Determining the Effect of the Age of the Environment on the Incidence of Cellular Senescence

Abstract

The goal of our project was to determine whether or not there was a causative relationship between the age of the environment and the incidence of cellular senescence. We used the protein p16 as a marker for senescence; this protein is localized to the nucleus when a cell exits the growth cycle (and thus becomes senescent). We made our cells--mouse myoblasts--senesce by UV irradiating them. In the assay we developed, cells were irradiated with increasing intensity, creating a kill curve which spanned from healthy proliferating cells, to DNA-damaged non-proliferating p16-expressing cells, to dead cells. The assay determined the optimal irradiation level for p16 expression. In the experimental procedure, we placed irradiated cells treated with optimal radiation and non-irradiated proliferating cells in wells containing blood serum from old or young mice (thus simulating environmental age). In order to measure nuclear p16 expression, we immunostained and imaged the wells, and ran a Western Blot. Our results indicate that the older environment decreases and younger environment increases the incidence of senescence cells. This counter-intuitive finding not only yields insight into novel ways to address illnesses related to blood loss and abnormal cell growth, but also leads to new and groundbreaking questions that can be investigated in future research.

Determining the Effect of the Age of the Environment on the Incidence of Cellular Senescence

1 Introduction

Cells are complex systems consisting of hundreds of components that work together in harmony to carry out specific tasks. An essential part of all cells is the cell cycle control system, a regulatory set of checkpoints that ensures that only properly functioning cells survive and duplicate. One of these checkpoints, located in the G1 phase of the cell cycle, is passed only by cells that are fully equipped to divide and function. When cells can't pass this checkpoint, they either die or become senescent—a state in which they don't divide, differentiate, or die. It is well established that the percent of senescent cells present in any organism increases with age. Although cellular senescence is a healthy, normal cell regulating mechanism, it also has undesired side effects because senescent cells exude harmful substances that damage their environment. Environments with greater percentages of senescent cells have cell behavior that deviates from the normal, including cancerous growth, immune malfunctions, and many other symptoms of increasing organismal age [1].

Although the correlation between senescence and age is established, we do not know whether there's a causative relationship: do older or younger environments affect the incidence of cellular senescence? We predicted that the causation would follow the pattern of correlation; in other words, older environments would encourage and younger environments would discourage the incidence of cellular senescence. Fully understanding the relationship between senescence and age could lead to developments in current treatments to life-threatening illnesses such as delayed immune system responses, extensive injury, and cancers by providing a way to increase cell proliferation and reduce unregulated cell growth.

The first step in investigating this relationship was creating an assay for the p16 gene expression, which has been established to be a good indicator of cellular senescence. An assay consists of tests performed on groups of cells to determine their behavior under different conditions. We tried two assays: the first involved the use of cells immortalized with the EJ-RAS gene; they also contained the p16 promoter driven 3MR construct that is a tri-fold gene reporter of senescence. The EJ-RAS gene causes the cells to grow uncontrollably, similar to cancer cells[2, 3]. The p16 promoter will be turned on in the nucleus if the cell senesces, and drive expression of the three genes of 3MR: *Renilla* luciferase (RL), red fluorescent protein (RFP), and herpes simplex virus- thymidine kinase (HSV-TK) [4]. In the assay, we created a "kill curve" to determine the optimal irradiation level for making cells senesce; we irradiated with different amounts of UV light, and quantified the amount of senescence using the 3MR genes. The goal was to establish a baseline for senescent cells. However, when conducting the test for the RL and HSV-TK, it became clear that the cells with the EJ-RAS gene were not cooperative - due to their cancerous mutation, which ignored the normal cell regulation checkpoints in the growth cycle, they did not senesce as expected when irradiated despite extensive DNA damage.

We conducted the assay again using primary myoblasts, which are normal muscle cells extracted from mice. Because the myoblasts did not have the 3MR construct, they didn't display the same p16 expression markers for senescence as the EJ-RAS cells; however, the myoblasts, which did contain the p16 protein, could be tested with very little modification of the previous assay. Due to the absence of RL and HSVTK, we used immunostaining and Western Blots to quantify the amount of p16 protein in the nuclei of groups of myoblasts radiated with increasing amounts of UV. All cells have a certain amount of p16 protein, but senescent cells generally have increased amounts of nuclearly-localized p16 protein. Immunostaining helped produce

images that could be analyzed for nuclear fluorescence; and Western blots quantitatively showed the amounts of localized p16 in groups of irradiated cells.

Senescence has not previously been studied with the goal of determining its relationship with age of the environment. The purpose of this research is to enhance the current understanding of the cell cycle, senescence, and their connection with age. It answers important questions: does putting senescent cells in a younger environment inhibit or encourage further propagation of senescence in that locus? Does senescence increase or decrease senescence?

2 Methods

The research conducted investigates the cause-effect relationship between senescence and age through the use of two sets of tests. The first set established a baseline for senescent activity under various levels of radiation, and the second manipulated the age of the environment. Two different types of cells were used in the research; each one was tested in different ways to suit its specific gene composition. The EJ-RAS cells, which acted as cancer cells, grew uncontrollably with no regulatory mechanisms, and thus did not senesce properly. Thus the myoblasts, normal mouse muscle cells, were used as a second trial.

