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Beginning of malignant tumor cells

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Beginning of Malignant Tumor Cells

An Undergraduate Honors College Thesis

in the

Department of Biomedical Engineering
College of Engineering
University of Arkansas
Fayetteville, AR

by

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SUMMARY

Despite advances in cancer treatments, cancer is still ranked the second leading cause of death. Cancers are started by cancer stem cells (CSCs). CSCs, also called tumor-initiating cells, are found in tumors and are responsible for the growth of tumors and the spread of cancer to other parts of the body. CSCs are very resistant, and cannot be killed by chemotherapy or radiation treatments. These cells can come from healthy stem cells. The environment surrounding the healthy stem cells and mature cells can cause mutations in the cell's genetics, resulting in development of CSCs. The environment can also increase tumor resistance to drugs. During preliminary experiments in our laboratory, we have found how the culture environment leads to mutation of human brain cancer cells. The cancer cells were also found to be drug-resistant and have the ability to change shape to resemble colonies of other stem cells.

In this project, we hypothesized that stressful conditions cause normal cells to change into CSCs capable of travelling throughout the body. To test the hypothesis, we attempted to examine which environmental changes cause conversion of cancer cells to CSCs. A U87 cell line was cultured in a MEM-FBS solution and concentrations of the antibiotic G418 were administered. The response of the cells to the G418 dosages was quantified by recording the cell confluency over several days. Changes in cell morphology were also recorded.

This work did not result in any CSC-like cells. Cells not treated with G418 proliferated to 100% confluency, but all dosages of G418 resulted in at most 8% cell confluency. Cell clusters formed in some of the wells of the cell plates. Future work should use different antibiotic concentrations to find where the cell confluency eventually remains constant.

INTRODUCTION

Background

Cancer is still the second leading cause of death, despite advances in surgery, radiotherapy, and chemotherapy for cancer treatment. According to the American Cancer Society, there are an estimated 13.7 million cancer survivors alive in the US, and that number will increase to almost 18 million by 2022 (1). Based on the results from recent studies, the cure for cancer requires eliminating the subset of cells that support tumor growth, which is believed to be cancer stem cells (CSCs) (10). Therefore, there is an urgent need to understand the process of how normal and healthy cells develop into CSCs, and why advanced tumor cells are likely to invade other organs.

CSCs are cancer cells found within tumors, and they retain characteristics associated with the normal stem cells. Even though chemotherapy and radiation can suppress or shrink cancer tumors, they do not kill CSCs, as CSCs are resistant to many anticancer agents and known to be strongly radiation resistant (2). CSCs are predicted to be responsible for tumor initiation and maintenance. CSCs not only support the growth and transmission of tumors, but also replenish the other cell types that constitute the tumor (13). CSCs do not necessarily act under the same internal and external factors as non-cancerous stem cells (13). They also drive metastasis, which is the major cause of death in advanced cancer (2-3). In addition, as tumor growth advances, there is a higher chance that new CSCs will be produced by the tumor (10). These new CSCs can continuously divide, unlike the differentiated cells that are also produced by the tumor (10). Chemotherapeutics are likely to only target the bulk cells that do not continuously divide which results in a relapse of tumor formation (13). Some researchers suggest a cancer therapy treatment that influences CSCs to produce non-dividing cells, instead of killing off the CSCs (10).

However, some researchers believe that all tumor cells, not just CSCs, can renew and continue tumor growth (13).

CSCs can be formed in many ways, and are derived from sources other than genetic mutations of non-cancerous stem cells (14). Adult stem cells can spontaneously change into CSCs, or a differentiated cell can dedifferentiate back into a stem cell by obtaining stem cell characteristics through mutation (12). As shown in Fig. 1., the initial CSC niche consists of a few

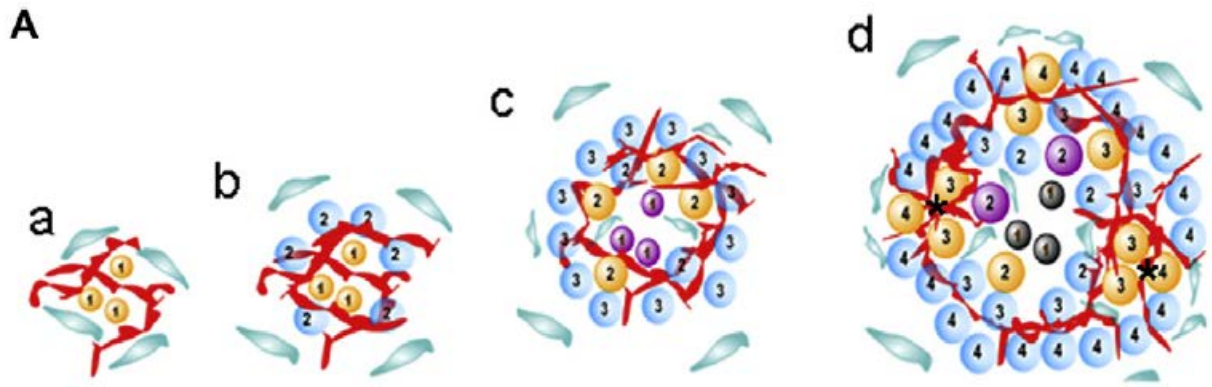

Figure 1. The CSC niche changes as the tumor grows (14).

