**UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL**
**INSTITUTO DE BIOCIÊNCIAS**
**CENTRO DE BIOTECNOLOGIA E DEPARTAMENTO DE BIOFÍSICA**

TRABALHO DE CONCLUSÃO DO CURSO EM CIÊNCIAS BIOLÓGICAS

# Nuclear Characterization of Tumor Stem Cells in Human Glioblastoma

## Caracterização Nuclear de Células Tronco Tumorais em Glioblastoma Humano

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# TRABALHO DE CONCLUSÃO

**Nuclear Characterization of Tumor Stem Cells in Human Glioblastoma**

Trabalho desenvolvido no Departamento de Biofísica do Instituto de Biociências e no Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul, como parte dos requisitos necessários para a obtenção do Título de Bacharelado em Ciências Biológicas pela Universidade Federal do Rio Grande do Sul.

**Kyle Edward Sunshein**
**Professor/ Supervisor: Dr. Guido Lenz**

## FIPSE/CAPES

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Kyle E. Sunshein is an undergraduate student of Biology (Pre-Medicine) at The Ohio State University, Columbus, OH, United States of America, selected for an exchange training period at UFRGS, within the Collaborating Program and Exchange Brazil-USA (FIPSE/CAPES 048/06) promoted by the Fund for the Improvement of Postsecondary Education (FIPSE) of the U.S. Department of Education and *Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior* (CAPES) of the Brazilian Ministry of Education.

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Kyle E. Sunshein é estudante de graduação em Biologia (Pre-Medicina) da *Ohio State University*, Columbus, OH, Estados Unidos da América, selecionada(o) para um período de intercâmbio e treinamento na UFRGS, junto ao Programa de Colaboração e Intercâmbio Brasil-Estados Unidos (FIPSE/CAPES 048/06) promovido pelo *Fund for the Improvement of Postsecondary Education* (FIPSE) do Departamento de Educação Norte-Americano e pela Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) do Ministério da Educação Brasileiro.

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# List of Abbreviations

| | |
|---|---|
| ºC | graus Celsius |
| CNS | Central Nervous System |
| CSC | *Cancer Stem Cells* |
| DMEM | Dulbecco's Modified Eagle Medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| EDTA | Ethylenediamine tetraacetic acid |
| EGF | Epidermal growth factor |
| FBS | Fetal Bovine Serum= |
| FGF | Fibroblast growth factor |
| GBM | glioblastoma multiform |
| µg | unit of measurement: microgram |
| mg | unit of measurement: milligram |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| LIF | Leukemia inhibitory factor |
| µl | unit of measurement: microliter |
| µM | unit of measurement: micromolar |
| ml | unit of measurement: milliliter |
| mm | unit of measurement: millimeter |
| mM | unit of measurement: millimolar |
| nm | unit of measurement: nanometer |
| NMA | Nuclear morphology analysis |
| ODC | ornithine decarboxylase complex |
| WHO | World Health Organization |

## Abstract

Gliomas are tumors of the central nervous system that possess glial characteristics and have been distinguished as being extremely aggressive, highly invasive, as well as neurologically destructive. Gliomas possess certain characteristics that help ensure their successful formation and development, such as: evasion of apoptosis, unlimited replication, the capacity to proliferate without external stimuli, the ability to grow despite external suppressive mechanisms, and a high resistance to both chemo and radiotherapy. Recent evidence suggests that gliomas are heterogeneous tumors comprised of both differentiated cells and a type of undifferentiated cells known as cancer stem cells (CSCs), which are thought to be directly involved in the high resistance to treatments and therapies. The specific objective of this study was to characterize the nuclei of CSCs transfected with a marker called ZsGreen to verify the presence of mitosis and nuclear irregularities characteristic of apoptosis, mitotic catastrophe, and senescence in either the CSCs or their neighboring cells with or without the presence of growth factors. ZsGreen is a green fluorescent protein that can be selectively viewed under fluorescence microscopy in cells with low proteasome activity due to the proteasomal recognition of the ornithine decarboxylase (ODC) degradation sequence. U87 glioma cells were cultured under standard conditions using both medium with 5% FBS and with growth factors (CSC culture medium). Four wells of a 24-well plate were each seeded with 50,000 cells, while two wells were transfected with ZsGreen and the other two were used as control groups without transfection. One control well and one transfected had medium with FBS while the other two wells were supplemented with basic Fibroblast Growth Factor (FGF), Epidermal Growth Factor (EGF), Leukemia Inhibitory Factor (LIF), as well as B27 supplement. Hoechst was also utilized with these U87 cells in order to view them under fluorescent microscopy and analyze their nucleus size and form using the NMA plugin of the ImageJ program. Although the transfection and nuclear staining were successfully performed, various factors provided difficulties in analyzing these photos and compiling the results. These factors included: unclear photo resolution, interference from cell debris, insufficient experimental population, among others. In order to conduct further studies concerning CSC research, major changes should be made to enhance the efficiency and practicality of this project, such as focusing on a specific irregularity (senescence, for example) and trying to induce that irregularity through specific *in vitro*

treatment methods. More reliable methods of data analysis could also be utilized regarding the general topic of CSC research such as flow cytometry or Fluorescence Activated Cell Sorting (FACS).

## Resumo

Gliomas são tumores do sistema nervoso central caracterizados como sendo extremamente agressivos, altamente invasivos, assim como neurologicamente destrutivos. Eles apresentam características que ajudam a garantir a sua formação e desenvolvimento, tais como: evasão da apoptose, replicação ilimitada, capacidade de proliferar sem estímulos externos, capacidade de crescer apesar dos mecanismos externos de supressão e uma alta resistência a quimio e radioterapia. Evidências recentes sugerem que gliomas são tumores heterogêneos composto por células diferenciadas e células indiferenciadas conhecidas como células tronco tumorais (CSCs), que estariam diretamente envolvidas na alta resistência aos tratamentos. O objetivo deste estudo foi caracterizar os núcleos de CSCs transfectadas com ZsGreen para verificar a presença de mitose e irregularidades nuclear característica de apoptose, catástrofe mitótica e senescência em tanto as CSCs quanto as suas células vizinhas, com ou sem a presença de fatores de crescimento. O plasmídeo do ZsGreen é uma proteína fluorescente acoplada com uma sequência degradada pelo proteassoma (ornitina decarboxilase) que pode ser visto através de microscopia de fluorescência em células com atividade proteassomal baixo. Células de glioma U87 foram cultivadas em condições normais utilizando o meio com 5% SFB ou com fatores de crescimento (meio específico para CSC). Quatro poços de uma placa de 24 poços foram semeados com 50.000 células, enquanto dois poços foram tranfectados com ZsGreen, os outros dois foram usados como grupo controle sem tranfecção. Um poço de controle e um poço transfectado tinham meio DMEM *Low glucose* com SFB enquanto os outros dois poços continham meio DMEM F12 suplementado com FGF, EGF, LIF e B27. A coloração nuclear com Hoechst também foi utilizada com estas células U87, a fim de exibí-los através de microscopia fluorescente e analisar seu tamanho e forma usando o plugin NMA do programa ImageJ. Embora a transfecção e coloração nuclear foram realizados com sucesso, vários fatores causaram dificuldades em analisar as fotos e compilação dos resultados. Estes fatores incluem: indefinição da resolução das fotos, a interferência de restos celulares, a população experimental insuficiente, entre outros. A fim de realizar estudos adicionais na pesquisa de CSCs, mudanças devem ser feitas para melhorar a eficiência e praticidade deste projeto, como se concentrar em uma irregularidade específica (como senescência) e tentando induzir essa irregularidade através de métodos de tratamento específico *in vitro*.

# 1 Introduction

## *1.1 Gliomas*

Gliomas are tumors of the central nervous system (CNS) and are the most common type of primary brain tumor. They have been identified as being extremely aggressive, highly invasive, as well as neurologically destructive, making them one of the deadliest of human cancers (Maher, 2001). It has also been observed that glioma cells (Figure 1) possess a high resistance to both chemo and radiotherapy, making them extremely difficult to treat (Bredel, 2002).

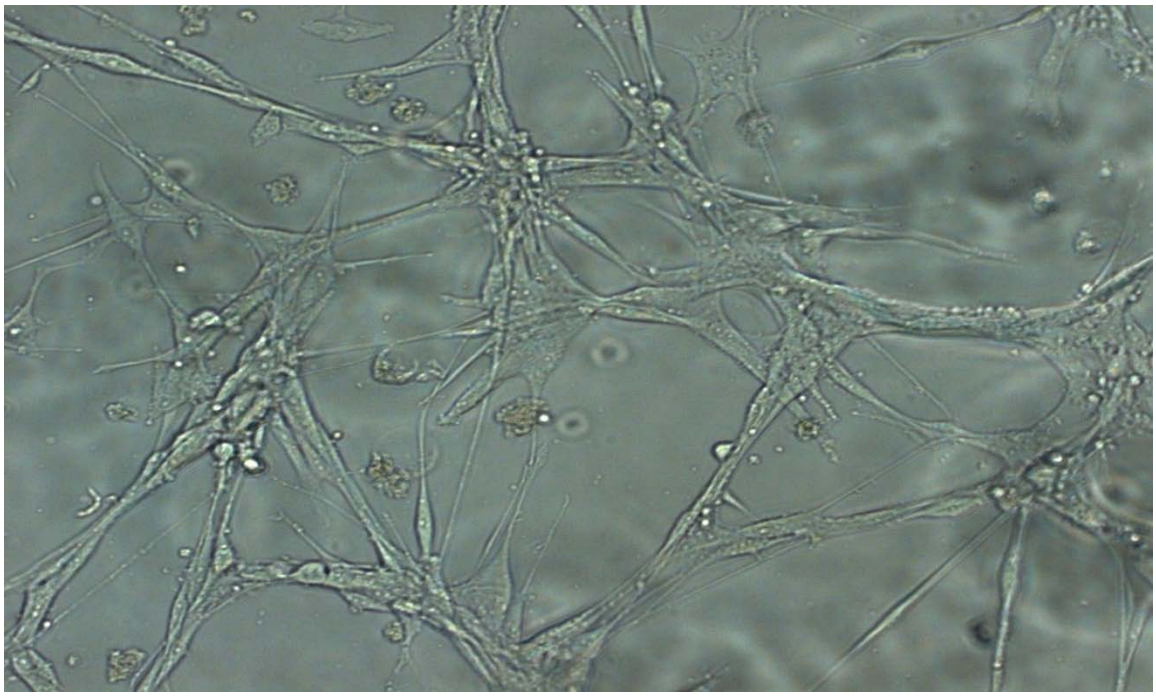


**Figure 1 – U87 glioma cells, cultured *in vitro*, shown in visible light (observed under an optic microscope).**

Like all other tumors, gliomas possess certain characteristics that help ensure their successful formation and development. These include: evasion of apoptosis, unlimited replication, the capacity to proliferate without external stimuli, the ability to grow despite external suppressive mechanisms such as the immune system, and the aptitude to spread to healthy tissues (Hanahan, 2000; Rich, 2004).