In both the assays and the experiment, the first step was to irradiate the cells with different amounts of UV; this damaged the DNA so that cells would exit the cell cycle and senesce[5]. We used a Stratagene Stratalinker model 2400 UVC (~250 nM) irradiator with a photodetector to calibrate the received dose. Radiation was administered in a range of doses to different batches of cells grown in a controlled environment to create a kill curve, which provided valuable insight into the optimal range of radiation for inducing senescence.

The methods used for the EJ-RAS cells had 2 main stages: (1) irradiating cells with UV light, and (2) quantifying amounts of RFP.

The myoblasts, which were cooperative in the assay and thus also used in the experiment, lacked the 3MR construct and were evaluated for senescence using immunostaining. The immunostaining process we used can be divided into three parts: (1) irradiating samples (and determining the optimal UV radiation dose), (2) determining the quantity of nuclear p16 in each sample through a Western, and (3) evaluating the percent nuclear localization in each sample through epifluorescence imaging. The immunostaining process in the assay and experiment were nearly identical; the only difference was that environmental conditions were manipulated in the experiment.

2.1 EJ-RAS

The EJ-RAS line of cells is genetically modified to include the EJ-RAS gene in the DNA (gift of the Campisi lab) [1]. This gene not only makes the cells grow without regulation, much like cancer cells, but also makes them carry the p16-3MR reporter construct.

The test conducted determined the percentage of senescent cells in each of the plates irradiated with UV light and intended to build a kill curve. To determine which plates had the highest likelihood of containing senescent cells, we created and analyzed a table of confluences (below).

	5 days after plating (% confluences)	8 days after plating (% confluences)	11 days after plating (% confluences)
Plate 1 (0 J/m^2)	80%	90% (100 mm plate)	80% (100 mm plate)
Plate 7 (16 J/m^2)	15%	25%	10%
Plate 8 (32 J/m^2)	5%	5%	15%
Plate 10 (64 J/m^2)	0%	0%	0%

Table 1: This table contains qualitative data collected while culturing EJ-RAS cells. All reported values are assumed to be on 35 millimeter plates unless stated otherwise.

Plate 1 served as a control; it was not irradiated. Plates 7 and 8 maintained about the same degree of confluence, considering that the estimation involved approximately a 10% margin of error. A constant confluence indicates that the cells are neither dying nor growing over time; thus, they are most likely senescent. Therefore, we predicted that an irradiation between 16 J/m^2 and 32 J/m^2 was the optimal range for inducing senescence in EJ-RAS cells.

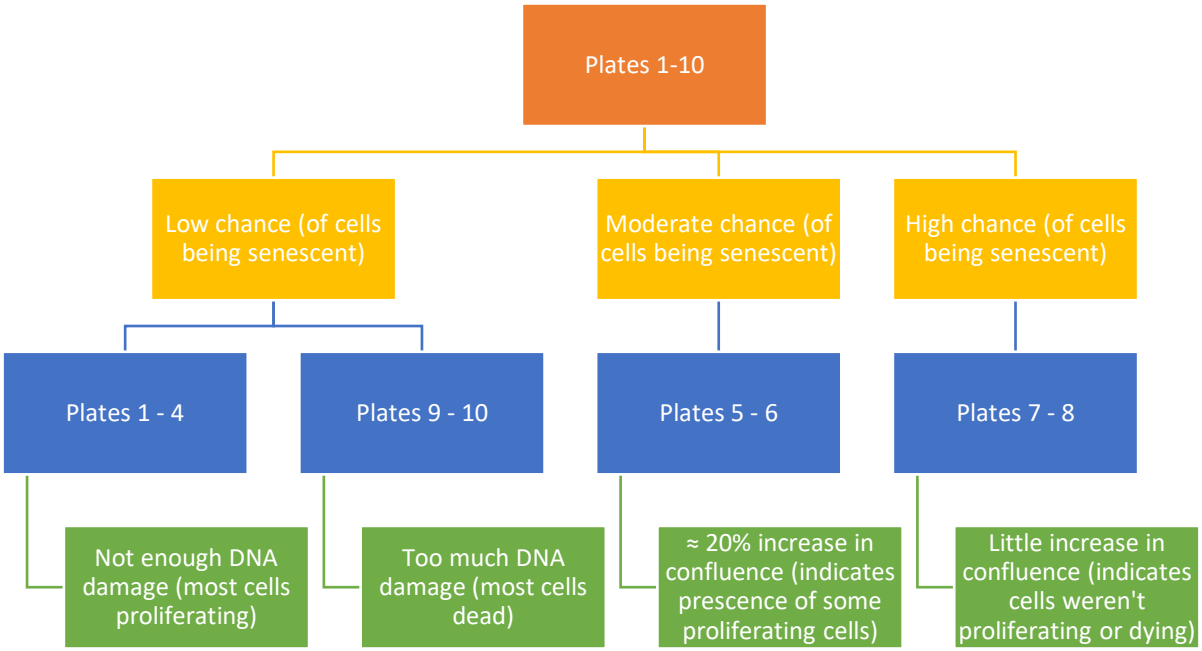


Figure 1: This flowchart demonstrates how we used the information from Table 1 to determine that Plates 7 and 8 were the ideal candidates for having senescent cells.

Once we determined that plates 7, 8, and 1 (the negative control) would be analyzed for senescence, we used fluorescence microscopy to identify the senescent cells in each plate. Corresponding brightfield and epifluorescent images were taken for comparison for every cell. We carefully and systematically screened each plate, making sure to take pictures in such a way that the ratio of regular to fluorescent cells in the plate was reflected in our images. By using scientific python, we calculated the mean RGB value of the pixels in the cell region of each fluorescent image, we determined the brightness of the red and thus quantified the amount of red fluorescent protein (RFP) in each specific cell.