differentiating CSCs (14). As the tumor continues to develop, the placement of blood vessels (red), differentiated cells (blue), and non-differentiated cells (yellow) continues to change. Recent studies suggest that environments surrounding cells and tissues have great impact on cell mutation and tumorigenesis. For instance, hypoxic (low oxygen tension) environment may induce mouse neural and human mesenchymal stem cells to undergo genomic variability, and the genomic alteration leads to the foundation of a subpopulation of CSCs (4). Additional evidence of tumorigenesis is that increased expression of CSC markers, including CD133 and HIF2α, in a normal stem cell population can be detected after low oxygen tension treatment (6-7). CD133 is a marker of human brain tumors, and its expression is used to purify glioma-initiating CSCs by

fluorescence (17). Also, grade 3 gliomas have an abundance of CSCs according to their stemness signatures (17).

In addition, an acidic environment increases the resistance of glioma cells to multiple drugs, the expression of CSC markers, and tumor formation (8). Very recently, a process called epithelial-mesenchymal transition (EMT) was discovered. EMT does not occur in normal adult cells because it is silenced, but the inducers of EMT in cancer cells are overexpressed (16). The plasticity of CSCs allow this process to occur and it is when epithelial cells lose their defining characteristics and obtain mesenchymal properties, and it may imply the formation of another metastatic tumor (16). These cells show increased invasiveness and resistance to programmed cell death. The EMT process allows cells to migrate through blood and lymphatic vessels to invade different organs (9). Because tumors can become vascular and can affect the rate of angiogenesis, angiogenesis is a major exit route from the initial tumor site into blood circulation (11). Therefore, EMT can be a critical route of tumor metastasis and an origin of carcinoma subpopulations.

Rational

As mentioned above, CSCs are tumor-initiating cells. They are deliberated to originate from a mutation in normal stem cells. In our preliminary experiments, we found that a population of drug-resistant human brain cancer cells can be readily formed under certain culture conditions. These cancer cells not only demonstrated resistance to antibiotics, but also modified their shape by forming colonies similar to human pluripotent stem cells. Thus, it was hypothesized that stress induces the formation of carcinoma cells from normal cells, and that somatic cells under stressful condition can be genetically mutated into CSCs that possess high migration capability.

The microenvironmental cues that stimulate the conversion of cancer cells into CSCs, which is the beginning of malignant tumor-initiating cells, were investigated using the following methods.

Significance

The results of this proposed work were intended to provide knowledge on malignant tumor formation as a subpopulation inside a tumor at its early stage; a CSC line established in this study was used as an *in vitro* model for drug discovery that is specifically targeting CSCs.

MATERIALS AND METHODS

Research and lab work took place at Dr. Sha Jin's lab at the University of Arkansas Engineering Research Center. Time was spent reading up on current research related to the project. In addition, time was spent in the lab to learn basic lab procedures, including culturing cells, immunostaining (Appendix B), and DNA transfection (Appendix C).

Culture medium for the U-87 MG cells (Appendix A) was prepared by combining 117 ml of MEM and 13 ml of FBS in a 125 ml bottle, and was kept refrigerated at -20°C. U-87 MG cells were obtained from a liquid nitrogen freezer in the lab, and warmed in a 37°C water bath. The medium was also warmed in a 37°C water bath. The cells were transferred to a 15 ml tube, and 4.5 ml of medium was added. The cells were centrifuged for 5 min at 1.3 rpm. After centrifuging, the supernatant was removed from the tube, and 2 ml of medium was added. The cells and medium were pipetted several times to mix. The cells were transferred to a 75 cm^2 flask, and 11 ml of medium was added. The flask was stored in a CO_2 incubator for two days.

After two days, the cells were viewed under a microscope and the cells appeared to be attached to the bottom of the flask. The medium was aspirated, and the cells were rinsed with 3 ml of DMEM. Next, 2 ml of Trypsin-EDTA was added to the cells, and the flask was returned to the incubator for 3 min. The flask was removed, and 4 ml of medium was added to the flask, and the contents were transferred to a 15 ml tube. The cells were centrifuged for 5 min at 1.3 rpm, then the supernatant was aspirated, and 7 ml of medium was added. The medium and cells were mixed by pipetting several times. Next, 1 ml of cells was transferred to a clean 75 cm^2 flask, and 11 ml of medium was added to the flask. The flask was returned to the incubator. The medium was removed from the flask every two days, and 12 ml of fresh medium was added.

To plate the remaining cells in the tube, 6 ml of medium was added to the cells and mixed, then 1 ml of cells were transferred to each well in a 12-well plate. Next, the antibiotic G418 (50 mg/ml) was added to the cells. The table below shows the volume of dose added to each well, and the concentration of G418 in each well. No antibiotic was added to 4C as a control.

	1	**2**	**3**	**4**
A	5 µl 0.25 mg/ml	5 µl 0.25 mg/ml	10 µl 0.5 mg/ml	10 µl 0.5 mg/ml
B	15 µl 0.75 mg/ml	15 µl 0.75 mg/ml	20 µl 1.0 mg/ml	20 µl 1.0 mg/ml
C	25 µl 1.25 mg/ml	25 µl 1.25 mg/ml	30 µl 1.5 mg/ml	0 µl 0 mg/ml

Table 1. G418 Doses.
This table shows the volume of G418 added to each well and the concentration of G418 in each well.