According to the World Health Organization (WHO) as of 2007, gliomas are classified by cell type, location, and grade. Cell type classifications include: gliomas from astrocytes,

oligodendrocytes, ependymal cells, and mixed gliomas. Location of gliomas is classified in relation to the cerebellar tentorium, or in other words, supratentorial (above the membrane) or infratentorial (below the membrane). The last category, grade, is used to classify the degree of malignancy of a tumor based on a scale of I-IV. WHO grade I tumors, also known as pilocytic astrocytoma, are the most benign type of gliomas that occur most often in children and young adults and can possibly be cured by complete surgical removal. WHO grade II tumors, often called fibrillary astrocytoma, are primarily benign tumors, although invasive, that can possibly evolve into more malignant tumor types. WHO grade III tumors, or anaplastic astrocytoma, are characterized by large-scale reversions of differentiation in affected cells as well as more extensive rates of recurrence. Lastly, WHO grade IV tumors, known as glioblastoma multiforme (GBM) are the most malignant and most invasive type of glioma (Louis, 2007; Maher, 2001).

The prognosis of patients diagnosed with gliomas can vary greatly depending on the grade of the tumor as well as the types of treatments administered. For patients with lower grade gliomas (types I and II), chances of survival are much greater due to more effective surgical removals and radiotherapies (Pignatti, 2002). As for patients with higher grade tumors (III and IV), the average survival time ranges from 2-3 years in best case scenarios to less than 40 weeks in worst cases, i.e. patients with GBM. Although ineffective towards long term survival, combinations of surgical procedures, chemotherapy, and radiation treatments can help increase survival in high-grade glioma patients from as little as a few months to as much as a few years (Hanahan, 2000).

## *1.2 Cancer Stem Cells*

Stem cells are defined as cells with the ability to both self-renew and differentiate into more specialized cell lines. In addition to totipotent embryonic stem cells, which are able to differentiate into all types of cells, tissue-restricted stem cells also exist in organs such as bone marrow, brain, skin, intestines, and the lungs in order to replenish cells during tissue homeostasis and injury repair (Fan, 2007).

Masses of high-grade tumor cells are comprised of a heterogeneous population, containing both differentiated and undifferentiated cells called tumor stem cells (cancer stem cells – CSC). These cells have very similar characteristics to the stem cells from the tissue of origin, such as being able to divide into either genetically identical daughter cells or more

specifically differentiated daughter cells, and have been found to be crucial in the establishment and development of tumors (Al-Hajj, 2004; Singh, 2004). There still exists some speculation about whether these cancer stem cells are the cells-of-origin of glioma initiation and progression, or just the results of the tumor formation (Fan 2007). However, some recent studies suggest that it is inaccurate to refer to CSCs as the cells-of-origin since they are separated from end-stage tumors, despite their ability to initiate new tumor formation (Wang, 2009). The generation of a tumorigenic cell requires consecutive gene mutations that disrupt various yet specific cellular pathways, and are much less likely to occur in mature, usually short lived cell types. Tissue-restricted stem cells and their immediate progenitors, however, are much more likely targets of these genetic mutations since they are characterized by having much longer life spans. Therefore, this supports the hypothesis that malignant tumors are originated from mutated stem cells (Fan, 2007).

In 2004, Singh conducted a study about the importance of tumor stem cells in the formation of gliomas using a transmembrane glycoprotein called CD133 as a marker of stem cells. This cell marker was used because it is normally expressed on the surface of neural stem cells. In his study, Singh injected immunodeficient mice with $CD133^+$ cells while injecting other immunodeficient mice with $CD133^-$ cells. He observed tumor formation only in the $CD133^+$ mice, indicating that these neural stem cells held some kind of importance of the development of cancer.

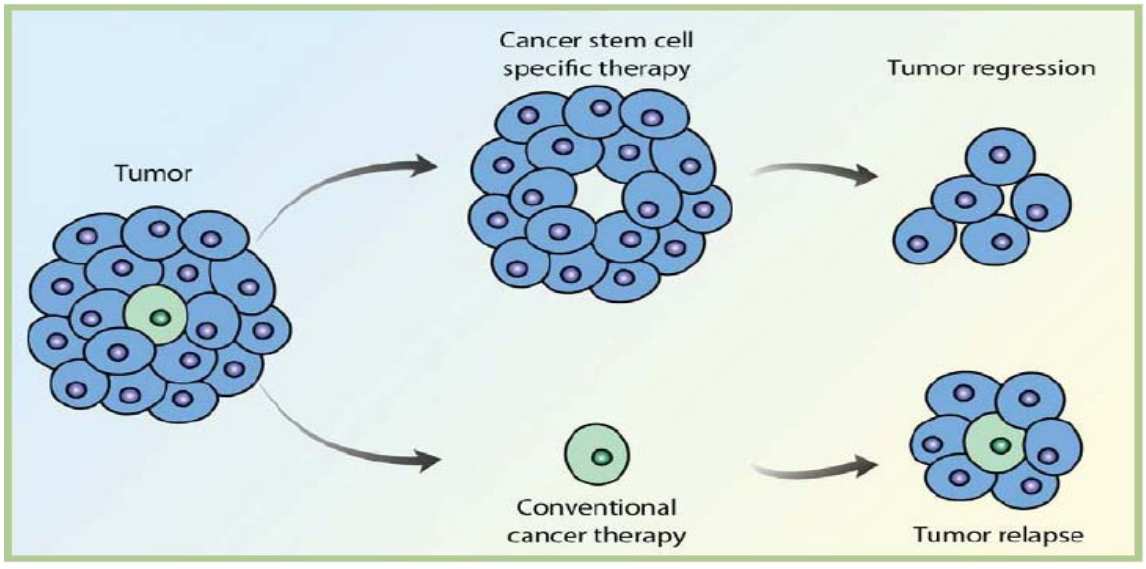



**Figure 2 – The hypothetical effects of conventional cancer therapy vs. cancer stem cell specific therapy on a heterogeneous tumor (Frank, 2008).**

The importance of CSCs in modern methods of cancer treatment cannot be ignored. For example, during chemotherapy, the majority of primary tumor cells may be destroyed, but if the tumor stem cells are not killed as well, then the tumor may likely relapse (Figure 2) (Jordan, 2004). The drug resistance and the regenerative capabilities of CSCs are what make them the primary problem in completely eradicating malignant tumors. Taking this into consideration, more extensive studies need to be conducted in order to more fully understand the complex mechanisms of these genetically unstable tumor-forming CSCs. Only then will it be possible to effectively develop more thorough methods of treatment for cancer patients.

## *1.3 Mitosis*

In biology, mitosis is the process by which a eukaryotic cell separates its duplicated genome into two identical halves. It is generally immediately followed by cytokinesis, which divides the cell membrane and cytoplasm to create two daughter cells that are genetically identical to the parent cell. Mitosis and cytokinesis together define the mitotic (M) phase of the cell cycle, which comprises about 10% of the entire cycle. This process is subdivided into different phases based on the activities and positions of the nuclear envelope, the chromosomes, and structures called microtubules. These phases are: prophase, prometaphase, metaphase, anaphase, and telophase. Each phase has its own characteristic form and is identifiable using microscopy ("Mitosis", 2010).

## *1.4 Apoptosis*

Programmed cell death, or apoptosis, is a genetically controlled process by which unnecessary or damaged cells undergo self-destruction when certain genes are activated, resulting in cell shrinkage, detachment and fragmentation of cellular bodies preserving the membrane integrity. It is necessary for normal development and homeostasis of all multicellular organisms, and it also plays a key role in many diseases such as cancer. Apoptosis is regulated by several factors and signals, both intracellular and extracellular, and is normally carried out in the cell by a family of proteases called caspases (Somani, 2010)

This cellular course of action is a crucial component of several bodily processes such as the immune response and embryonic development of organ systems. Taking this into account, abnormalities in cell death regulation can lead to several serious diseases. For example,

insufficient apoptosis in an organism is a primary constituent of cancer. Researchers today are constantly attempting to develop new methods to induce apoptosis in tumor cells in order to re-establish the balance of homeostasis in an organism (Elmore, 2007).

## *1.5 Mitotic Catastrophe*

There exists a type of cell death that only occurs during mitosis that is known as mitotic catastrophe. It is thought to be a result of a combination of deficient cell-cycle checkpoints, cellular damage, and attempts at aberrant chromosome segregation early in mitosis. Cells that fail to be destroyed after mitotic failure can possibly divide asymmetrically, creating consequent generations of aneuploid cells, posing the risk of oncogenesis and potentially the onset of cancer. This being said, mitotic catastrophe can be considered a safety mechanism against aneuploidization and oncogenesis (Castedo, 2004).

## *1.6 Cellular Senescence*

Normal somatic cells, like multicellular organisms as a whole, have a limited lifespan, i.e. a limited number of replications through the cell cycle that they are able to perform. Once a cell reaches its limit, it can undergo a process called cellular senescence, which is an irreversible arrest of cell proliferation that is also accompanied by changes in cell function (Campisi, 2001). Senescent cells are characterized by a sharp increase in size in comparison to normal cells, as well as fairly flat morphology and over expression of β-galactosidase (Bandopadhyay, 2005).