2.2 Myoblasts

The second type of cells used in this research was mouse myoblasts. Myoblasts are cells that elongate into tubular shapes when they differentiate; they form the primary structure of muscle fibers when connected end-to-end. These cells, which were taken from healthy mice, were more natural than the mutated EJ-RAS cells and thus showed promise of behaving predictably. The myoblasts didn't display the 3MR construct like the EJ-RAS cells did, but like all cells, they contained the p16 protein, which becomes nuclear when a cell senesces. This localization was quantifiable through immunostaining and a Western blot, making the myoblasts an appropriate choice for our assay.

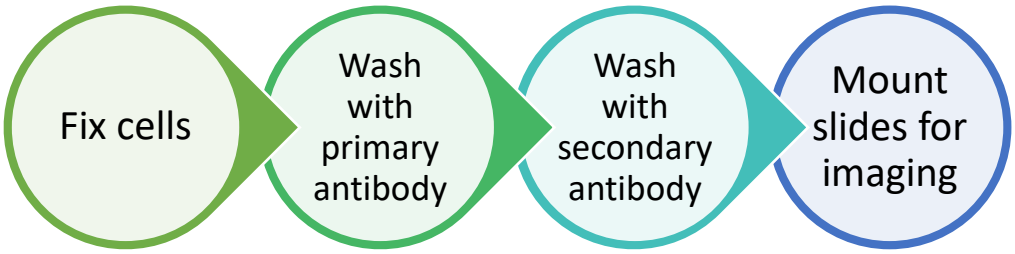
Each plate of myoblasts was irradiated at varying doses of UV while the cells were at ~50% confluence in order to maintain an even starting point. Based on the results from the EJ-RAS cells, the lowest dose (excluding the controls) was 12 J/m^2 and the highest was 48 J/m^2.

2.2.1 Assay Immunostaining

The first test conducted using the myoblasts was the assay immunostaining to determine a kill curve and establish baseline behavior under various conditions. This method is not to be confused with experimental immunostaining, in which we varied additional factors such as the age of the environment. Prior to being stained with antibody, the cells were plated onto 8-chamber slides and fixed with the ethanol fixation method, which keeps cellular structure and localization intact during the immunostaining procedure. Intact cell structure was crucial because the goal was to distinguish between nuclear p16 expression, a marker of senescence, and cytoplasmic p16 expression, a regular cell expression. Immunostaining entails the use of various antibodies to "mark" certain proteins in the cells, and it is commonly used for this purpose. The wells were each washed in the primary antibody (PA), rabbit anti-p16, which found and binded

to localized (nuclear) p16 protein. They were then washed in the secondary antibody (SA), anti-primary, which found and binded to the primary antibody. The SA was necessary because the compound is linked to a fluorophore that fluoresces red when exposed to green light—making it possible to image the wells. It is important to note that HOECHST DNA staining dye, which binds to nuclear DNA and fluoresces blue under UV light, was added in dilute concentrations to the secondary antibody. This made it possible to view just the nuclei under the microscope and more accurately determine whether or not the red fluorescence was nuclear. Once the wells were thoroughly washed, the slides were mounted to coverslips using Fluoromount (Sigma Aldrich), which helps delay fluorescent bleaching.

Figure 2: This flowchart summarizes the process used to prepare to cells for fluorescent imaging in assay immunostaining (Section 2.2.1) This procedure is also a part of the experimental immunostaining method (Section 2.2.3)



5 wells were used to create a kill curve with 5 levels of radiations in increments of 12 J/m^2. These irradiation levels illustrated a full spectrum of the kill curve, with the starting point of 0 J/m^2 indicating almost no cell death to the ending point of 48 J/m^2 indicating almost guaranteed cell death. Each of the 3 remaining wells served as controls: they received a different combination of primary and secondary antibodies in an effort to correct for and control various experimental factors. One well received nonspecific PA and specific SA, which would show nonspecific binding of rabbit antibody when secondary bound to it. One sample received

secondary antibody only, which would show any red fluorescence caused by non-specific secondary binding. The final sample received nothing at all; this was the negative control for any background cellular fluorescence (caused by, for instance, the tint of the microscope). A labeled diagram of the 8 well chamber slide is shown below.

Well 1: 12 J	Well 2: 24 J	Well 3: 36 J	Well 4: 48 J
Well 5: 0 J (nothing)	Well 6: 0 J (non-specific PA and specific SA)	Well 7: 0 J (specific PA)	Well 8: 0 J

Table 2: This table shows the positioning of the cells on the 8 well slide. Each well was imaged separately during data collection.

After immunostaining, we took pictures of cells from each well for analysis of the percentage of general and localized cell fluorescence. The images were exposed and edited uniformly in such a way that redness caused by the unwanted factors explained above was subtracted from the overall redness of the picture, showing purely p16 fluorescence. In each well, when enough cells were present, we analyzed a large data set of approximately 120 cells. We counted the total number of cells and compared that value to the number of cells expressing nuclear fluorescence. This allowed us to determine the degree of localization and thus the degree of senescence per plate.

2.2.2 Westerns

We also used the Western Blot technique to quantify the amount of nuclear p16 protein in some samples of cells. We could not use this method for all the plates because many did not have enough cells to produce enough protein for a Western.