The cells were observed daily for 26 days using a microscope, and the cell confluency was measured daily and recorded. Every other day, the medium was removed from each well, and 1 ml of fresh medium was added to each well, along with a dose of G418 following Table 1.

After 5 days, more cells were plated from the 75 cm^2 flask. The medium was warmed in a water bath, and the flask was removed from the incubator. The medium was aspirated, and the cells were rinsed with 3 ml of DPBS, then 2 ml of Trypsin-EDTA was added. The flask was placed back in the incubator, and after 3 min it was removed and 4 ml of medium was added. The cells were transferred to a 15 ml tube and centrifuged for 5 min at 1.3 rpm. The tube was removed from the centrifuge and the supernatant was aspirated, then 7 ml of medium was added to the tube. The cells were plated in a 6-well plate by removing 3 ml of the cells and distributing 0.5 ml of those cells to each well in the 6-well plate. Next, 1.5 ml of medium was added to each

well. Next, the antibiotic G418 (50 mg/ml) was added to the cells. The table below shows the volume of dose added to each well, and the concentration of G418 in each well. No antibiotic was added to A3 and B3 as a control.

	1	**2**	**3**
A	5 µl 0.125 mg/ml	10 µl 0.25 mg/ml	0 µl 0 mg/ml
B	5 µl 0.125 mg/ml	10 µl 0.25 mg/ml	0 µl 0 mg/ml

Table 2. G418 Doses.
This table shows the volume of G418 added to each well and the concentration of G418 in each well.

The cells were observed daily for 21 days using a microscope, and the cell confluency was measured daily and recorded. Every other day, the medium was removed from each well, and 1 ml of fresh medium was added to each well, along with a dose of G418 following Table 2.

Finally, for both plates, the average percent cell confluency was calculated for the different dosages. Scatter plots of the averages were created to observe the changes in percent cell confluency.

RESULTS

Only the cells that received no G418 (control) continued to grow and fill the well to 100% confluency. Initially, some of the control cells died, but they quickly recovered and continued to grow and formed tumor spheroids until the plate was full to 100% confluency. The tumor spheroids looked very similar to the one depicted in Fig. 2.

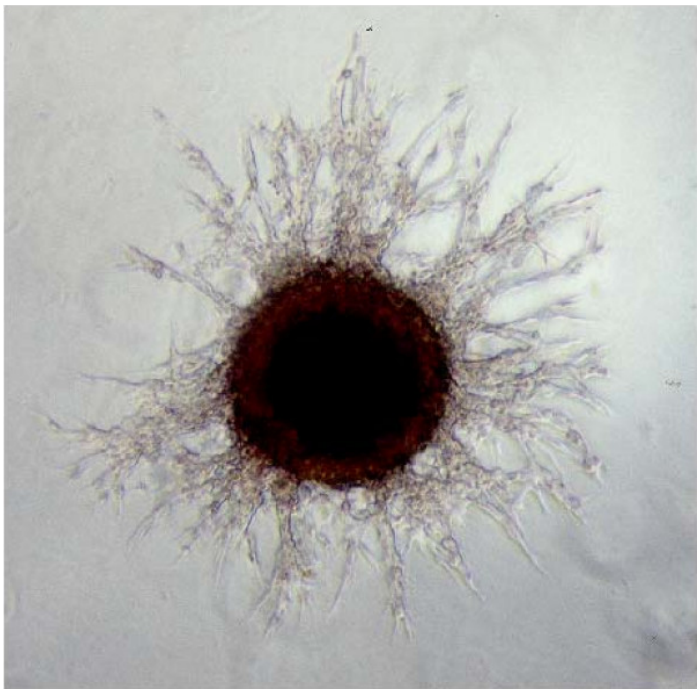

Figure 2.Tumor Spheroid (15).
The control cells that received no antibiotic grew until they reached 100% cell confluency. Tumor spheroids formed in the wells that looked similar to this one.

The tables of percent cell confluency are in the appendix. The table of confluencies for the 12-well plate is in Appendix D, and the table for the 6-well plate is in Appendix E. Below is the graph (Fig. 3) of percent cell confluencies observed for the 12-well plate. From the graph, cells appeared to die faster initially with increased G418 concentration. However, after about Day 7, the cell confluency appeared to decline at about the same rate until Day 26.

