threshold to select only for cells with an amount of senescence staining that was above the threshold.

Immunofluorescent Staining and Analysis:

Cells were seeded in 6-well plates containing coverslips coated in poly-D-lysine. Adherent cells were crosslinked using 4% formaldehyde for 10 minutes and then underwent 3 washes with 1X PBS. The slides were placed on a coverslip with 100 μ L blocking buffer, consisting of 10% goat serum and 0.5% Triton in PBS, to block non-specific binding of the primary antibodies. The slides were then incubated at room temperature for 1 hour. The coverslip was then carefully transferred to a new slide with 100 μ L of the primary antibody solution, consisting of 5% goat serum, 0.25% Triton in PBS, and a 1:500 dilution of the primary antibody to γ -H2AX (Abcam- ab26350). The sample was incubated at 4° C overnight. After overnight incubation, 3 washes with 1X PBS were performed. Next, 150 μ L of secondary antibody mix, 2 drops of Alexa-fluor-488 goat anti-mouse (Invitrogen) per mL, was added to each well and incubated for 30 minutes. The cells underwent 3 washes with 1X PBS once again. Finally, 2 drops of Slow fade Diamond Antifade Mountant (Molecular Probes) were placed on a clean glass slide and the coverslip was transferred onto the mounting media. The cells were incubated in a dark place overnight. Cells were imaged with a Leica SP8 confocal microscope equipped with a 40x oil immersion objective (Leica) using the same settings for every image. Image quantification was done by measuring the fluorescence intensity of γ -H2AX inside the nucleus divided by the fluorescence intensity of DAPI in ImageJ.

Morphology Analysis:

Each cell line was imaged using the Corning CytoSMART Cell Counter on the day of passage. A cell count was also recorded with each passage using the Corning CytoSMART and a 1:1 mixture of cells in media and trypan blue. Image quantification was done by dividing the area of the cells by the major axes produced by an ellipse fit in ImageJ.

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