Senescence can be induced by either “natural cause” or other detrimental factors, such as double stranded DNA damage. This “natural cause”, or in other words, aging, is a result of the shortening of telomeres, the repetitive DNA sequences and specialized proteins that cap the ends of linear chromosomes. Telomeres maintain the integrity and structure of eukaryotic genomes by preventing the degradation or fusion of chromosome ends. The shortening of these telomeres is the result of incomplete replications of this DNA due to 3’ overhangs at both telomere ends, and is supposedly the main reason for aging (Campisi, 2001).

The process of cellular senescence, from an evolutionary aspect, could very well have developed as an anti-cancer or tumor-suppressive mechanism. A combination of specific genetic mutations is required for tumor formation, which in theory should take a substantial amount of

time. Thus, giving cells a shorter, limited lifespan is one of the safest ways to prevent these mutations from occurring (Campisi, 2007).

## *1.7 Nuclear Analysis*

Through the marvels of modern technology, scientists nowadays have developed various techniques that allow the nuclei of specific cells to be identified and studied individually. One of the most commonly used of these methods is nuclear staining, which is defined as using a certain compound or substance to selectively dye the nucleus of a cell in order to be analyzed more efficiently (Raju, 2006). Examples of these selective compounds include Hoescht 33342 and DAPI, two widely used stains that bind to double stranded DNA and fluoresce brightly during fluorescence microscopy ("DAPI Nuclear Counterstain", 2011). This fluorescence allows each individual nucleus to be studied in order to verify the presence or absence of irregularities.

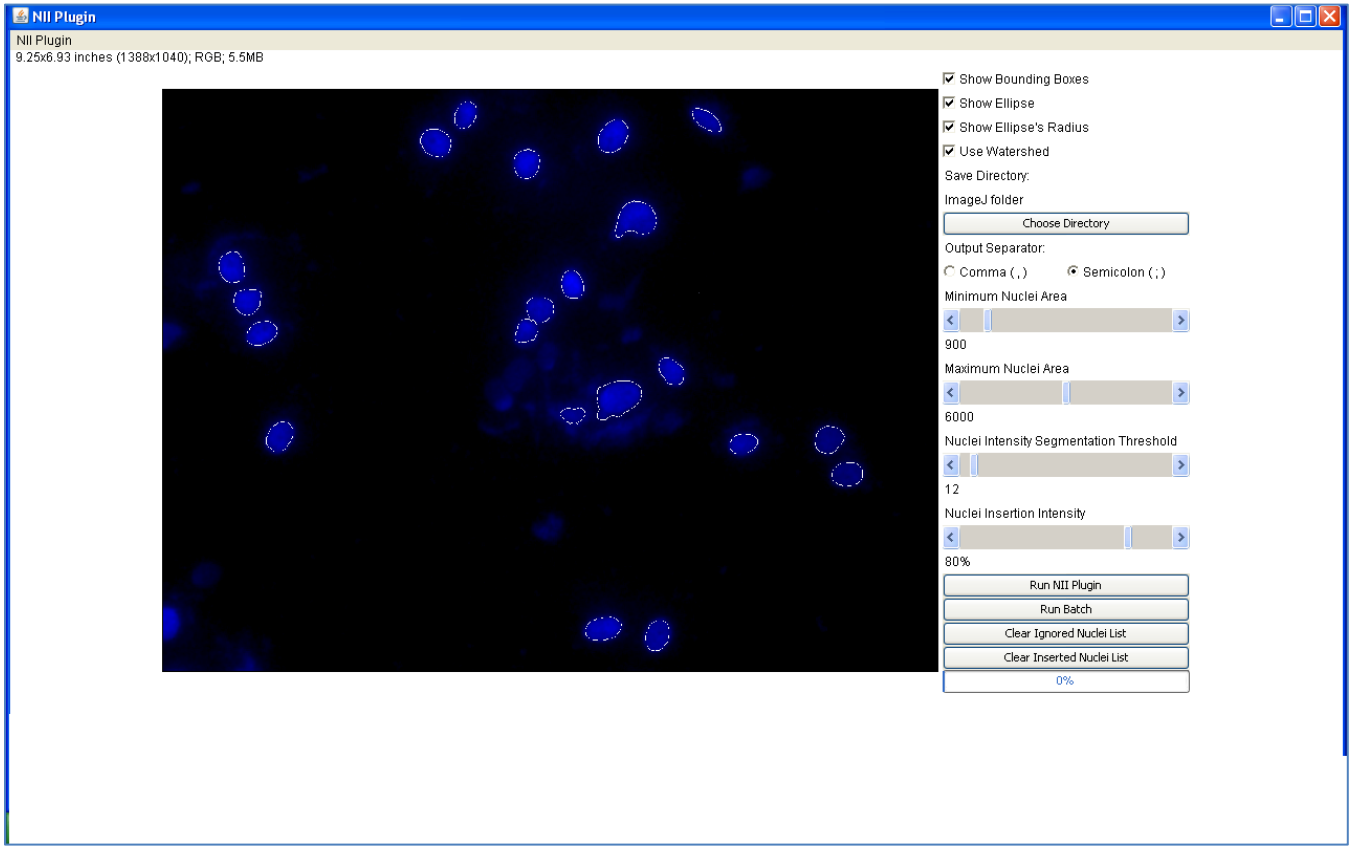



**Figure 3 – A screenshot of the NMA plugin from the ImageJ image processor program. A blue fluorescence photo of U87 cells dyed with Hoechst nuclear stain is being analyzed based on the parameters of total nuclei area and general nuclei form.**

Another useful tool in the field of nuclear analysis is the ImageJ program. This image processor (shown above in Figure 3), when combined with nuclei staining, can be used to analyze numerous cells simultaneously based on several factors pertaining to their nuclei. The information from this program can be utilized in NMA analysis by measuring the size and shape of each individual cell in a sample, allowing easy identification of abnormalities (Collins, 2011).

## *1.8 ZsGreen*

ZsGreen is a green fluorescent protein that, when fused with an ornithine decarboxylase degradation sequence (ODC) in a cell, can form a specific protein that is able to be observed through fluorescence microscopy (Vlashi, 2009), as seen below in Figure 4. The specific amount of ZsGreen able to be expressed depends on both the cell type as well as proteasomal activity. The proteasome, a large protein complex present in all eukaryotic cells, is involved in the progression of the cell cycle and its degrading activity controls the lifetime of most cellular proteins, including many regulatory proteins. Specifically, 26S proteasome has been found to degrade ODC and consequently ZsGreen during stages of high activity. Thus, depending on the cell type, low proteasomal activity can result in a buildup of ZsGreen protein in transfected cells (Pajonk, 2010).

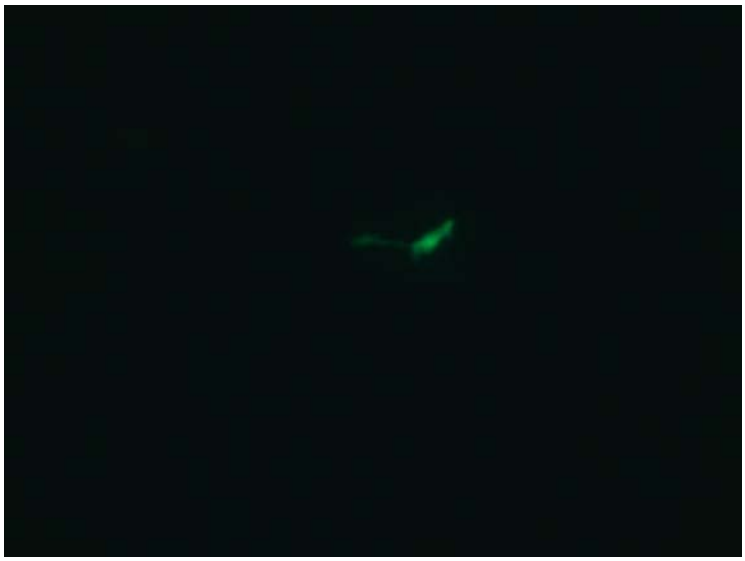


**Figure 4 – A U87 glioma cell, cultured *in vitro*, transfected with ZsGreen plasmid and viewed under fluorescent light (using an optic microscope).**

Vlashi et al. in 2009 used the ZsGreen-ornithine decarboxylase complex (ZsGreen-ODC) to detect the presence of CSCs in real time. In their work, they showed that cells in a differentiated state show high proteasomal activity, which in turn degrades the ZsGreen-ODC,

thus showing no fluorescence. However, CSCs showed fairly low proteasomal activity, which allowed the eventual buildup of ZsGreen-ODC, causing fluorescence to be maintained. This allowed them to analyze the presence of these cells using both microscopy and flow cytometry. Techniques such as this are invaluable to the research of CSCs since they easily pinpoint their location and allow their cellular morphology to be visibly analyzed.

## *1.9 Growth Factors and Cytokines*

According to Dorland's Medical Dictionary (Anderson, 2011), a growth factor is a protein, hormone, or any other naturally occurring substance that promotes cellular growth, differentiation and proliferation. While individual functions of each different type of growth factor varies, they typically act as signaling molecules between cells, including many kinds of cytokines for example. The fibroblast growth factor family (FGF) is perhaps one of the largest and most diverse families of growth factors involved in soft-tissue growth and regeneration (Basilico, 1992). FGF is an extremely versatile growth factor that acts on numerous tissue types including endothelial, muscular and neuronal. On top of that, it can also promote adipocyte differentiation, induce macrophage and fibroblast IL-6 production, stimulate astrocyte migration, and, most importantly to this experiment, prolong neuronal survival. Being recognized for their proliferative capabilities, FGF is considered to play a significant role in both development as well as tumorigenesis (Baird, 2001).