A large portion of our Western methods was conducted as described by the blotting membrane manufacturer (Amersham Hybond NC #RPN3032D), and Enhanced

Chemiluminescent (ECL) reagent manufacturer (Avansta #K-12045). In addition, our protein quantification was performed as described by reference # (y) on the Bradford assay, using the reagent manufacturer's protocol (Gbiosciences #786-676).

To harvest the protein from the cells, we first washed the cells on plates 5, 6, and wild Types 5 and 6 gently in PBS. We added sodium dodecyl sulfate to lyse the cells and dislodged the cells using a protein scraper. We froze the lysate that was produced after centrifuging and discarding the supernatant.[6]

To normalize the amount of protein in each sample, we performed a serial dilution of the protein lysates harvested previously. A solution of Bovine Serum Albumin (BSA) was used as a standard. Once the dilution was performed for the samples, we obtained absorbance values for each by using a spectrophotometer to measure absorbance at 595 nm. These values were analyzed to yield a calibration curve for BSA, from which the concentrations of other proteins were obtained.[7]

We loaded appropriately sized samples into the Western and then ran it. We used a voltage of 20V, and the proteins in each sample separated by size. We used a nitrocellulose membrane for the transfer and obtained 4 membrane samples; we washed each membrane in primary and secondary antibodies to mark the p16 protein for imaging. Throughout the process, we made sure to keep the membranes pristine, so as not to introduce contaminant proteins before imaging.[6]

We imaged the membranes under white Epifluorescence and analyzed the images to yield results.[6]

2.2.3 Experimental Immunostaining

The process for the experimental immunostaining, which aimed to determine a relationship between cellular senescence and environmental age, was identical to that for the assay immunostaining procedure except for the medium conditions the cells were grown in. Some cells were placed in old serum, serum extracted from old mice. Similarly, others were placed in young serum. Both the old and young mice were born and raised in the lab; they were healthy, and identical in all aspects except for their age (this was in order to minimize any effect not caused by age and maximize the extent to which our experiment was controlled). The layout of the 8-well slide containing the cells and their corresponding environments is shown below:

Well 1: 0 J/m^2 Growth Medium	Well 2: 20 J/m^2 Growth Medium	Well 3: 0 J/m^2 Young Mouse Serum	Well 4: 0 J/m^2 Old Mouse Serum
Well 5: 0 J/m^2 Conditioned Medium (normal cells placed in irradiated cells' medium)	Well 6: 20 J/m^2 Old Mouse Serum	Well 7: 20 J/m^2 Young Mouse Serum	Well 8: 0 J/m^2 Growth Medium IgG:(nonspecific primary antibody)

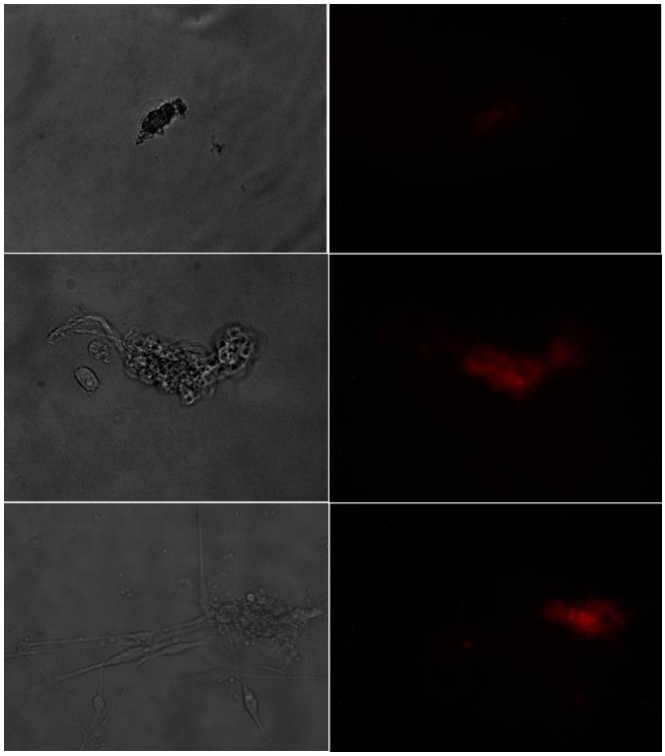
Table 3: This table shows the positioning of the cells on the 8 well slide. Each well was imaged separately during data collection. Wells 3 and 4 make up ES 1, while Wells 6 and 7 make up ES 2.

All irradiated cells were irradiated with 20 J/m^2 because this was approximated to be the optimal senescence point by the kill curve in the assay -- the point at which the greatest percentage of cells were senescent without significant depletion in cell numbers. Well 1 was the negative control; the nuclear p16 expression was expected to be negligible and the cytoplasmic fluorescence was expected to serve as a baseline for background senescence. Well 2 was the positive control; a large percentage of these cells were expected to express the senescence marker. Well 8 was the background, as seen in the assay. Wells 3 and 4 made up Experiment Set

1 (ES 1), which aimed to explore the effect of serum age on healthy cells. The prediction was that old serum would induce more senescence—old serum is more senescent, and it is natural that the causation would follow—and create—the correlation we observed. Wells 6 and 7, which made up ES 2, were a repeat of ES 1 with stressed cells, and the same prediction held. Well 5 was an experimental variable of its own -- the medium was not old or young but conditioned with 50% irradiated cell medium, and thus "stressed." The expectation was that conditioned medium would give rise to a greater number of senescent cells than the regular medium (the negative control), as substances secreted by senescence are pervasive in the environment and free to interact with healthy cells. We expected it to have fewer number of cells than the positive control, however, in which nearly all of the cells would be senescent.