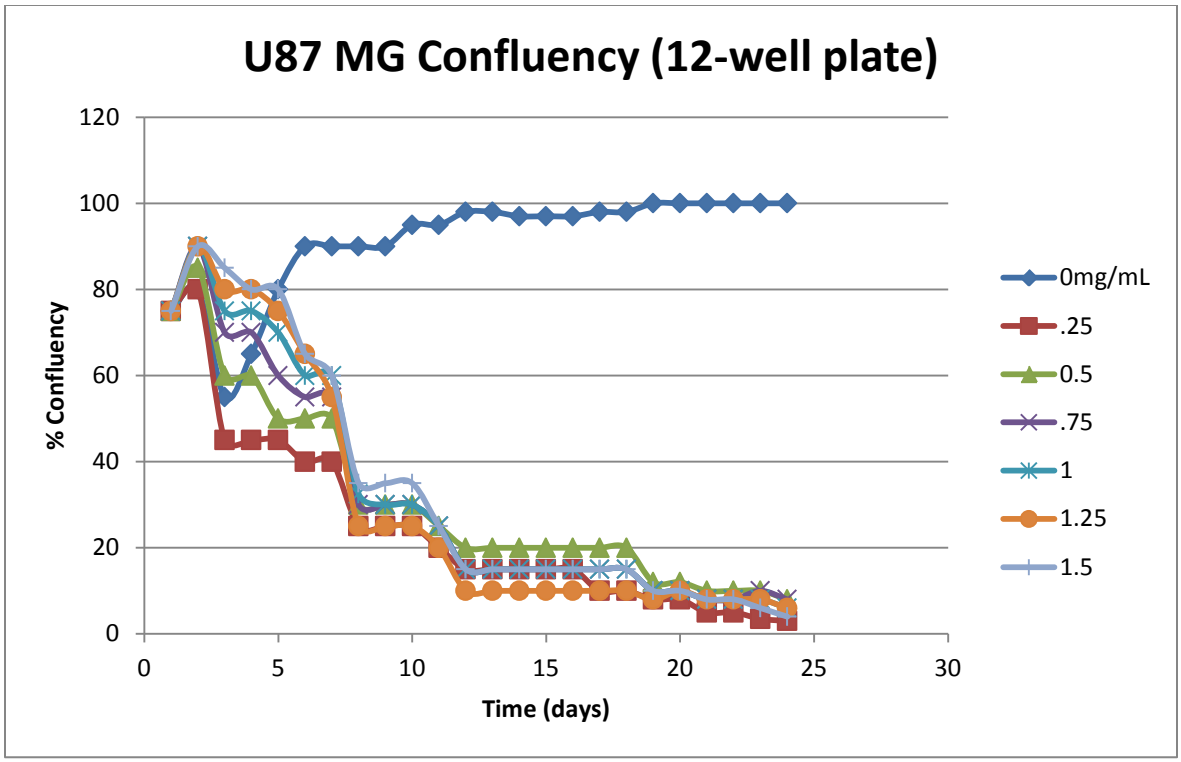

Figure 3. Percent Cell Confluency.
Most of the cells died, except for those in the control that were administered no antibiotic.

Overtime, the cells that received 5-20 µl of G418 in the 12-well plate stayed small and round, and formed small clusters. The cells that received 25-30 µl of G418 stayed small and round, and did not form clusters.

Below is the graph (Fig. 4) of percent cell confluencies observed for the 6-well plate. The results from the 6-well plate were similar to the results of the 12-well plate. Only the cells receiving no G418 survived, and the cells in the remaining wells appeared to decline at about the same rate. However, there was not a sharp decline until Day 9, compared to Day 7 for the 12-well plate. The cells that received 5 µl of G418 in the 6-well plate had a morphology that resembled glial cells, and several clusters formed. The cells receiving 10 µl of G418 were more round and few looked similar to glial cells. Also, fewer clusters of cells formed.

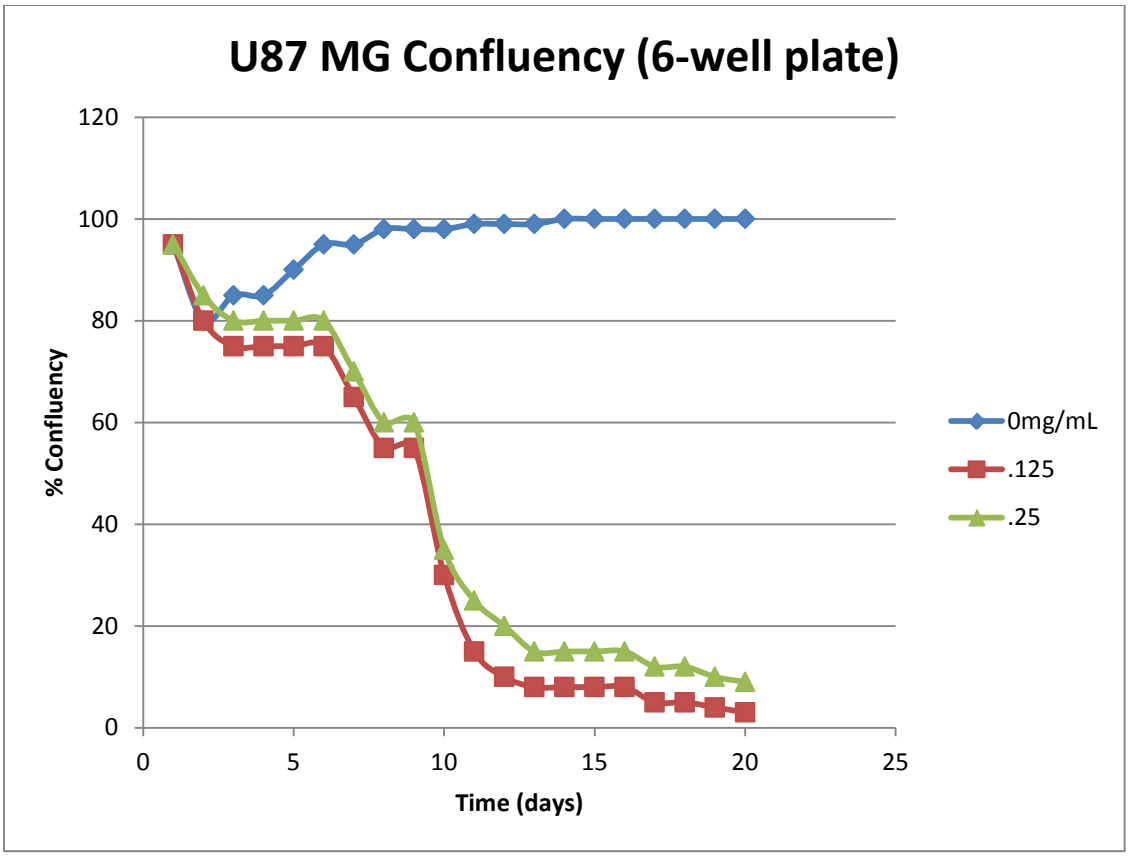

Figure 3. Percent Cell Confluency.
Most of the cells died, except for those in the control that were administered no antibiotic.