Another important type of growth factor is the epidermal growth factor (EGF), which is a lightweight polypeptide that results in proliferation, differentiation and survival of its target cells. It performs its respective functions by utilizing the EGF receptor (EGFR), which is a transmembrane glycoprotein that also belongs to the tyrosine kinase receptor family (Fallon, 1984). EGF and EGFR have both been implemented in the causes of tumor formation, and in fact, inhibition of EGFR activity in tumor cells has shown to decrease tumor growth by blocking the cascade of cell signals that lead to overall cell growth and proliferation (Herbst, 2003).

One more significant molecule that effects cell growth and development is leukemia inhibitory factor (LIF). This cytokine receives its name based on its capability to induce differentiation in myeloid leukemia cells, and it has been related to several bodily processes such as embryogenesis, bone metabolism, and more relevant to this study, neuronal development (Hu, 2007). Kawahara et al. in 2009 found that the actual removal of LIF from an embryonic stem cell

culture caused the cells to differentiate, while still maintaining their pluripotency and proliferative abilities. In this they concluded that LIF contributed to maintaining these stem cells in an undifferentiated state.

# 2 Objectives

## *2.1 General Objective*

To observe the differences in morphology and activity of CSCs as compared to that of differentiated tumor cells.

## *2.2 Specific Objectives*

To characterize the nuclei of CSCs transfected with ZsGreen to verify the presence of irregularities during the maintenance of pluripotency while also using growth factors, in addition to examining whether or not these CSCs or their adjacent cells show any signs of mitosis, apoptosis, mitotic catastrophe, or senescence.

# 3 Materials and Methods

## *3.1 Cell Culture*

The tissue culture bottles were acquired from Techno Plastic Products (TPP). The reagents used to make the cancer stem cells culture solutions are listed as the following: Dulbecco's Modified Eagle Medium Low Glucose and DMEM Nutrient Mixture F-12 (Gibco BRL), basic Fibroblast Growth Factor (FGF – 20 ng/mL), Epidermal Growth Factor (EGF – 20 ng/mL), Leukemia Inhibitory Factor (LIF – 10 ng/mL), B27 Supplement (0.1 x/mL), $NaHCO_3$ (Merck), 1% Penicillin/Streptomycin (Gibco BRL), 0.1 % Fungizone (Gibco BRL), with an adjusted pH of 7.4

The culture medium for maintaining cells was prepared with DMEM Low Glucose, $NaHCO_3$ 44 mM, HEPES 7.68 mM, 1% Penicillin and Streptomycin, 0.1% fungizone, with an adjusted pH of 7.4 and with supplementation of 5% fetal bovine serum (FBS). The culture medium for maintaining the undifferentiated cells was prepared with DMEM F-12, $NaHCO_3$ 14.28 mM, 1% Penicillin and Streptomycin, 0.1% fungizone, with an adjusted pH of 7.4 and with supplementation of FGF, EGF, LIF and B27. The CMF solution, pH 7.4, used in the preparation of trypsin and the washing of the cells prior to trypsinization, contained $NaH_2PO_4$ 8.0mM, NaCl 138 mM, KCl 2.68 mM. All solutions used in cell culture were prepared in distilled water that was autoclaved and sterilized by filtration in 0.22 mm pored Millipore membrane.

The cell line used was human glioma U87, which was obtained from ATCC (American Type Culture Collection – Rockville, Maryland, USA). The cells were cultivated in DMEM Low Glucose with 5 % FBS or DMEM F-12 with factors medium containing antibiotics and fungizone. The cells were kept in an incubator at 37°C with a humidified atmosphere and 5% $CO_2$. The culture medium was changed every 2-3 days.

## *3.2 Transfection*

A 24-well plate was seeded with 50,000 U87 cells in the configuration as shown in Figure 5. The plasmid that was transfected was ZsGreen (2.216 μg/μl), and Lipofectamine 2000 (Invitrogen) was used as a transfection reagent in a 1:1 ratio (1 μg of plasmid DNA: 1 μL

Lipofectamine). After combining the plasmid and Lipofectamine, the solution was then thoroughly mixed using a Vortex shaker for 10 seconds. An additional 15 minutes of waiting took place in order for the micelles containing the plasmid to form completely (Figure 6).

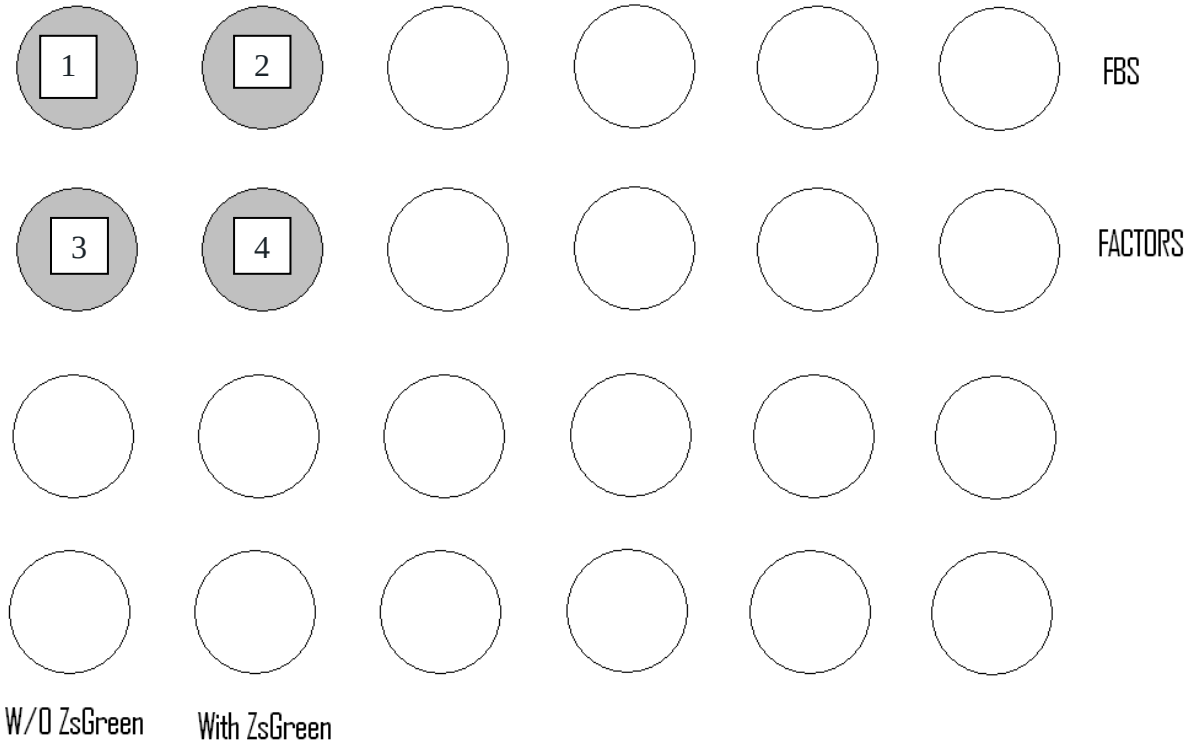



**Figure 5 – The specific plating configuration of the tested cells is shown. Each grey circle represents one well of the 24-well cell plate that contained U87 cells. The labels on the bottom of the figure denote the two columns, namely, transfected cells and the control cells. The labels on the right of the figure signify the presence of Fetal Bovine Serum (FBS) and growth factors in the cell solutions of their respective rows after transfection had been completed.**

Meanwhile, 150 µl of fresh DMEM Low medium with 5% FBS was added in each of the four wells, followed by 40 µl of transfection mix being added to the two transfection positive wells. After an initial 4 hours, all media was changed accordingly to the format of Figure 5, with either FBS or growth factor treatments, and these cells were incubated for five days to allow the transfection process to fully develop before any further action was taken.

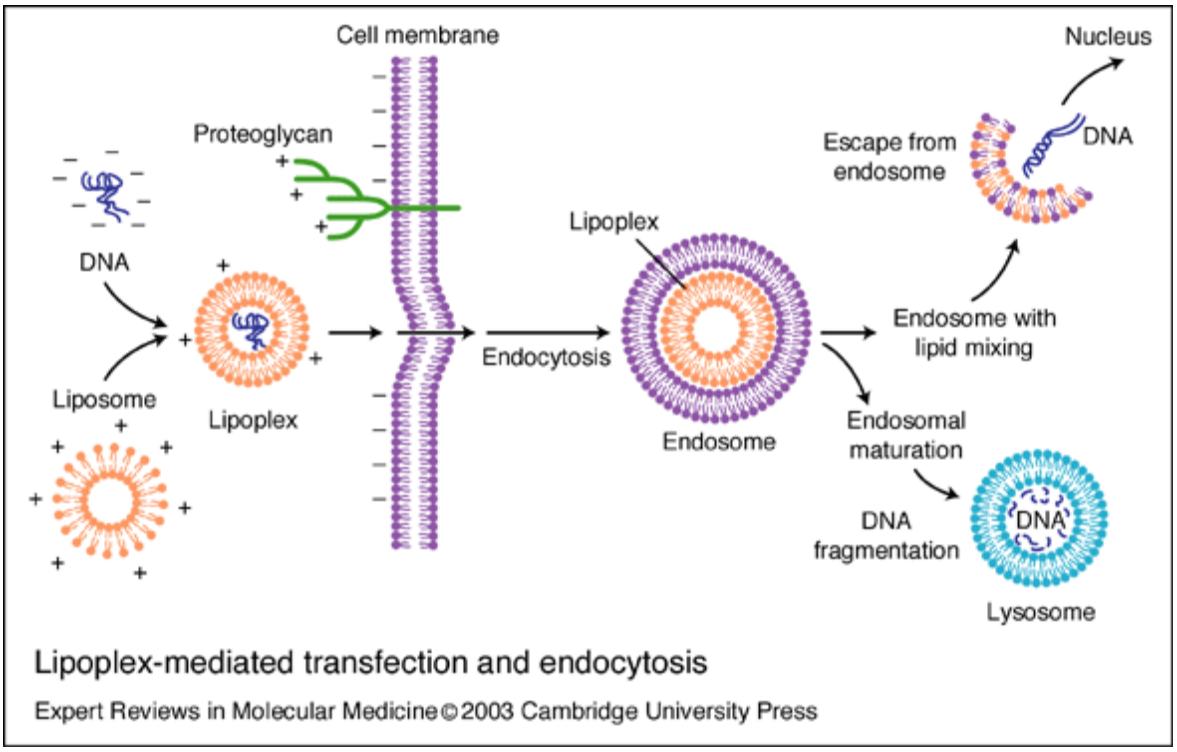



**Figure 6 – A summarization of the process of transfection, or the introduction of foreign nucleic acids into a cell. The positively charged liposome used in this experiment, Lipofectamine 2000, facilitated the entrance of ZsGreen into the target cells by creating a lipoplex micelle that could easily be engulfed by endocytosis (Parker, 2003).**

## *3.3 Nuclear Staining and Microscopy*

The nuclei of the tested cells were then stained using Hoechst dye (2 μg/mL), and after 6 hours were then viewed under an inverted fluorescence Carl Zeiss microscope. Using the Axiovision microscope program, photographs were taken of the cells from each well using both visible light as well as UV fluorescent light. For the wells containing transfection positive cells, three photos were taken at each position, one to see the cells in visible light, another to view the green fluorescence (ZsGreen), and the last to view the blue fluorescence (Hoechst). For the wells containing the control cells, which were transfection negative, only a total of three photos per well were taken, one for each type of light.