The slide was prepared as described in the assay, and 3 images were taken for each group of cells from each well. The first picture in the set of three was a phase contrast image, to indicate the location of the cells. The second was a nuclear fluorescence image to show the location of the nuclei. The third was the red fluorescence, given off by the excited secondary antibody bound to the primary anti-p16.

Figure 3: Paneling representing corresponding Brightfield (Left) and Epifluorescence (Right) images for Plates 1 (Top), 7 (Middle), and 8 (Bottom).



3 Results and Discussion

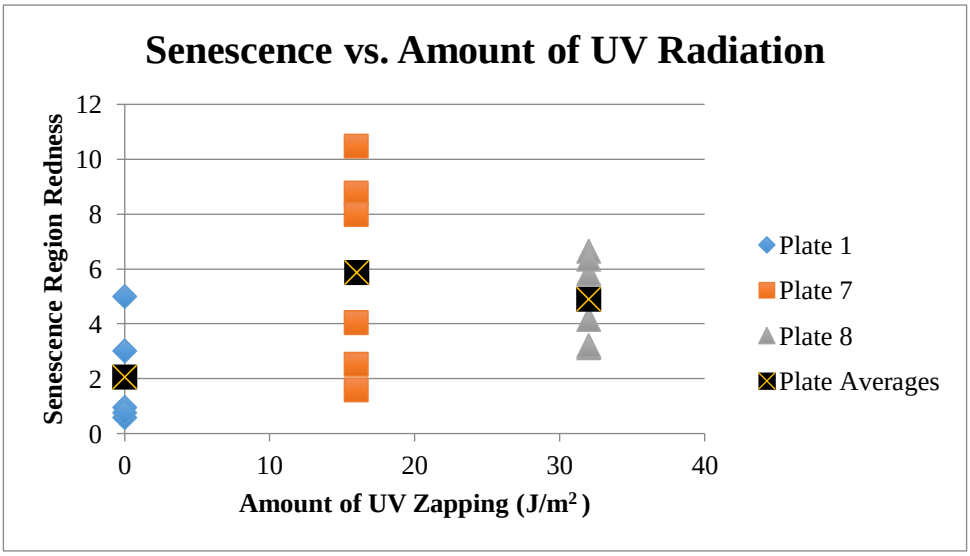
3.1 EJ-RAS (RFP Visual Testing) Assay

The images in Figure 3 are representative; they reflect the average

brightness of the images from each respective plate. Plate 1 has very faint fluorescence, indicating that when the EJ-RAS cells received no radiation and were allowed to grow and behave as they would under normal conditions, very few were senescent. Since senescence is a normal regulatory method in cells, it reasonable that it was not zero even in the control plate. The average cell in Plate 7 and 8 was much brighter, indicating that more of their cells were senescent. However, we expected plate 8 cells to be much brighter than plate 7 ones—a much greater percentage of the plate should have been senescent, increasing the average brightness. The fact that this did not occur indicates that the EJ-RAS cells did not respond appropriately to the cell-regulation mechanisms—at 32 J/m^2, despite much more extensive DNA damage, they grew just as prolifically as they did at 16 J/m^2.

The results from the senescence analysis of the EJ-RAS cells indicated that the EJ-RAS cells were not cooperative. The rate of death and the corresponding rate of senescence did not increase with irradiation. Graph 1 shows the average brightness of the red in senescent cells for plates 1, 7, and 8. The senescence (and death rate, indicated by the percent confluence in the

Graph 1: The results of the EJ-RAS assay. The brightness of the p16 marker as a function of amount of irradiation.