DISCUSSION

Different doses of the antibiotic G418 were administered to U87-MG cells at an attempt to observe the transformation of the cells to CSC-like cells. CSC-like cells were not observed, since all of the cells that received G418 continued to die until they reached low percent cell confluencies. For CSC-like cells, the percent cell confluency would have stayed constant after an initial decrease. The 6-well plate was an attempt to dilute the G418 in attempt to find a concentration where the cells would overcome the antibiotic and stay at a constant percent confluency. Possible errors were made in estimating percent confluency, since it was measured by eye, and dead cells may have been confused with live cells.

Future work should test different G418 concentrations, or different antibiotics to find which antibiotic and concentration will cause the cells to resemble CSCs. Also, any cell clusters formed should be analyzed. Future work could increase knowledge on how malignant tumors grow and spread, and potentially lead to new cancer treatment strategies.

ACKNOWLEDGMENTS

I would like to thank my thesis advisor, Dr. Sha Jin, for allowing me to work in her lab as an undergraduate and gain lab experience. The time I spent in lab learning new techniques and running my own experiments was very valuable and will help in future lab work.

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ATCC™

Product Information Sheet for ATCC® HTB-14™

Cell Line Designation: U-87 MG
ATCC Catalog No. HTB-14™

Table of Contents:

Cell Line Description

Organism: *Homo sapiens* (human)
Tissue: brain; glioblastoma; astrocytoma
Age: 44 years
Tumor Stage: classified as grade IV as of 2007
Gender: male
Ethnicity: caucasian
Morphology: epithelial
Growth properties: adherent
Tumorigenic: yes, Tumors developed within 21 days at 100% frequency (5/5) in nude mice inoculated subcutaneously with 10(7) cells
AntigenExp: Blood Type A, Rh+
DNA profile (STR analysis):
D5S818: 11,12
D13S317: 8,11
D7S820: 8,9
D16S539: 12
vWA: 15,17
THO1: 9.3
TPOX: 8
CSF1PO: 10,11
Amelogenin: X
Depositors: J. Ponten
Comments: This is one of a number of cell lines derived from malignant gliomas (see also ATCC HTB-15™ and ATCC HTB-16™) by J. Ponten and associates from 1966 to 1969.
Mycoplasma contamination was eliminated in September 1975.

Note: ATCC has confirmed that the ATCC® HTB-14™ cell line is male in origin based on STR, Y-chromosome paint and Q-band assays. However, based on the current literature, the cell line is still of glioblastoma origin and the discrepancy of gender is not unusual. It is possible that the cell line was misidentified in the depositor's original publication.

Karyology: This is a hypodiploid human cell line with the modal chromosome number of 44 occurring in 48% of cells. The rate of higher ploidy was 5.9%. Twelve markers were common to all cells, including der(1)t(1;3) (p22;q21), der(16)t(1;16) (p22;p12), del(9) (p13) and nine others. The marker der(1) had two copies in most cells. There was only one copy of normal X. N1, N6 and N9 were not found.

Note: Cytogenetic information is based on initial seed stock at ATCC. Cytogenetic instability has been reported in the literature for some cell lines.

Biosafety Level: 1

Appropriate safety procedures should always be used with this material. Laboratory safety is discussed in the following publication: *Biosafety in Microbiological and Biomedical Laboratories,* 4th ed. HHS Publication No. (CDC) 93-8395. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention. Washington DC: U.S. Government Printing Office; 1999. The entire text is available online at www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Use Restrictions

These cells are distributed for research purposes only. ATCC recommends that individuals contemplating commercial use of any cell line first contact the originating investigator to negotiate an agreement. Third party distribution of this cell line is discouraged, since this practice has resulted in the unintentional spreading of cell lines contaminated with inappropriate animal cells or microbes.

Handling Procedure for Frozen Cells

To insure the highest level of viability, thaw the vial and initiate the culture as soon as possible upon receipt. If upon arrival, continued storage of the frozen culture is necessary, it should be stored in liquid nitrogen vapor phase and not at –70°C. Storage at –70°C will result in loss of viability.

SAFETY PRECAUTION: ATCC highly recommends that protective gloves and clothing always be used and a full face mask always be worn when handling frozen vials. *It is important to note that some vials leak when submersed in liquid nitrogen and will slowly fill with liquid nitrogen. Upon thawing, the conversion of the liquid nitrogen back to its gas phase may result in the vessel exploding or blowing off its cap with dangerous force creating flying debris.*

1. Thaw the vial by gentle agitation in a 37°C water bath. To reduce the possibility of contamination, keep the O-ring and cap out of the water. Thawing should be rapid (approximately 2 minutes).