## *3.4 ImageJ Analysis*

The nuclei were analyzed using the ImageJ image processor program, utilizing the Nuclear Morphology Analysis (NMA) plugin which quantifies the number of cells in the population that possess characteristics of mitosis, apoptosis, mitotic catastrophe, as well as senescence based solely on nuclei morphology. After analyzing each of the blue fluorescent photos (obtained through Hoechst staining) using ImageJ, the data was converted to a Microsoft Excel document in order to display the specific ratios of regular and irregular cells in graphical form. Numerical data collected from NMA was used to sort each observed nucleus into categories signified by certain letter codes. These nuclei categories were coded as the following: Normal (N), Mitotic (S - small), Apoptotic (SR – small and regular), Senescent (LR – large and regular), Small and Irregular (SI), Large and Irregular (LI), and Irregular (I).

Each photographed cell was plotted on a section of a scatter plot graph based on the normality of its nuclear size and shape. Every section of the scatter plot represents a certain biological cellular condition, and therefore each cell categorized into a section showed signs of that respective condition. For example, cells grouped into the upper left section of the plot (large sized and regular shaped nuclei) were classified as being senescent. Likewise, the other sections of the plot were represented by the following biological conditions: normal size and shape – normal nuclei, small size and normal shape – apoptotic nuclei, small size and irregular shape – mitotic nuclei, large and irregular shape– mitotic catastrophe.

# 4 Results and Discussion

The following section displays the sequences of analyses that were performed after the transfection process was completed. First, the microscope photos were analyzed visually as well as through the ImageJ program/NMA plugin. Second, the data taken from the analyzed blue fluorescent nuclei was compiled into graphical form and used to compare and contrast the differences between the nuclei from the different wells.

## *4.1 Photo Analysis*

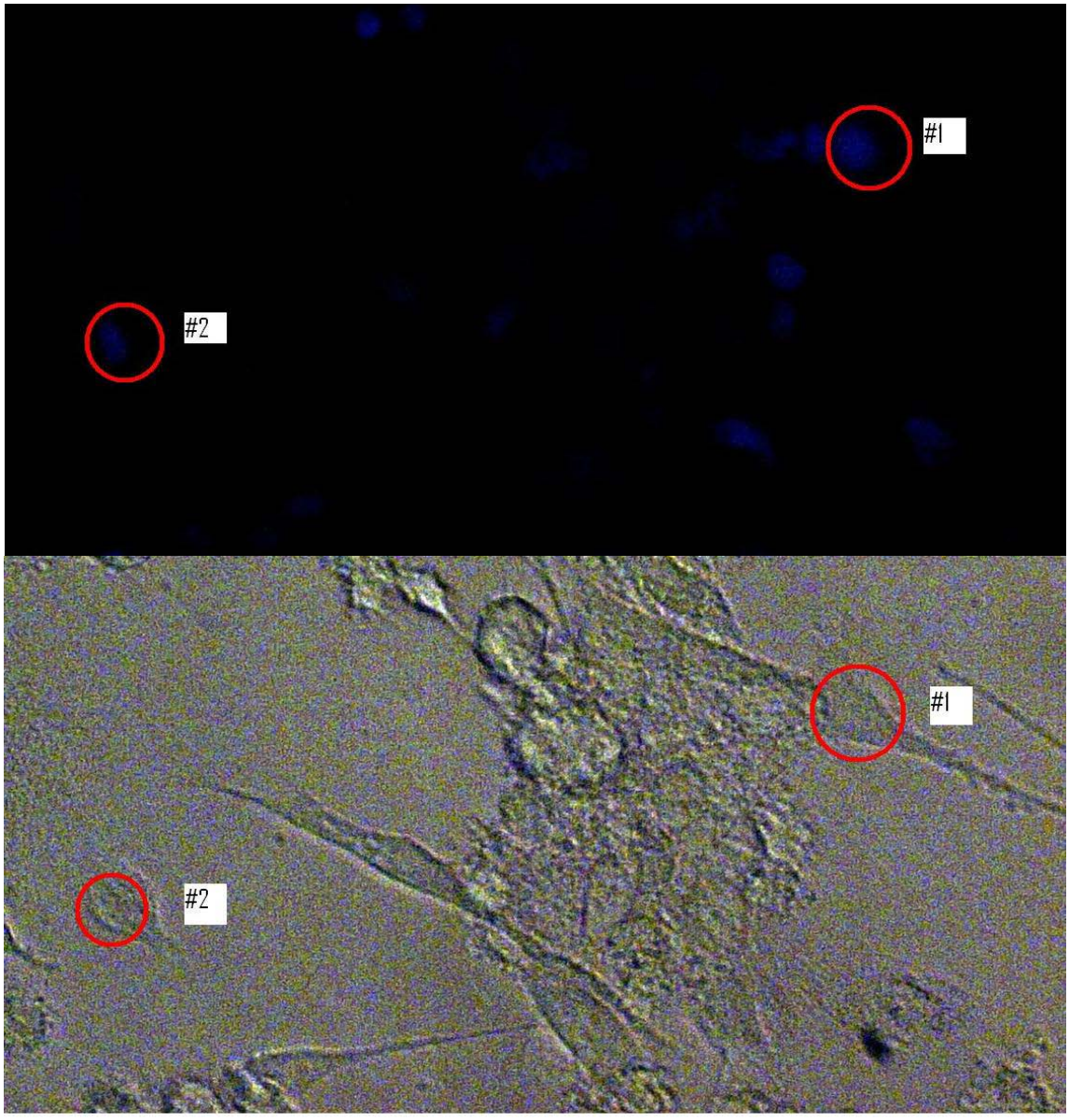


**Figure 7 – Well 1 (transfection negative, without growth factors) microscope photos, with blue fluorescence shown on top and visible light shown on the bottom. Cells 1 and 2 were labeled in order to provide comparable nuclei to specific cells in the visible photo. These photos showed the least amount of clarity out of any of the wells, which proved difficult to analyze via NMA plugin.**

Figure 7 above shows the microscope photos taken from the double-negative control cells in well 1. These cells were not tranfected with ZsGreen or treated with any growth factors; they were only cultured in medium with FBS and should therefore represent the normal form and size of U87 cells *in vitro*. Labeled cells #1 and 2 are two of the more distinguishable cells in both photos based on nuclear appearance and membrane visibility.