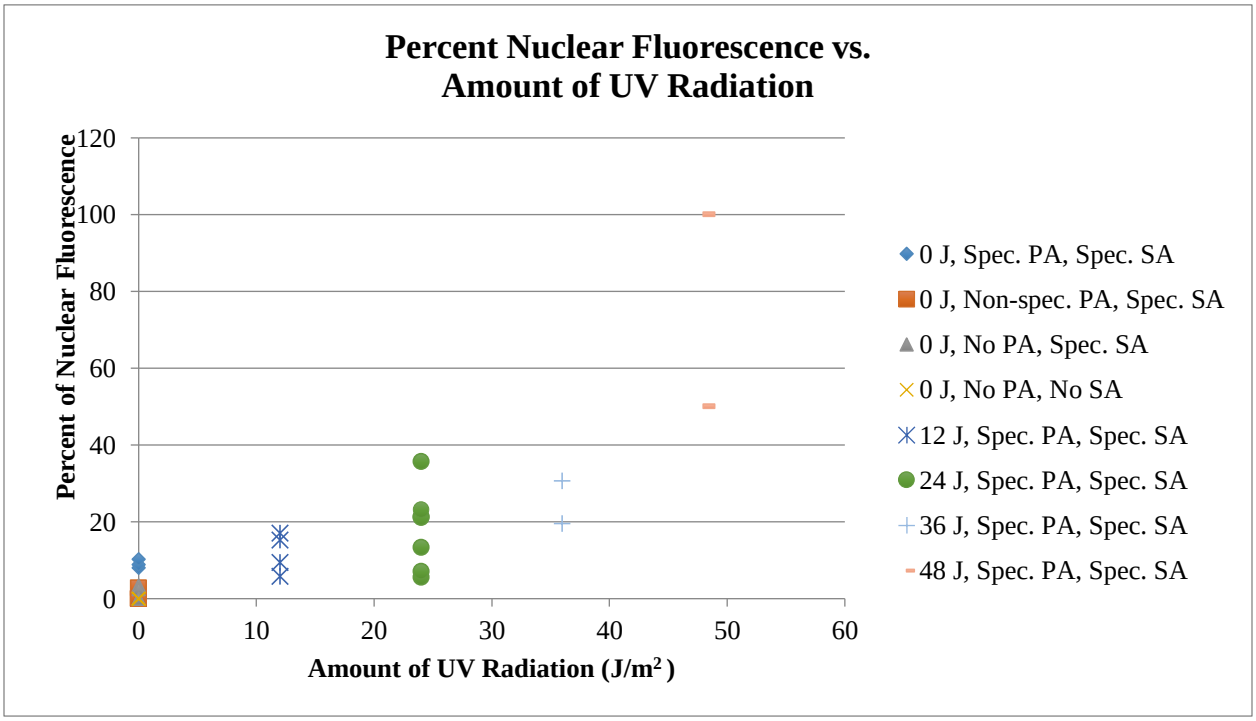


methods) at 16 J/m^2 and at 32 J/m^2 is approximately equal, making a kill curve impossible to determine. Overall, the inconsistency of the EJ-RAS cells made them an ineffective cell type for our purposes—thus, we attempted the same methodology on more conventional cells.

3.2 Myoblasts

3.2.1 Immunostaining Assay

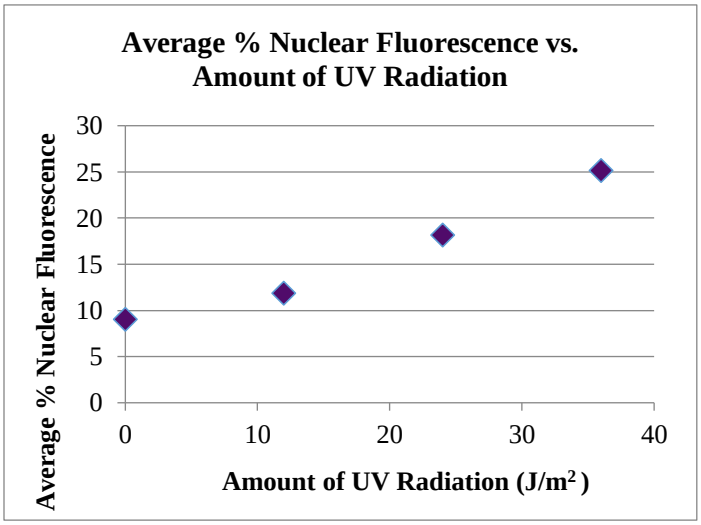
Graph 2: The results of the myoblast immunostaining assay. The brightness of the p16 marker as a function of amount of irradiation.



The wells that were not irradiated served as control plates to the fluorescence that arose naturally from various sources, such as the background of the microscope and nonspecific antibody binding. The non-irradiated well that was washed with both primary and secondary antibody was the negative control; it represented the amount on nuclear p16 in healthy, proliferating cells. All the other wells show the effects of increasing irradiation on p16 expression (senescence). The assay immunostaining on the myoblasts revealed that higher amounts of radiation greatly increased the percent senescence. This is because the DNA was so

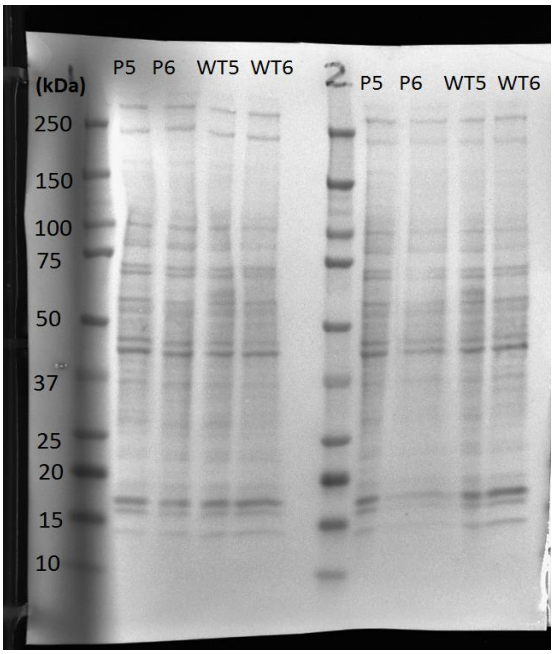
damaged that the cells were largely incapable of growing—they either died or senesced. Lower dosages did not damage the DNA so extensively or pervasively, explaining the lower senescence rates. In all of the images taken for the myoblasts, a cell with nuclearly localized fluorescence (fluorescence centered in the nucleus of the cell) was defined as senescent, while a cell with cytoplasmic senescence (diffused fluorescence throughout the cell interior) was defined as normal. Each data point in Graph 2 represents the percentage senescence for one picture taken from the well indicated. Each data point in Graph 3 indicates the average nuclear fluorescence of all such pictures. For 0 J/m^2, the average is of the well washed with both antibodies (the control).

Graph 3: The results of the immunostaining assay, summarized (averages only).