2. Remove the vial from the water bath as soon as the contents are thawed, and decontaminate by dipping in or spraying with 70% ethanol. *All of the operations from this point on should be carried out under strict aseptic conditions.*

3. Transfer the vial contents to a centrifuge tube containing 9.0 ml complete culture medium. and spin at approximately 125 xg for 5 to7 minutes.

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Or contact your local distributor.

4. Resuspend cell pellet with the recommended complete medium (see the specific batch information for the culture recommended dilution ratio). and dispense into a 25 cm^2 or a 75 cm^2 culture flask. *It is important to avoid excessive alkalinity of the medium during recovery of the cells. It is suggested that, prior to the addition of the vial contents, the culture vessel containing the complete growth medium be placed into the incubator for at least 15 minutes to allow the medium to reach its normal pH (7.0 to 7.6).*

5. Incubate the culture at 37°C in a suitable incubator. A 5% CO_2 in air atmosphere is recommended if using the medium described on this product sheet.

Handling Procedure for Flask Cultures

The flask was seeded with cells (see specific batch information) grown and completely filled with medium at ATCC to prevent loss of cells during shipping.

1. Upon receipt visually examine the culture for macroscopic evidence of any microbial contamination. Using an inverted microscope (preferably equipped with phase-contrast optics), carefully check for any evidence of microbial contamination. Also check to determine if the majority of cells are still attached to the bottom of the flask; during shipping the cultures are sometimes handled roughly and many of the cells often detach and become suspended in the culture medium (but are still viable).

2. **If the cells are still attached,** aseptically remove all but 5 to 10 ml of the shipping medium. The shipping medium can be saved for reuse. Incubate the cells at 37°C in a 5% CO_2 in air atmosphere until they are ready to be subcultured.

3. **If the cells are not attached,** aseptically remove the entire contents of the flask and centrifuge at 125 xg for 5 to 10 minutes. Remove shipping medium and save. Resuspend the pelleted cells in 10 ml of this medium and add to 25 cm^2 flask. Incubate at 37°C in a 5% CO_2 in air atmosphere until cells are ready to be subcultured.

Subculturing Procedure

Volumes used in this protocol are for 75 cm^2 flask; proportionally reduce or increase amount of dissociation medium for culture vessels of other sizes.

1. Remove and discard culture medium.

2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin-0.53mM EDTA solution to remove all traces of serum which contains trypsin inhibitor.

3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C C to facilitate dispersal.

4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting.

5. Add appropriate aliquots of the cell suspension into new culture vessels.
   Subcultivation Ratio: 1:2 to 1:5

6. Incubate cultures at 37°C.

Note: For more information on enzymatic dissociation and subculturing of cell lines consult Chapter 10 in Culture of Animal Cells, a manual of Basic Technique by R. Ian Freshney, 3rd edition, published by Alan R. Liss, N.Y., 1994.

Medium Renewal

2 to 3 times weekly.

Cultured 2/2/13
Prepared 8/7/13

Complete Growth Medium

The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium, Catalog No. 30-2003.
To make the complete growth medium, add the following components to the base medium:

- fetal bovine serum to a final concentration of 10%

This medium is formulated for use with a 5% CO_2 in air atmosphere.
ATCC tested fetal bovine serum is available as ATCC Catalog No. 30-2020.

Cryoprotectant Medium

Complete growth medium described above supplemented with 5% (v/v) DMSO. Cell culture tested DMSO is available as ATCC Catalog No. 4-X.

Additional Information

Additional product and technical information can be obtained from the catalog references and the ATCC Web site at www.atcc.org, or by e-mail at tech@atcc.org.

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(additional references are available in the catalog at www.atcc.org)

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Appendix B

Cell Signaling TECHNOLOGY®

Immunofluorescence General Protocol

IMPORTANT: Please refer to the **APPLICATIONS** section on the front page of the datasheet to determine if this product is validated and approved for use on cultured cell lines (**IF-IC**), paraffin-embedded samples (**IF-P**), or frozen tissue sections (**IF-F**). Please see product datasheet for appropriate antibody dilution and unmasking solution.

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **10X Phosphate Buffered Saline (PBS):** To prepare 1L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1L dH_2O. Adjust pH to 8.0.
2. **Formaldehyde**, 4%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4°C in dark, dilute in PBS for use.
3. **Blocking Buffer:** (1X PBS / 5% normal goat serum (#5425) / 0.3% Triton X-100):
   To prepare 10 ml, add 2.5 ml 10X PBS, 1.25 ml normal serum from the same species as the secondary antibody (e.g., normal goat serum, normal donkey serum) and 21.25 ml dH_2O and mix well. While stirring, add 75 µl Triton X-100.
4. **Antibody Dilution Buffer** (1X PBS / 1% BSA / 0.3% Triton X-100):
   To prepare 40 ml, add 4 ml 10X PBS and 120 µl Triton X-100 to 0.4 g BSA. Bring to final volume of 40 ml with dH_2O and mix well.
5. **Fluorochrome-conjugated secondary antibody** (recommended secondary antibodies)
   NOTE: When using any primary or fluorochrome-conjugated secondary antibody for the first time, titrate the antibody to determine which dilution allows for the strongest specific signal with the least background for your sample.
6. **Prolong® Gold Antifade Reagent** (Invitrogen, Eugene, OR, cat# P36930)

Reagents specific to IF-P application:

1. **Xylene**
2. **Ethanol**, anhydrous denatured, histological grade, 100% and 95%.
3. **Antigen Unmasking:**
   a. **For Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1L add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) to 1L dH_2O. Adjust pH to 6.0.
   b. **For EDTA:** 1 mM EDTA: To prepare 1L add 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$) to 1L dH_2O. Adjust pH to 8.0.