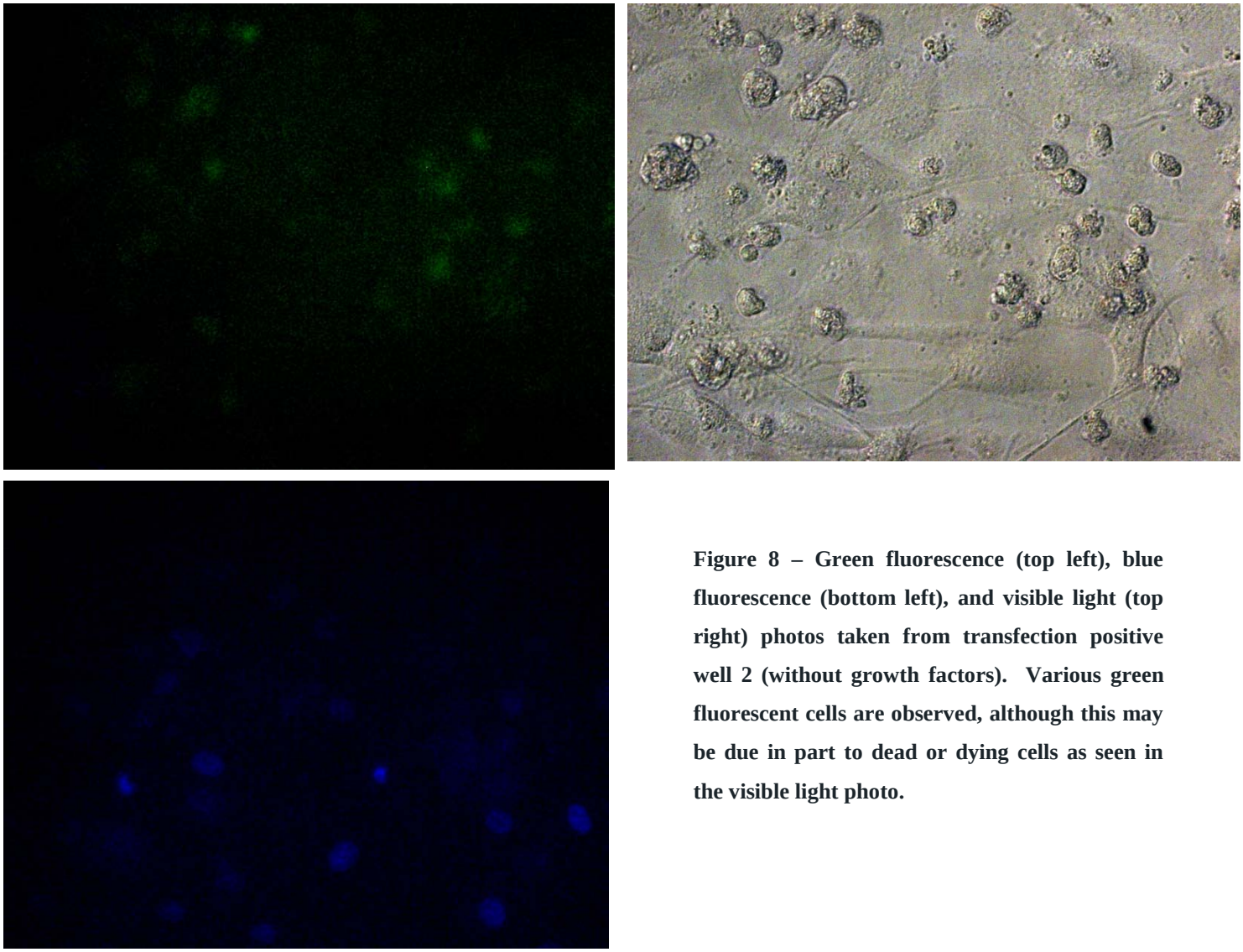
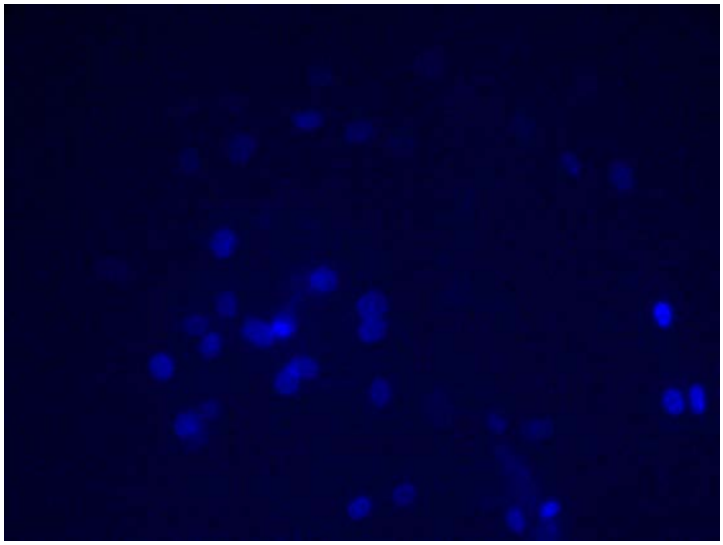


**Figure 8 – Green fluorescence (top left), blue fluorescence (bottom left), and visible light (top right) photos taken from transfection positive well 2 (without growth factors). Various green fluorescent cells are observed, although this may be due in part to dead or dying cells as seen in the visible light photo.**

An overall weak green fluorescence can be seen in the top left photo of Figure 8, which was taken from the transfection positive, growth factor negative well 2. Although ZsGreen is primarily used as a stem cell marker, it is unlikely that the concentrations of green fluorescence shown in the above figure represent actual cancer stem cells. Based on the shriveled appearance of these cells in the visible light photo as well as their lack of distinguishable nuclei in the blue fluorescence photo, these cells were most likely dead or dying at the time these photos were taken. A reasonable explanation for these cells expressing the most fluorescence out of this particular group was that their proteasomes stopped functioning, therefore leaving the cells unable to degrade ZsGreen and causing visible buildups of this fluorescent protein (Vlashi, 2009). The presence of dead cells and debris in these wells made it much more difficult to distinguish actual CSCs from the normal U87. Thus, the photos from well 2 were not ideal to classify CSC morphology.

The photos taken from transfection negative, growth factor positive well 3 were much clearer and easier to analyze compared to the previous two wells. Referring to Figure 9 below, the blue fluorescent nuclei are much better defined with regards to shape, size, and brightness.



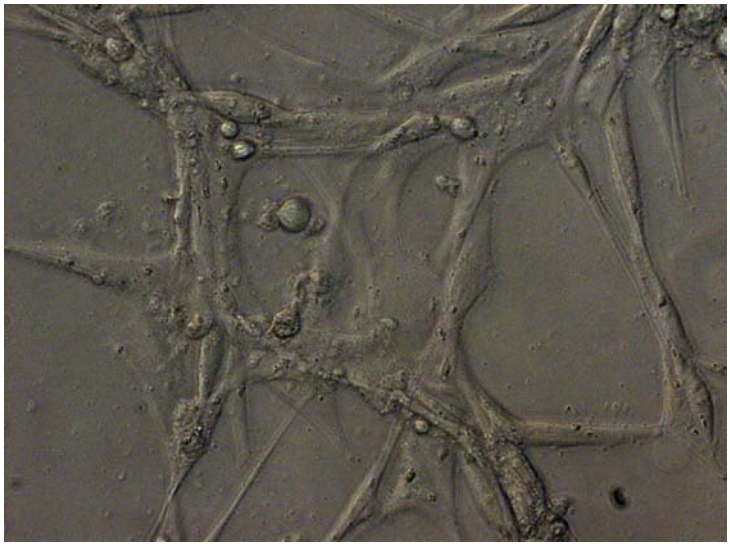


**Figure 9 – Microscope photos from transfection negative, growth factor positive well 3, with blue fluorescence (top) and visible light (bottom). Elongated morphology and overall healthier looking appearance was most likely caused by the addition of growth factors**

Notice how the cells express a more elongated and cylindrical morphology as compared to those from the first two wells, as well as the absence of abundant cell debris. This was most likely due to the presence of growth factors in this specific cell group, which are known to prolong cellular lifespan as well as to promote astrocyte development (Basilico, 1992; Herbst, 2003).

The final group of tested cells from well 4 (Figure 10) were positive for both ZsGreen transfection as well as the presence of growth factors. Looking at the green fluorescent photo, a single concentration of fluorescence can be seen on the cell on the right sided as well as a few smaller concentrations on the left side. Comparing this to the cells shown in the visible light picture, it can be inferred that these particular cells may be CSCs based on their morphology as well as the possible sphere forming ability of the left sided cells.

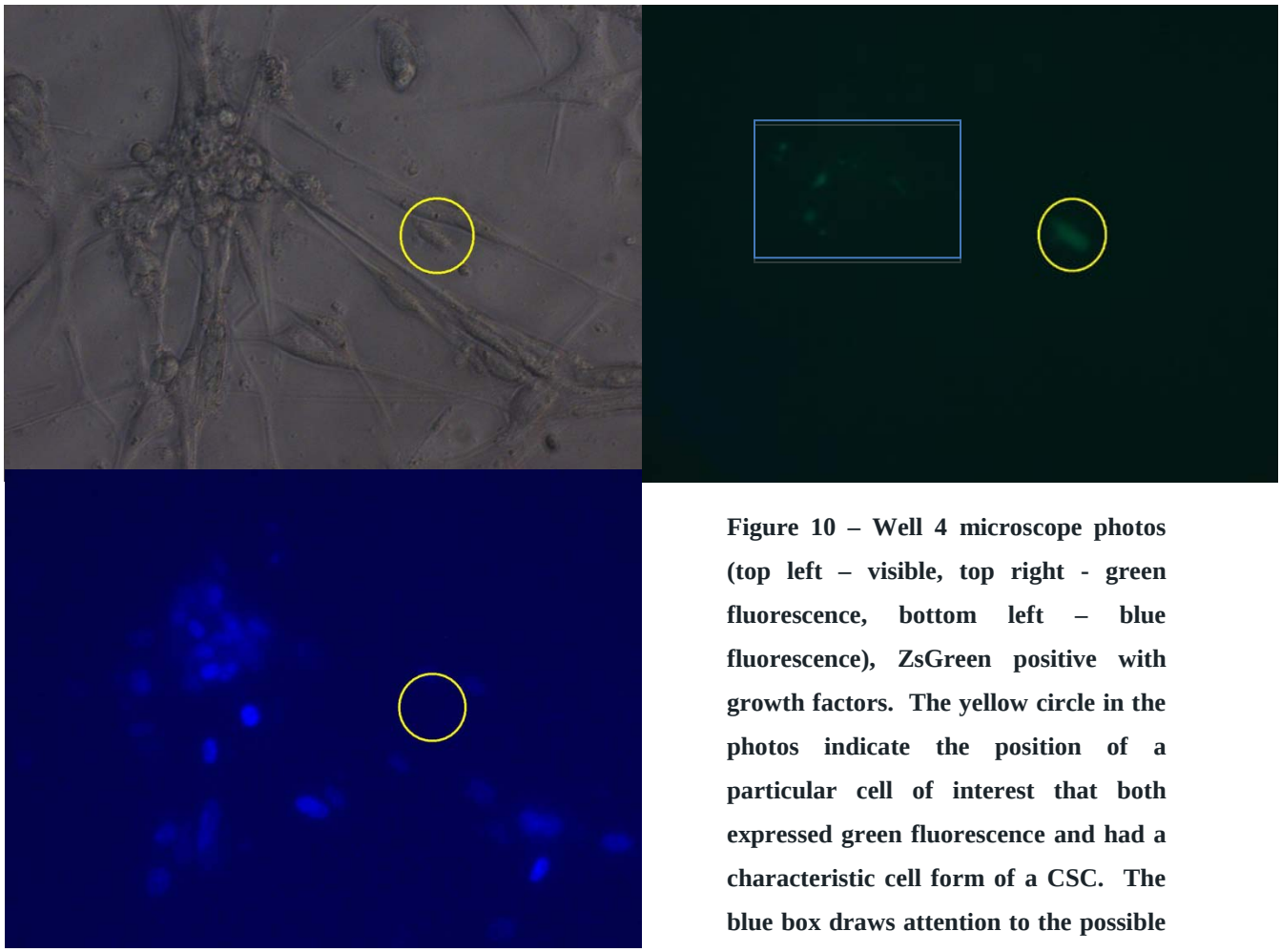
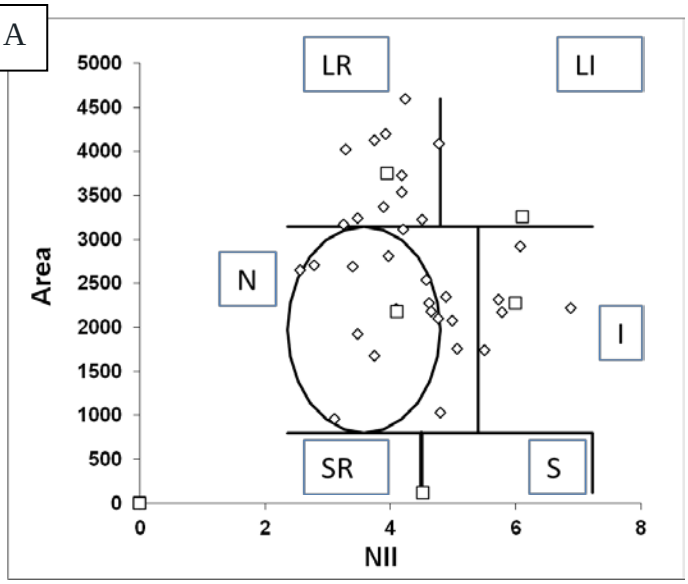


**Figure 10 – Well 4 microscope photos (top left – visible, top right - green fluorescence, bottom left – blue fluorescence), ZsGreen positive with growth factors. The yellow circle in the photos indicate the position of a particular cell of interest that both expressed green fluorescence and had a characteristic cell form of a CSC. The blue box draws attention to the possible presence of CSCs in the sphere forming as seen in the visible light photo.**

## *4.2 Graphical Analysis*

Using all of the blue fluorescence photos taken from the wells, the following graphs and tables were assembled based on the parameters of area, aspect, radius, and roundness of each cell. Certain pre-established values and formulas determined the degree of irregularity of each analyzed nucleus and allowed them to be classified into categories. The photos from well 1 weren't clear enough and did not contain a sufficient amount of well-defined nuclei for an accurate graphical representation to be made. Thus, only graphical data from wells 2, 3, and 4 have been included below.

A




B

| | Symbol | Number of Nuclei | % of nuclei |
|---|---|---|---|
| Normal | N | 17 | 47.2 |
| Mitotic | S | 1 | 2.8 |
| Apoptotic | SR | 0 | 0.0 |
| Small and Irregular* | SI | 0 | 0.0 |
| Senescent | LR | 11 | 30.6 |
| Large and Irregular* | LI | 1 | 2.8 |
| Irregular* | I | 5 | 13.9 |
| | | 35 | 100.0 |

C

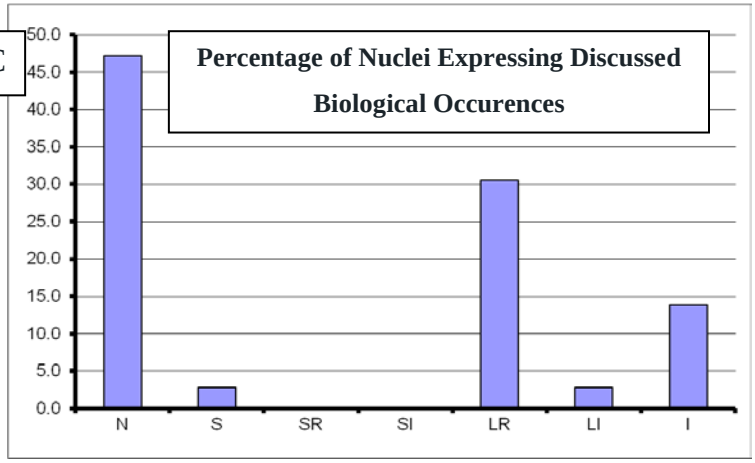
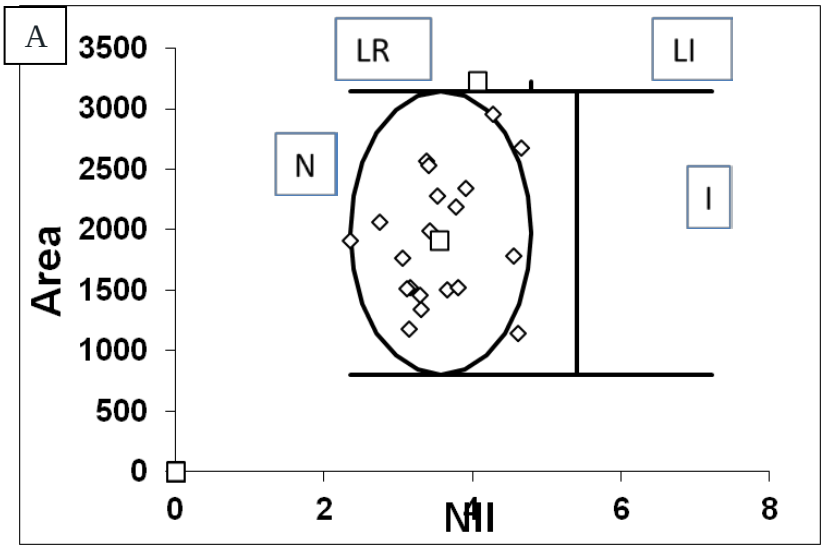



**Figure 11 – Overall graphical representation of well 2 nuclei morphology. A) Each point represents one nucleus. The X-axis represents the overall normalness of the nuclear form, while the Y-axis represents the area of each of the nuclei. The ellipse represents the standard size and shape of normal U87 cells. Each section of the graph separated by black lines represents a certain irregularity based solely on nuclear size and shape. The letter codes correspond to the specific biological occurrence shown in the part B. B) Overall count of each categorized cell (signified by letter codes) in table form, based on collected values. C) Overall percentage of categorized cells (by letter codes) in graphical form.**

A




B

| | Symbol | Number of Nuclei | % of nuclei |
|---|---|---|---|
| Normal | N | 20 | 95.2 |
| Mitotic | S | 0 | 0.0 |
| Apoptotic | SR | 0 | 0.0 |
| Small and Irregular* | SI | 0 | 0.0 |
| Senescent | LR | 1 | 4.8 |
| Large and Irregular* | LI | 0 | 0.0 |
| Irregular* | I | 0 | 0.0 |
| | | 21 | 100.0 |

C

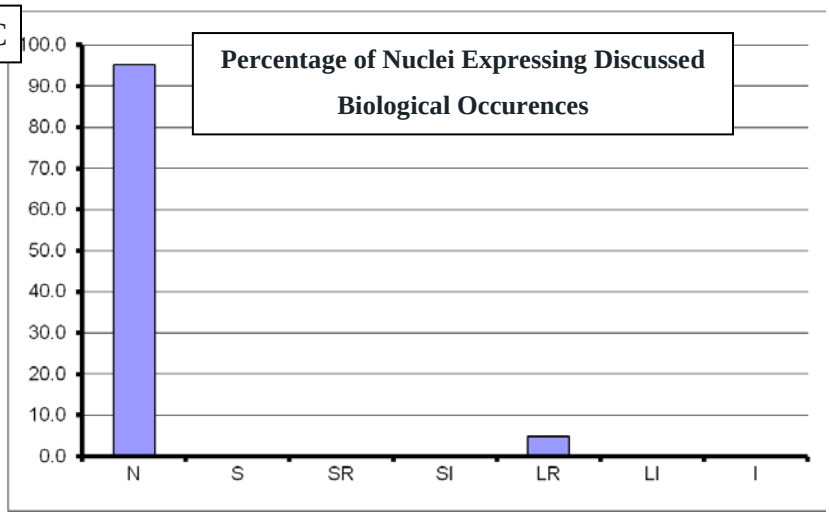
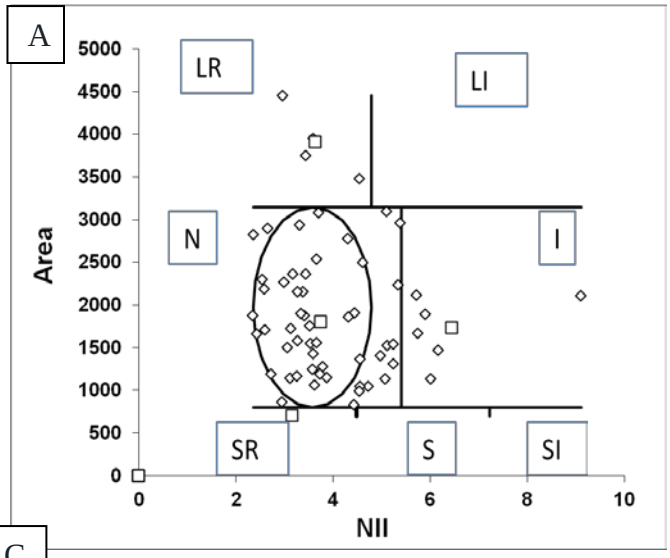



**Figure 12 – Graphical analysis of nuclei from well 3. The same standards apply for parts A, B, and C as they did in the previous figure.**

A




B

| | Symbol | Number of Nuclei | % of nuclei |
|---|---|---|---|
| Normal | N | 50 | 82.0 |
| Mitotic | S | 0 | 0.0 |
| Apoptotic | SR | 1 | 1.6 |
| Small and Irregular* | SI | 0 | 0.0 |
| Senescent | LR | 4 | 6.6 |
| Large and Irregular* | LI | 0 | 0.0 |
| Irregular* | I | 6 | 9.8 |
| | | 61 | 100.0 |

C

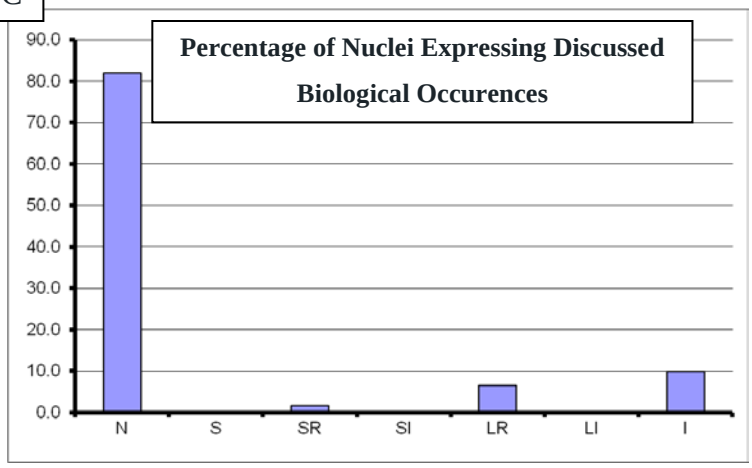



**Figure 13 – Graphical analysis of nuclei from well 4. The same standards apply for parts A, B, and C as they did in the previous two figures.**

To summarize the collected statistical results: 47.2 % of the nuclei photographed from well 2 were found to be typical while 30.6 % were calculated to be senescent, 13.9 % to be irregular, 2.8 % mitotic and 2.8 % large and irregular. The ImageJ program found 95.2 % of the photographed nuclei from well 3 to be of normal size and shape while 4.8 % of them were senescent. The well 4 photographed nuclei were found to be 82.0 % normal, 9.8 % irregular, 6.6 % senescent, and 1.6 % apoptotic.

## *4.3 Expected Outcomes/ Ideal Results*

In a hypothetically ideal research setting, without interference from any unforeseen or unknown factors or variables, the observed results from this experiment would differ

substantially from how they turned out in reality. Primarily, the microscope photographs of the tested cells would have been much clearer and much easier to analyze, with well-defined plasma and nuclear membranes visible. Both the ZsGreen transfection and Hoechst stain would function more efficiently, allowing the cells to express a higher degree of fluorescence so that their membranes and nuclei could be clearly distinguished from one another using the NMA plugin of the ImageJ program. This increase in fluorescence would also assist in viewing the same cell in each of the three photo types (blue fluorescence, green fluorescence, and visible) in order to characterize certain cell traits and compare it to adjacent cells.

Another factor that would be absent from an ideal version of this experiment would be the presence of debris and dead cells in the wells, which would interfere with photo and graphical analysis by providing inaccurate representations of both green and blue fluorescence (as seen in Figure 8). Each location of each photo would contain a large concentration of fluorescent cells as to maximize the variability of each sample, as well as to have as much significant data as possible to include in the graphical figures.

When originally organizing and formulating the specific methods for this experiment, certain outcomes were expected to be observed after transfection and analysis. For example, one of the purposes of transfecting and staining these cells was to visibly identify any signs of mitosis, apoptosis, mitotic catastrophe, and senescence in the CSCs or their neighboring cells. In an ideal experiment, these processes and abnormalities would be expected to be easily identifiable and classifiable at first glance. Cells undergoing mitosis would display mitotic spindles that indicate the onset of one of the later, more easily visible mitotic stages such as anaphase or telophase (“Mitosis”, 2011). Apoptotic cells would show obvious signs of cell shrinkage and nuclear fragmentation (Elmore, 2007). Cells undergoing mitotic catastrophe would exhibit clear chromosomal breaks and gross nuclear alterations (Vitale, 2011), while senescent cells would show an apparent increase in size and significant flattening of form (Bandyopadhyay, 2005). As well as being clearly visible through microscopy, in an ideal research setting, these signs would be easily recognized by the NMA plugin and therefore would be subject to quantification via graphical analysis.

## *4.4 Experimental Difficulties*

After discussing the ideal version of this experiment, now let us focus on the realistic outcomes that were observed from this project as a whole. Several factors contributed to the non-ideality of this research proposal. Primarily, the overall objective of this project was somewhat vague to begin with, making it fairly difficult to work towards a specific goal or outcome. The sole intention to "characterize nuclei of CSCs transfected with ZsGreen to verify the presence of irregularities" provides us with one primary setback: why would we expect these irregularities without trying to specifically induce them? The various growth factors were added to the cells in order to keep them healthier and maintain the pluripotency of the CSCs, making them easier to transfect with ZsGreen. In other words, no specific treatments were administered to these cells that would likely cause the fore mentioned irregularities, yielding very low results with respect to cells in mitosis, mitotic catastrophe, senescence, or apoptosis. A reasonable follow-up to this project would be to treat these cells with chemotherapeutic agents in order to observe the responses of CSCs vs. non-CSCs, thereby examining the processes of CSC selective resistance.

One key problem that was observed during the analysis phase was the clarity of the fluorescent photos. It was especially difficult to focus on the individual fluorescent cells by following the previously mentioned procedure of taking multiple photos at the same location using different UV lights. Having to constantly change the levels of contrast, brightness, and other settings made it fairly problematic to maintain a regular level of focus between the green and blue fluorescence photos. Thus, there existed a fair amount of variability from photo to photo in regards to the overall brightness and clarity of the fluorescent cells. For example, in a photo taken from the transfection positive well 4, the ZsGreen is clearly visible in the green fluorescence photo. However, when that exact same cell from the exact same location was viewed with blue fluorescence, no nucleus was visible in the photo, therefore defeating the purpose of studying it through NMA analysis (Figure 10).

A separate issue dealing with photo clarity was revealed while using the ImageJ program and the NMA plugin to convert the photographic data into graphical figures. Before calculating the area of each of the photographed blue fluorescent nuclei, the program would first recognize the individual nuclear membrane of each cell in the photo. Since many of the nuclei showed varying levels of clarity, the program could not always accurately distinguish their exact size and shape in each photo. Referring to Figures 11, 12 and 13, some cells were identified as being irregular or perhaps even senescent based on their calculated area and shape. Although these

could be accurate assessments, there is also the possibility that these observations were only caused by the false recognition of unclear and unfocused nuclei. The most effective way for guaranteeing accurate and significant results in this type of experiment would be to maximize the clarity of each analyzed photo.

Another problem that was encountered while conducting this experiment was the issue of a small experimental population. Data from only one single test using 4 wells of cells was recorded and used in photo and graphical analysis. Performing multiple test series using the same transfection reagents would have provided more accurate and diverse results to analyze. Using only one small set of data runs the risk of finding less statistically significant information, since it does not effectively represent the results from a larger experimental population. To cite a specific example from this experiment, only one blue fluorescence photo was taken from the transfection negative wells, wells 1 and 3 (Figures 7 and 9, respectively), with less than thirty fluorescent nuclei visible in each of them. It was difficult to form an effective graphical figure to represent such a small amount of data, especially compared to wells 2 and 4 which had each had significantly more photos taken of them. An equal amount of photos should have been taken of each well, both transfection positive and negative, for the sole purpose of having sufficient data to analyze via graphical analysis.

On top of all this, only a fairly narrow time frame was available to fully perform the proposed experiment. After all the training and preparation, only about three months were left to perform all transfections and analyses. To cover all subjects thoroughly enough to come up with sufficient, viable results from an experiment such as this one should require a longer period of time to allow multiple testing series, as well as allowing room for error and re-trial.

## *4.5 Future Improvements and Proposed Expansion*

To improve on these numerous difficulties while still maintaining focus on cancer stem cell research, a new study should be formed based on the previous information and results using more efficient and applicable methods and materials. Instead of transfecting U87 cells for the sole purpose of their visual observation, the cells should be treated with some kind of irregularity-inducing agent while using the transfection plasmid to observe the activity of CSCs in response to this treatment. This way, the study would provide a much more practical approach

in finding methods that specifically target CSCs while still using some of the previously mentioned procedural methods and information. ZsGreen was an effective marker for identifying CSCs and could be utilized again in further studies, as well as the possibility of co-transfection with other plasmids or anything else that can be used to mark the presence of the neural stem cell marker CD 133 (Singh, 2004). Nestin, an intermediate filament protein, could be utilized to evaluate the mitotic ability of these cells (Michalczyk, 2005) while other transfection methods could be explored, such as viral insertion of genetic material.

To maximize the efficiency of this new study on CSCs, it would be best if a single, primary subject was the main focus of the entire experiment so as to not complicate the procedure and process of analyzing the results. In other words, instead of studying multiple irregularities in U87 cells, it would be the researcher's best interest to focus on only one such as cell senescence. Treatments such as the cancer preventing resveratrol (Baur, 2006) or anything else that induces oncogene-induced senescence could be used to test the activities and responses of CSCs (Marin, 2010).

To expand more on how the procedure could be enhanced, the new study should be comprised of multiple test series (or a larger experimental population) under each category (control, double positive, etc.) as to ensure that sufficient data exists to analyze and provide accurate graphical representations of the results. Also, as the NMA plugin from the ImageJ program could not effectively analyze the microscope photos due to their low clarity and resolution, new analysis methods could be implemented in future studies to provide more reliable results. Techniques such as confocal microscopy and Fluorescence Activated Cell Sorting (FACS) have been shown to be extremely effective in the analysis of tranfected cells and could therefore be very useful in providing a way to isolate CSCs, analyze their size and form, and organize their data into a well prepared scatter plot interpretation (Ormerod, 2008; "FACS Methodology", 2001).

# 5 Perspectives

In order to enhance our studies on CSCs, their morphology, and their general activities while still utilizing previous techniques and learning from past mistakes, it is necessary to:

- Conduct more relevant and applicable studies on CSCs by focusing on specific *in vitro* treatments that could be CSC specific.
- Focus on a specific irregularity, such as cellular senescence, and conduct more in-depth experiments that aim to induce said irregularity in tumor cells
- Utilize a larger experimental population by performing multiple test series.
- Explore other cellular analysis techniques such as confocal microscopy and Fluorescence Activated Cell Sorting, as well as the effectiveness of possible co-transfection of ZsGreen with other cellular markers

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