The overall trend makes logical sense: the higher the dosage of irradiation received by the cells, the higher the likelihood that any surviving cells would become senescent. We determined that an irradiation level between 12 and 24 J/m^2 was optimal—a significant number of cells were senescent, but a significant number were also still alive.

Figure 4: Image containing 2 of 4 imaged Western membranes, analyzed for results.



3.2.2 Westerns

Figure 4 shows an image of the nitrocellulose membrane that the gel was transferred to. Each lane, which corresponds to a plate from the irradiated culture from Figure 3, contains protein bands that represent the amount of different proteins in each plate. The proteins can be identified by their size, which is measured in kilo-Daltons using the pre-stained marker to the very right. The amount of p16 protein in each plate was affected by two variables: the number of cells on the plate (a greater number of cells would increase overall protein concentration), and amount of irradiation. However, we were only interested in the latter relationship. In order to isolate the effect of irradiation, we normalized the amount of protein in each lane using actin. Actin, a protein that stabilizes at 42kD, has a linear relationship with cell count and is unaffected by irradiation. Thus, we determined the actin ratios between lanes consequently modified the p16 ratios. The adjusted p16 bands, which only reflected the changes in p16 expression due to irradiation, yielded the following results: although the p16 band in plate five is slightly darker than the p16 band in plate 6, the actin band is much darker in latter. If the p16 is adjusted as per the actin, it is obvious that the cells in plate 6 have a higher percent confluence than those in plate 5. WT5 and WT6, the negative controls, both had bands much lighter than 5 or 6.

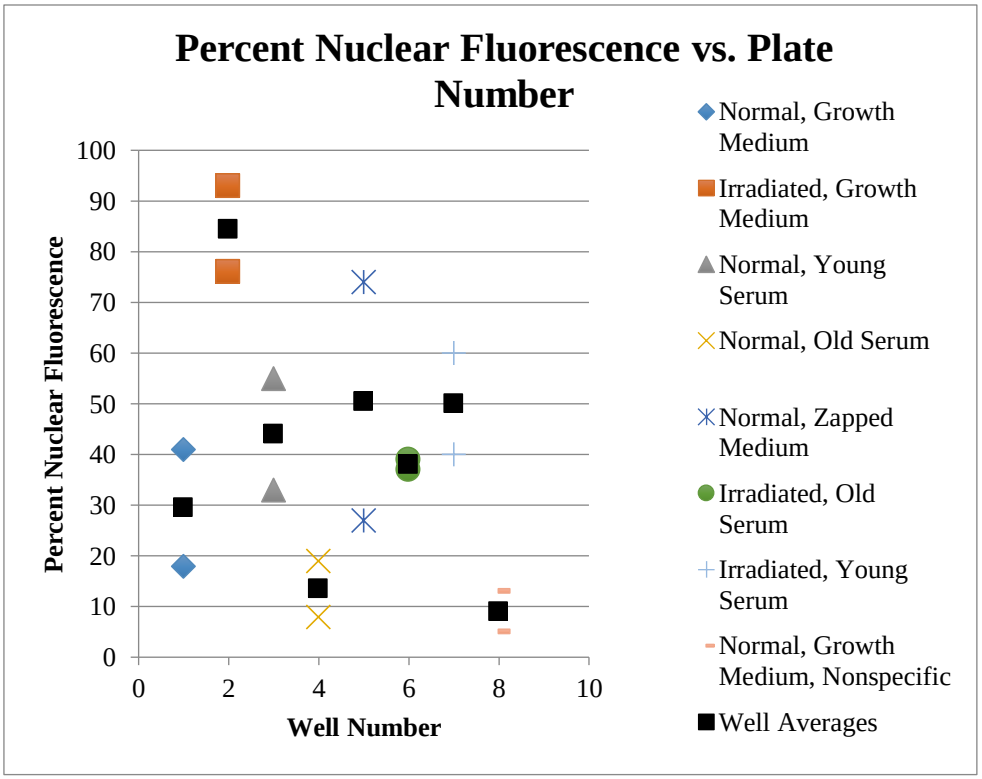
3.3.3 Experimental Immunostaining

As seen in Graph 4, the positive control and negative controls both worked as expected: well 1, which contained normal cells in growth medium, had a very low percent nuclear fluorescence and well 2, which contained irradiated cells in growth medium, had a very high percent nuclear fluorescence.

Well 8 contained normal cells washed with non-specific primary antibody, which binds with generic proteins. As explained in the assay, this detected the background fluorescence that was not caused by p16, establishing a baseline for how brightly fluorescent a cell must be to be considered senescent. Well 8 had a percent fluorescence of approximately 9%, which, when the 10% margin of error is taken into account, goes to almost 0%; this, too, is consistent with what we expected.

Well 5, the experimental well, matched the predictions, with a percent fluorescence between the positive and negative controls. However, in both ES 1 and ES 2, the percent fluorescence in the old serum is clearly greater than that in the young serum -- the exact converse of the expectation.

Graph 4: The results of the experimental immunostaining. Two pictures, each with approximately 200 cells, were taken per well.

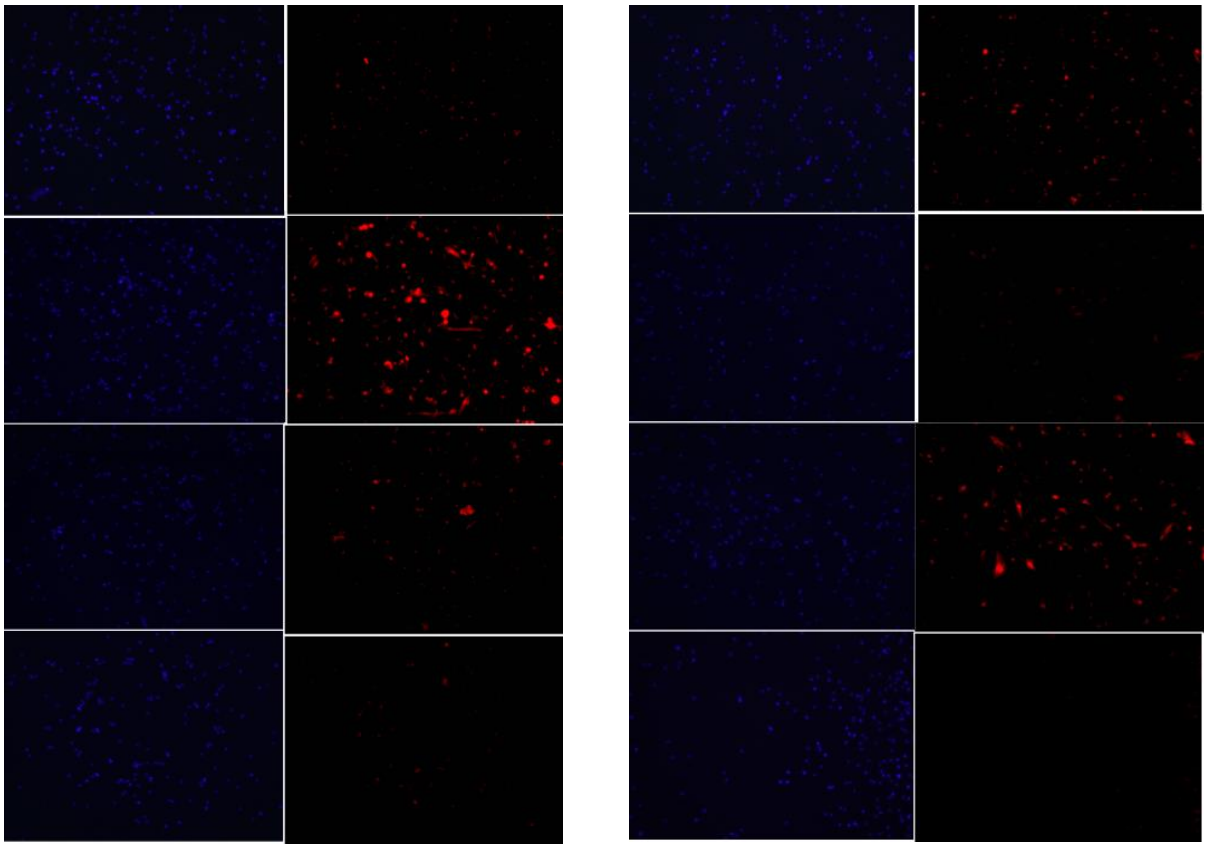


Although it is possible that there was some undetected experimental error, it is unlikely because our results were robust: we quantified approximately five hundred cells for every plate, and repeated the quantification several times. The data set was large, and the accuracy of the three controls indicate that there was no significant experimental error. Our results were conclusive in showing

that older environments decrease and younger environments increase incidence of cellular senescence. This finding is both groundbreaking and counterintuitive; investigation into the relationship between cellular senescence and age has not been done before, and the findings are unexpected -- leading to the question of why.

Figure 5: Paneling showing images taken from each of 8 wells. These pictures represent the pictures that had the higher percent fluorescence for each well. The well numbers line up with the images seen below.

Well 1: Non – irradiated, growth medium
Well 2: Irradiated, growth medium
Well 3: Non – irradiated, young serum
Well 4: Non – irradiated, old serum
Well 5: Non – irradiated, conditioned medium
Well 6: Irradiated, old serum
Well 7: Irradiated, young serum
Well 8: Non – irradiated, growth medium (non-specific primary)



4 Future Work and Conclusion

Our research concluded that an older environment decreases the incidence of cellular senescence. We originally predicted the opposite because we expected the causation to mirror the

correlation. However, our experiment indicates that although older environments have more senescent cells present in them, they decrease the actual incidence of senescence. The unexpectedness of the finding suggests that there may be some vital connection between the cell cycle and age that we don't understand. This research is the first step in uncovering something interesting and potentially profound.

To confirm that this pattern is universal, the same experiment can be repeated in living mice to test if the age-senescence relationship holds in non-isolated environments. If it stands true, then the experiment can continue to be repeated in progressively higher mammals and eventually humans.

This new line of experimentation our research instigated will not only help uncover new information about senescence, a critical biological response, but also open new ways to approach certain illnesses.

As we stated in the introduction, senescent cells release harmful substances that damage their environment. Old environments, which have greater percentages of senescent cells, have cell behavior that diverges from the normal, including cancerous growth and immune malfunctions. However, knowing that older serum also decreases the incidence of senescent cells—in other words, knowing that the correlation could be reversed by the causation relationship—could make environments behave younger and thus reduce the effects of age-induced, senescence-related disorders.

In conclusion, this research has uncovered an unexpected relationship that has important repercussions in the future of experimentation dealing with age and senescence and our understanding of the cell cycle's relation with age.

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