B. Specimen Preparation

I. Cultured Cell Lines (IF-IC)

NOTE: Cells should be grown, treated, fixed and stained directly in multi-well plates, chamber slides or on coverslips.

1. Aspirate liquid, then cover cells to a depth of 2–3 mm with 4% formaldehyde in PBS.
   NOTE: Formaldehyde is toxic, use only in fume hood.
2. Allow cells to fix for 15 minutes at room temperature.
3. Aspirate fixative, rinse three times in PBS for 5 minutes each.
4. Proceed with Immunostaining (Section C).

II. Paraffin Sections (IF-P) Appendix B

NOTE: Do not allow slides to dry at any time during this process.

1. **Deparaffinization/Rehydration:**
   a. Incubate sections in three washes of xylene for 5 minutes each.
   b. Incubate sections in two washes of 100% ethanol for 10 minutes each.
   c. Incubate sections in two washes of 95% ethanol for 10 minutes each.
   d. Rinse sections twice in dH_2O for 5 minutes each.

2. **Antigen Unmasking:**
   NOTE: Consult product datasheet for specific recommendation for the unmasking solution.
   a. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0, then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.
   b. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.

3. **Proceed with Immunostaining (Section C).**

III. Frozen/Cryostat Sections (IF-F)

1. **For fixed frozen tissue proceed with Immunostaining (Section C).**
2. **For fresh, unfixed frozen tissue, please fix immediately, as follows:**
   a. Cover sections with 2–4% formaldehyde in PBS.
   NOTE: Formaldehyde is toxic, use only in fume hood.
   b. Allow sections to fix for 15 minutes at room temperature.
   c. Rinse slides three times in PBS for 5 minutes each.
   d. Proceed with Immunostaining (Section C).

C. Immunostaining

NOTE: All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

1. Block specimen in Blocking Buffer for 60 minutes.
2. While blocking, prepare primary antibody by diluting as indicated on datasheet in Antibody Dilution Buffer.
3. Aspirate blocking solution, apply diluted primary antibody.
4. Incubate overnight at 4°C.
5. Rinse three times in PBS for 5 minutes each.
   NOTE: If using primary antibodies directly conjugated with Alexa Fluor® fluorochromes, then skip to step C8.
6. Incubate specimen in fluorochrome-conjugated secondary antibody* diluted in Antibody Dilution Buffer for 1–2 hours at room temperature in dark.
7. Rinse in PBS as in step 5.
8. Coverslip slides with Prolong® Gold Antifade Reagent.
9. For best results, examine specimens immediately using appropriate excitation wavelength. For long-term storage, store slides flat at 4°C protected from light.

***Recommended Secondary Antibodies:**

Anti-Rabbit

- Anti-Rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 488 Conjugate) #4412
- Anti-Rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 555 Conjugate) #4413
- Anti-Rabbit IgG (H+L), $F(ab')_2$ Fragment (Alexa Fluor® 647 Conjugate) #4414

Anti-Mouse

7:30
2 mL & 20µL GHTR

Protocol-at-a-Glance

Xfect™ Transfection Reagent Protocol-at-a-Glance

(PT5003-2)

This protocol is provided for transfection with Xfect Transfection Reagent (Cat. Nos. 631317 & 631318). Use this procedure to transfect DNA into mammalian cells in a 6-well format. For other formats, please see Table I on page 2 of this protocol. Transfections can be carried out entirely in the presence of serum.

A. Notes

1. Storage & Handling
   - Thaw Xfect Polymer (100 µg/µl) at room temperature just prior to use. Once thawed, store Xfect Polymer at 4°C for up to 12 months.
   - Thaw Xfect Reaction Buffer at room temperature just prior to use. Vortex after thawing. Once thawed, store Xfect Reaction Buffer at 4°C for up to 12 months.
   - After each use make sure that the cap for the Xfect Polymer is closed tightly and return to the supplied foil pouch containing desiccant.
2. **Mock transfections:** Use a plasmid that does not contain your gene of interest. You must include a source of nucleic acids to assemble with the Xfect Polymer.

B. Transfection Protocol

1. Prepare cells for transfection.

   Adherent cells: One day prior to the transfection, plate cells in 1 ml of complete growth medium so that the cells will be 50–80% confluent at the time of transfection.

   Suspension cells: Just prior to preparing complexes (step 2), plate 5×10^5–1.25×10^6 cells in 1 ml of growth medium.

2. Thoroughly vortex Xfect Polymer.
3. For each transfection sample, prepare two microcentrifuge tubes:

Concentration of DNA: 2.06µg/µl

Tube 1 (Plasmid DNA)		Tube 2 (Polymer)	
3.64 (7.5µg) µl	(5 µg) Plasmid DNA†	1.5 µl	Xfect Polymer (always use 0.3 µl of Xfect Polymer per 1 µg of plasmid DNA)
96.36 µl	Xfect Reaction Buffer	98.5 µl	Xfect Reaction Buffer
100 µl	Total Volume	100 µl	Total Volume

2.25 µl
97.75 µl
100µl

NOTES:

- *These quantities are per well of a 6-well plate.* Please see Table I on page 2 for other formats.
- It is crucial that the Xfect Polymer does not remain in aqueous solution for longer than 30 min at room temperature.

†5 µg of plasmid DNA works best for most cell lines. However, the first time you use Xfect, we recommend testing 2.5 µg, 5 µg, and 7.5 µg. Using less than 2.5 µg per well in a 6-well plate may result in a low transfection efficiency. See Figure 1 on page 2.

4. Vortex each tube well to mix.
5. Add the Polymer solution to the DNA solution and vortex well at a medium speed for 10 sec.
6. Incubate the samples for 10 min at room temperature to allow nanoparticle complexes to form.
7. Add the entire 200 µl of nanoparticle complex solution (Step B.5) dropwise to the cell culture medium from Step B.1. Rock the plate gently back and forth to mix.

   NOTE: It is not necessary to remove serum from your cell culture medium. It is normal for the medium to change color slightly upon addition of the nanoparticle complex solution.

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8. Incubate the plate at 37°C for 4 hr to overnight.

   NOTE: 4 hr incubation with Xfect-DNA nanoparticles is sufficient for optimal transfection. Incubation overnight is possible for convenience but does not generally increase transfection efficiency. If you have sensitive cells we recommend incubating for no more than 4 hr.

9. Remove nanoparticle complexes from cells by aspiration, replace with 2 ml fresh complete growth medium, and return the plate to the 37°C incubator until time of analysis. Peak expression is typically reached 48 hr posttransfection.

Table I. Scaling Xfect Transfections Up or Down

Culture Vessel	Surface Area/ Well	Growth Medium	DNA	DNA Dilution Volume (in Xfect Reaction Buffer)	Xfect Polymer Volume	Polymer Dilution Volume (in Xfect Reaction Buffer)
24-well plate	2 cm^2	250 µl	0.5–1 µg	25 µl	Always use 0.3 µl of Xfect Polymer for every 1 µg of plasmid	25 µl
12-well plate	4 cm^2	500 µl	1–2.5 µg	50 µl		50 µl
6-well plate	10 cm^2	1 ml	2.5–7.5 µg	100 µl		100 µl
10 cm dish	60 cm^2	10 ml	20–40 µg	600 µl		600 µl

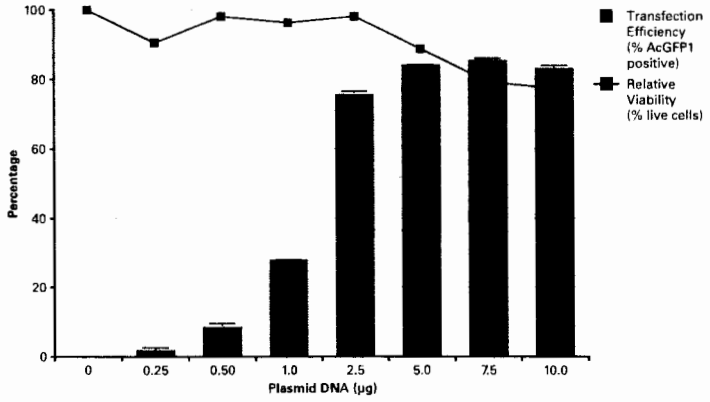

Figure 1. Transfection of Hela Cells in a 6-well plate using Xfect. Increasing the amount of DNA + Xfect Polymer significantly increases efficiency without affecting viability. 5 µg of plasmid DNA works best for most cell lines.

Appendix D: Daily Average Percent Confluency for each G418 Dose (12-well plate)

Well (dosage of G418)(mg/mL)	Day 0	Day 1	Day 2	Day 3	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21	Day 22	Day 23	Day 24	Day 25	Day 26
0	75	80	55	65	80	90	90	90	90	95	95	98	98	97	97	97	98	98	100	100	100	100	100	100
0.25	75	80	45	45	45	40	40	25	25	25	20	15	15	15	15	15	10	10	8	8	5	5	3.5	3
0.5	75	85	60	60	50	50	50	30	30	30	25	20	20	20	20	20	20	20	12	12	10	10	10	8
0.75	75	90	70	70	60	55	55	30	30	30	25	15	15	15	15	15	15	15	10	10	8	8	10	8
1	75	90	75	75	70	60	60	32.5	30	30	25	15	15	15	15	15	15	15	10	10	8	8	8	6
1.25	75	90	80	80	75	65	55	25	25	25	20	10	10	10	10	10	10	10	8	10	8	8	8	6
1.5	75	90	85	80	80	65	60	35	35	35	25	15	15	15	15	15	15	15	10	10	8	8	6	4

Appendix E: Daily Average Percent Confluency for each G418 Dose (6-well plate)

Well (dosage of G418)(mg/mL)	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Day 15	Day 16	Day 17	Day 18	Day 19	Day 20	Day 21
0	95	80	85	85	90	95	95	98	98	98	99	99	99	100	100	100	100	100	100	100
0.125	95	80	75	75	75	75	65	55	55	30	15	10	8	8	8	8	5	5	4	3
0.25	95	85	80	80	80	80	70	60	60	35	25	20	15	15	15	15	12	12	10	9

This thesis is approved.

Thesis Advisor:

Thesis Committee: