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Characterization of Cytokeratin 8 in Cancer

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Characterization of Cyokeratin 8 in Cancer

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ABSTRACT: A Cytokeratin 8 (K8)/Green Fluorescent Protein (GFP) fusion construct was created to better understand the behavior of K8 within cancer cells. This intermediate filament (IF) protein is a member of the cytoskeletal gene family, along with actin and tubulin. IFs are normally expressed in a tissue-specific and differentiation-dependent manner, in which their role is more supportive than essential to the cell. Such roles include rigidity of cellular shape, protein trafficking, cellular locomotion, and cell-signaling platforms. K8 mutation, over-expression, and aberrant post translational modifications have been observed in various carcinoma cell lines to be the cause of several phenotypes, including apoptosis inhibition, drug resistance, transformation, Mallory-Denk body formation, localization at the plasma membrane, and secretion of the protein.

To study these abnormal phenotypes, the K8 gene was isolated and inserted into the GFP over expression vector. Transfecting this vector into HeLa cells allowed for the study of K8 within a well-defined cervical cancer cell line. This study was intended to provide answers to K8's localization at the plasma membrane in carcinoma cell models while avoiding criticisms to previous immunohistochemical localization studies. A cellular model of K8 processing that exhibits established phenotypes found in the literature was thus created and has the potential to address several paramount questions related to K8's role in supporting the development and progression of cancer. It could also be utilized as an assay for the discovery of K8 filament formation inhibitors, which may prove useful in combination with current chemotherapeutics. The model could also be used to provide weight to diagnostics, such as the CAncer REcognition test, which utilizes antibodies against K8 as biomarkers for malignancy via an Enzyme-Link ImmunoSorbent Assay.

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INTRODUCTION

Early cancer detection and disease monitoring methods are crucial to patient survival. When a tumor is still in its tissue of origin, the disease is far more manageable and treatments produce more optimistic prognoses; for this reason, it is paramount to identify the presence of cancer cells as early as possible. Unfortunately, modern medicine is strongly focused on treatment and less on prevention. Only when both are utilized in an efficient and timely manner can future generations hope to see a decline in the morbidity and mortality caused by this disease.

Most cancers arise through genetic mutations in growth regulatory circuits resulting in uncontrolled proliferation or defects in programmed cell death pathways. While these mutations can be the cause of the disease, they can also lead to its detection and treatment. Subtle yet significant surface alterations, or tumor markers, can be found on carcinoma cells which differentiate them from the surrounding normal tissue. Having the ability to differentiate normal cells from those which have transformed is a rational foundation for the development of a diagnostic test for the presence of the disease.

One such alteration can be found in cytokeratin 8's (K8) aberrant localization in several carcinoma cell lines. K8 is a cytosolic protein and a member of the Intermediate Filament (IF) family. IFs, together with actin and tubulin, define the cytoskeleton of eukaryotic cells, with IFs representing the largest of the cytoskeletal gene families. IFs are expressed in a tissue-specific and differentiation-dependent manner, and they serve various purposes, including rigidity of cellular shape, intracellular organization of proteins, transport, locomotion of the cell, scaffolding functions, and cell signaling platforms (Strnad et al. 2008). Since these proteins assist in such a variety of ways, they frequently interact with numerous kinases, adaptor, and apoptotic proteins.

Keratin nomenclature breaks down the group into two sub-types based on their pH: acidic keratins known as type I and basic keratins known as type II. Type I and type II keratins bind with each other to create heteropolymer filaments. Specific keratin pairs are preferentially expressed in individual cell types (Strnad et al. 2008); one commonly expressed type I/type II pair of interest is K8 and K18. This keratin pair is observed in almost all single-layered epithelia cells (Strnad et al. 2008).

IF proteins' supportive rather than essential intracellular roles allow for mutations to go unchecked and lead to cellular characteristics such as chemotherapeutic resistance (Lau & Chiu 2007; Wang et al. 2008; Liu et al. 2008), transformation (Raul et al. 2004; Gilbert, Loranger, Daigle, & Marceau 2001; Gires et al. 2006), and an aggregation of the K8 into inclusion bodies known as Mallory-Denk Body formations (Gires et al. 2006; Ku & Omary 2000). So how can we use the mutation of K8 to our advantage in the detection of cancer?

Previous studies discovered a high titer of IgM antibodies within cancer patients which bound specifically to an epitope found on K8 named LT-11. These studies led to the initial development of an Enzyme Linked ImmunoSorbent Assay (ELISA) diagnostic termed the CAncer REcognition (CARE) test (Thornthwaite et al. 2004). The assay utilizes the Anti-K8 antibodies found selectively in cancer patient serum as a sensor for tumor presence; but, in order to present itself to the immune system, intracellular K8 must somehow be localizing at the cell surface or releasing itself from the cell due to lysis or secretion. Investigations exploring how K8 was being presented to the immune system, and why it was considered foreign only in the case of tumor cells, needed to be carried out.

To better understand this phenomenon a model system was generated to study the localization of K8 within a cancer cell. Localization studies had been previously carried out (Gires et al. 2005), but with substantial criticisms. In said study, "non permeabilized" cells were subjected to staining using anti LT-11 antibodies and imaged via fluorescence microscopy. Results showed a strong signal at the plasma membrane, signifying the presence of K8 at the cell's surface. Unfortunately, the study's use of potentially membrane destabilizing immunostaining techniques could have generated false signals of the protein's presence at the cell surface. By utilizing a green fluorescent protein (GFP) tag bound to K8, our new model avoided the need for immunostaining and allowed us to image the protein directly. It also created a platform which could be utilized to explore new questions regarding the aberrant behavior of K8.

It is of great importance to develop a K8 over-expression cellular model that exhibits the same phenotypes as the various carcinomas documented in the literature. Our study is thus the creation of a model system in which K8's biochemical characteristics within cancer can be

observed. These studies aimed to empower potential diagnostics utilizing K8 and develop an assay which could be used for the discovery of novel drug inhibitors of K8/K18 formation.

METHODS AND MATERIALS

Target Gene Isolation

The K8 gene was generated utilizing a colon cancer (HCT-116) cell line cDNA library, created in our laboratory by graduate student Jose Salvatico. First *K8-F* (5'- GGT TCT CCG CTC CTT CTA GG -3') and *K8-R* (5'- CTC CTG TTC CCA GTG CTA CC -3') primers containing no restriction enzyme (R.E.) sites were used in a polymerase chain reaction (PCR) to generate the K8 gene. PCR profile was as follows: 30 cycles (95 °C, 15 sec, 51–61 °C gradient, 30 sec, 68 °C, 1 min 30 sec). All PCR reactions were carried out using Eppendorf's Master Cycler Gradient thermocycler. The elongation time needed for the K8 gene was calculated using the following ratio: approximately 1 Kb per 60 sec (K8 = 1.4 Kb ≈ 1 min 30 sec). Annealing temperatures ranging from 51 to 63 degrees were used to test for the primer's optimum conditions. PCR product yields were determined by loading samples onto a 1% agarose gel, separating samples via electrophoresis and staining the gel with Ethidium bromide (EtBr). The greatest and purest yield (brightest signal band) was found at 54.3 °C. The PCR product taken from this tube was purified via the QIAquick PCR purification kit, provided by QIAGEN (Valencia, CA).

Next, custom K8 inserts were created for both the EGFP-C and EGFP-N plasmids from BD Biosciences (San Jose, CA). The K8-N insert was generated using *CK8R1-NF* (5'- A TAT GAA TTC GCC ACC ATG TCC ATC AGG GTG ACC CAG -3') and *Krt8N-BamH1-Rev* (5'- T ATT GGA TCC AA CTT GGG CAG GAC GTC AGA -3') primers. The K8-C insert was generated using *Krt8C-EcoR1-Rev* (5'- T AAT GAA TTC A ATG TCC ATC AGG GTG ACC CAG-3') and *ck8BH1-CR* (5'- T TAT GGA TCA CTT GGG CAG GAC GTC AGA AAG TGA -3'). These primers were placed in separate tubes with the K8 template DNA, and PCR was carried out with the following profile: (95 °C, 15 sec, 54.3 °C, 30 sec, 68 °C, 1 min 30 sec). PCR product yields were then observed on a 1% agarose gel. The PCR products were then purified via QIAquick PCR purification. Both K8-C and K8-N inserts were then R.E. digested using EcoR1 and BamH1

in React 3 buffer provided by Invitrogen (Carlsbad, CA) at 37 °C for 1 ½ hr.

Digested samples were then purified via Phenol/Chloroform and Ethanol (P/C/Ethanol) precipitation. Each DNA sample was adjusted to 110 µl total volume by adding de-ionized H₂O (dH₂O). This process was followed by the addition of 30 µl of Phenol/Chloroform. The samples were centrifuged at 14,000 rpm for 5 min, the top 100 µl of solution were transferred to a new tube and the following were added: 10 µl 3 M NaOAC, 1 µl glycogen and 300 µl 100% ethanol kept at -20 °C. The tubes were then incubated at -80 °C for 15 min. DNA was pelleted by centrifugation at 13,000 rpm for 15 min at 4 °C and supernatant was removed. The pellet was then resuspended in 100 µl of 70% ethanol kept at -20 °C and re-pelleted at 14,000 rpm for 5 min at 4 °C. Supernatant was removed and pellet was allowed to dry at room temperature. DNA was finally resuspended in 15 µl of dH₂O.

Ligation

K8-C and K8-N were inserted into either EGFP-N or EGFP-C plasmids to create K8/GFP-N and GFP/K8-C. First the DNA concentrations of both the plasmids and inserts were measured at 260 nm by loading 2 µl of sample with 98 µl of dH₂O using TECAN's GENios 96 well plate reader (Männedorf, Switzerland). Then EGFP-C was mixed with K8-C and EGFP-N was mixed with K8-N in the following ratios (Insert to Plasmid): 6:1 and 12:1. Ligation was carried out at room temp (approximately 22.5 °C) for 1 hour. Ligated products were then purified via P/C/Ethanol Precipitation and re-suspended in 20 µl of dH₂O (plasmids were maintained at a high concentration to increase transformation efficacy).

Transformation

K8/GFP-N and GFP/K8-C were transformed into XL-Gold *Escherichia coli* Ultra Competent cells provided by Stratagene (La Jolla, CA) via heat shock. XL-Gold *E. coli* cells were first thawed on ice and treated with β-mercaptoethanol. Both the 6:1 and 12:1 ligation products were mixed together, placed with cells and allowed to set for 30 min on ice. Cells were then placed in a dry bath at 42 °C for 30 sec then allowed to grow in Luria-Bertani (LB) media for 45 min in an incubator shaker at 37 °C. Cells were pelleted, plated on Kanamycin (Kan) selective plates and incubated over-night. Colonies were selected and placed in 3 mL of LB (Kan) media for 15 hrs in a shaker incubator at 37 °C.

Screening of Colonies

Plasmids from cultured cells were harvested via QIAprep Spin Mini Prep Kit, provided by QIAGEN. The isolated plasmids were digested with EcoR1 and BamH1 at 37 °C and analyzed on a 1% agarose gel to confirm insertions. Positive clones were found for GFP/K8-C, while all of the K8/GFP-N plasmids were negative for the K8 insert. The process of ligation and the aforementioned procedures were repeated for the K8/GFP-N plasmid using Max Efficiency® DH5- α -T1 competent *E. coli* cells (Invitrogen).

Plasmid Sequencing

Positive clones were chosen and grown in 3 mL of LB (Kan) media. These cultures were harvested and plasmid DNA concentrations were then measured at 260 nm with a 280 nm purity reference. GFP/K8-C and K8/GFP-N were sent to GENEWIZ (South Plainfield, NJ) for sequencing. Results came back 99% homologous to K8's sequence found in the National Center for Biotechnology Information database with one silent mutation.

Verification of Expression

Both GFP/K8-C and K8/GFP-N plasmids were transfected into HeLa (Cervical) and MDA-MB-231 (Breast Cancer) cell lines for the purpose of Western blot confirmation of K8/GFP fusion protein expression. First, both cell lines were grown to 95% confluency on a 6 well plate. Next, 1 μ g and 2 μ g of each plasmid were added to separate tubes containing 250 μ l of OPTI-MEMI media (1 μ g of a blank DNA was added to the 1 μ g plasmid tubes to keep DNA concentrations constant). A 8 μ l volume of Lipofectamine™ 2000 (Invitrogen) was mixed with 250 μ l of OPTI-MEMI media for each transfection well, then added to the DNA/OPTI-MEMI suspensions and incubated at room temperature for 20 min. Growth media was removed from each well and 500 μ l of the Lipofectamine/plasmid mixture was added. HeLa cells were then given 1 ml of serum free RPMI, while MDA-MB-231 cells were given 10% FBS DMEM media. Plates were gently rocked to spread plasmid evenly and placed in the culture incubator for 24 hrs; 1 ml of 20% RPMI was given to the HeLa cell cultures after 5 hrs.

Media was removed from all transfection wells, then cells were trypsinized and pelleted in a microcentrifuge at room temperature. Cells were then washed with 1ml of phosphate buffered saline (PBS), and re-suspended in 50 μ l of 1x radio-immunoprecipitation assay buffer

containing ethylenediaminetetraacetate(EDTA)-free protease inhibitor cocktail (Roche Diagnostics). Tubes were placed on ice for 30 min then centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant (cytosolic fraction) was transferred to a new tube and the protein concentrations were measured via Bradford assay.

Protein samples were suspended in 5x SDS loading dye containing: 2.0 ml 1 M Tris-HCl pH 6.8, 0.8 g SDS, 4.0 ml 100% glycerol, 0.4 ml 14.7 M β -mercaptoethanol, 1.0 ml 0.5 M EDTA and 8 mg bromophenol Blue. Samples were heated at 95 °C for 5 min and approximately 10 μ g were loaded onto a .75 mm 10% polyacrylamide gel. Electrophoresis was carried out at 80 volts for 60 min then 150 volts for 25 min. Proteins were then transferred to a nitrocellulose membrane at 100 volts for 90 min. The membrane was then probed for the GFP and Actin (to act as a loading control) with the following antibodies (and their concentrations): Primary antibodies- (1:1,000 mouse α -GFP) and (1:10,000 Rabbit α -actin), Secondary antibodies- (1:1,000 Goat α -rabbit IgG, Horseradish Peroxidase(HRP)-conjugated) and (1:1,000 Goat α -mouse IgG, HRP-conjugated). Antibodies came from the following sources: Mouse α -GFP and Goat α -mouse IgG (Santa Cruz Biotechnology), Rabbit α -actin (Sigma) and Goat α -rabbit IgG, HRP-conjugated (Pierce). Remaining samples were stored at -80 °C. Twelve days later western blot procedures were repeated, utilizing those samples taken from cells transfected with 2 μ g of plasmid.

Slide Preparations

After Western blot analysis showed the presence of the fusion protein, confocal imaging of K8/GFP was carried out. Plasmids were transfected into the HeLa cell line, which was grown on glass cover slips. The transfected cells were prepared and sealed onto microscope slides and then imaged by confocal microscopy.

HeLa cell cultures were grown to 40% confluency on glass coverslips. Following this, 1 μ g of GFP/K8-C or K8/GFP-N plasmids were transfected into separate HeLa cultures (see *Verification of Expression Product* for procedure). The cover slips were transferred to a new 12 well plate, 2 ml of 2% Paraformaldehyde were added to each well and allowed to incubate at room temperature for 15 min. Wells were washed by decanting the supernatant, adding 3 ml of PBS, incubating for 2 min and removing the PBS; this process was done twice. To-Pro 3 Iodide (1:5000 dilution) in 1 ml PBS was

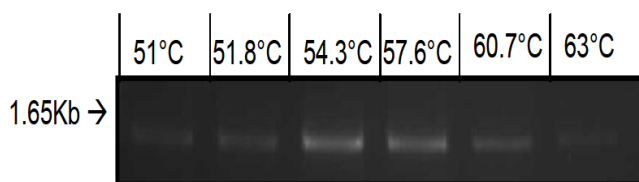
added to each well and incubated in darkness for 30 min to counter-stain DNA. The samples were kept in darkness for the rest of the preparations. Wells were washed and cover slips were mounted onto microscope slides using 10 μ l of Vectashield™ mounting solution minus DAPI (Plasmid). The cover slips were sealed using clear nail polish, and dried for 10 min. Slides were then stored at -20 °C until imaged. Imaging was carried out using the ZEISS Axiovert 100M confocal microscope and its associated software.

To evaluate K8/GFP localization at the plasma membrane, Z-stacking was performed. This technique utilizes the confocal microscope's ability to block out of focus light from the detector, therefore accurately depicting a single focal plane. Several focal planes, from the bottom of the cell to the top, are then captured and analyzed. These planes can then be processed and placed on top of each other, forming a three-dimensional image which provides better cellular structure detail.

RESULTS AND DISCUSSION

The K8 gene was cloned from a HCT-116 cell line cDNA library using PCR. This was carried out at varying annealing temperatures to find optimal conditions (high product purity and quantity). *Figure 1* shows an optimal product at 54.3 °C annealing temperature. This product was then inserted into both GFP-C and GFP-N vectors, and sent in for sequencing.

FIGURE 1: PCR OPTIMIZATION



PCR products created from addition of K8-F and K8-R primers with HCT-116 cDNA library, carried out at annealing temperatures from 51 to 63°C. Samples were loaded onto a 1% argose gel and run at 75volts for 60min. K8 sequence is 1.4Kb in length.

Sequencing results confirmed that both vectors contain copies of the K8 gene and had one silent mutation at nucleotide 680, making them 99% homologous with the sequence found in the NCBI database (*Figure 2*). Once these constructs were transfected into the cancer cell lines, HeLa and MDA-MB-231, the cytosolic protein fractions were analyzed for the presence of the desired fusion product.

FIGURE 2: SEQUENCING RESULTS FOR THE GENERATED K8 INSERT VERSUS THE K8 GENE FOUND IN THE NCBI DATABASE

```
[K8 in plasmid] ATC AAC TTC CTC AGG CAG CTG TAT GAA GAG GAG ATC CGG 699
                |||||
[K8 in NCBI]   ATC AAC TTC CTC AGG CAG CTA TAT GAA GAG GAG ATC CGG 699
```

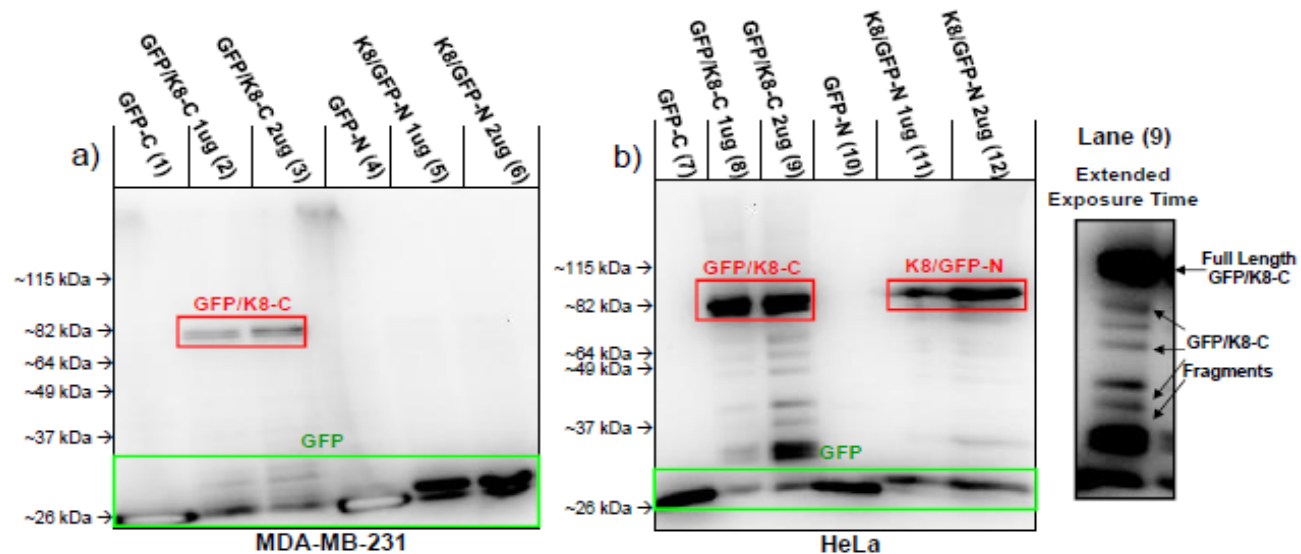
[K8 in plasmid]- CTG(DNA) → GAC(mRNA) → Asp(Amino acid translation)
[K8 in NCBI]- CTA(DNA) → GAU(mRNA) → Asp(Amino acid translation)

One silent mutation, illustrated by above codon translation, was found in both K8 inserts (K8-C and K8-N) making them 99% homologous with documented K8 sequence.

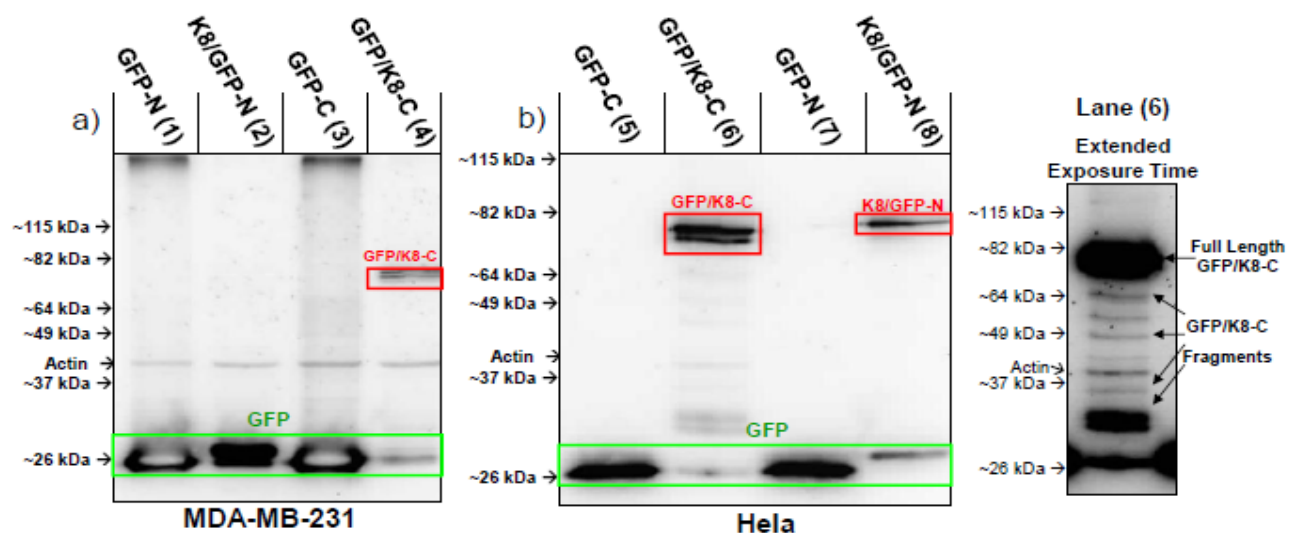
Western blot analysis showed a distinct difference in the post translational modification of K8/GFP-N and GFP/K8-C in the MDA-MB-231 cell line (*Figure 3*). The results show that these cells may be able to process K8/GFP-N more efficiently than GFP/K8-C, since K8/GFP-N samples showed little to no signal around the 80.5 kDa region. The K8/GFP was undoubtedly expressed in these cells since the fusion construct generates a single polypeptide and immunoreactivity was observed at approximately 27 kDa, the weight of GFP. Thus, the K8/GFP protein was expressed, but the K8 portion was preferentially lost due to degradation or secretion.

The results also show a difference in the post translational modification of the fusion protein in MDA-MB-231 cells compared to the HeLa. MDA-MB-231 cells may be able to proteolytically process K8 more efficiently than the HeLa cells. If HeLa had an intrinsically higher level of K18 expression, for example, this would facilitate the protection of the K8/GFP protein by promoting filament formation when the K8/GFP and K18 concentrations were approximately equal. It is well established in the literature that the formation of K8/K18 filaments helps to protect the two proteins from degradation (Gilbert, Loranger, Daigle, & Marceau 2001).

Further observations showed the presence of multiple K8/GFP fragments exclusively within HeLa cells transfected with the GFP/K8-C plasmid; such distinct fractions were not observed in the same concentration of protein sample taken from the K8/GFP-N transfected cells (*Figure 4*).

FIGURE 3: VERIFICATION OF GFP/K8-C AND K8/GFP-N EXPRESSION IN MDA-MB-231 AND HELIA WESTERN BLOT UTILIZING α -GFP.

Western blots were probed with both α -GFP and α -Actin. The GFP-K8 fusion product has an expected molecular weight of: $\sim 53.5\text{kDa}$ (K8) + $\sim 27\text{kDa}$ (GFP) = $\sim 80.5\text{kDa}$. A GFP product which has been cleaved off of K8 at $\sim 27\text{kDa}$ was observed in all the transfected cells, while no Actin bands were present at $\sim 39\text{kDa}$. Cell extracts from those transfected with the GFP plasmid were used as a GFP molecular weight marker. (a) The MDA-MB-231 cell line showed a decreased presence of GFP/K8-C compared to HeLa, potentially due to more efficient degradation/secretion of the K8 portion of the construct. (b) The HeLa cell line showed relatively high levels of expression for both GFP/K8-C and K8/GFP-N. A distinct GFP/K8-C fragmentation was also observed in lanes (8-9), while K8/GFP-N lanes (11-12) displayed little to no fragmentation bands.

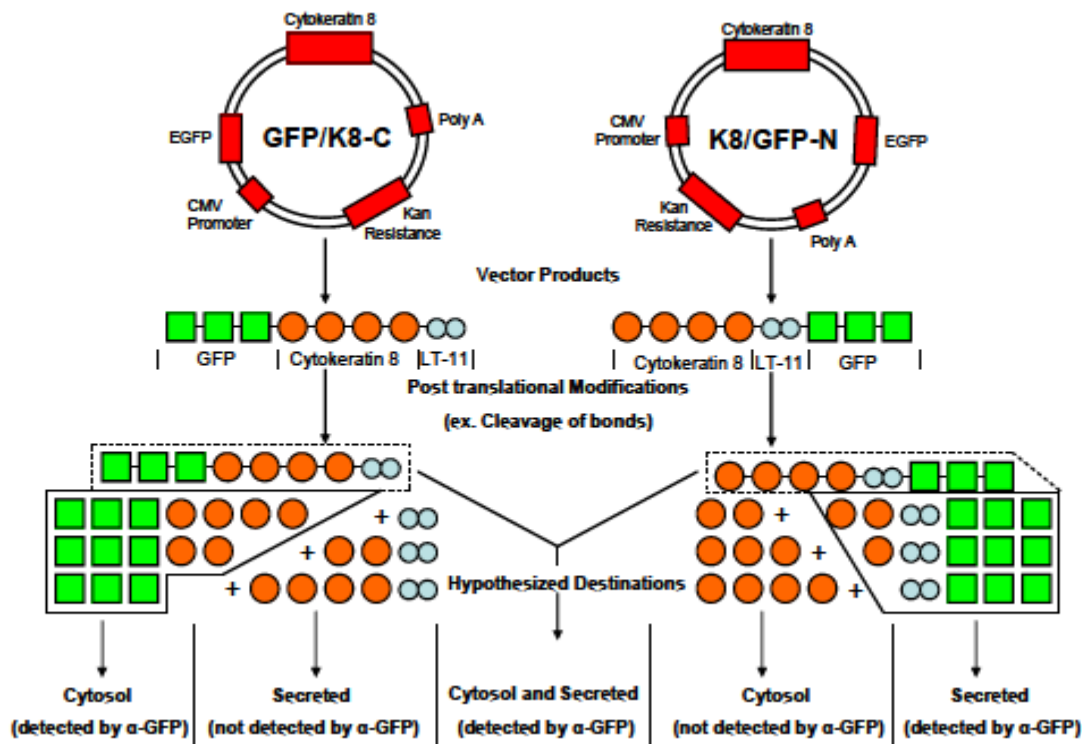
FIGURE 4: SECONDARY VERIFICATION OF GFP/K8-C AND K8/GFP-N EXPRESSION AND FRAGMENTATION

Western blot procedures were repeated after samples were frozen for two weeks to confirm previous expression results and GFP/K8-C exclusive fragmentation. All results, except for the presence of an Actin band at $\sim 39\text{kDa}$, were concurrent with the previous experiment. The GFP fusion product has a calculated molecular weight of $\sim 80.5\text{kDa}$. (a) The MDA-MB-231 cell line showed decreased GFP/K8-C presence in lane (4), no K8/GFP-N in lane (2) and the presence of an Actin band. (b) The HeLa samples continued to show both the GFP/K8-C and K8/GFP-N products, while fragmentation continued to remain exclusive to GFP/K8-C in lane (6). A distinct Actin band was not observable in the HeLa extracts, until an extended exposure of the blot was taken.

Previous literature supports the concept of extensive K8 post translational modifications, proteolytic cleavage (Ku, Zhou, Toivola & Omary 1999) and the secretion of full length K8 as well as its fragments in some forms of lung cancer (Yoko et al. 2002). Combining this information with the documented localization of K8's C-terminal epitope LT-11 at the cell surface (Gires et al. 2005) allowed for a model to be generated to help address the difference in the observed fragmentation (*Figure 5*). In this model, GFP's position in relation to K8 is important in understanding the difference in the detectable bands produced. From the literature (Gires et al. 2005; Yoko

et al. 2002) one could hypothesize that K8 is degraded and those protein fragments closest to the LT-11 epitope end up on the outside of the cell membrane, whether it be secreted or inserted. GFP/K8-C encodes for a protein with GFP at the N-terminus of K8 (furthest away from the LT-11 C-terminus epitope), producing fragments which remain in the cytosol that are detectable when probing cytosolic fractions with α -GFP. In the case of K8/GFP-N, GFP is encoded directly after the LT-11 epitope which will facilitate its export out of the cytosol, leaving only full length K8 detectable within the cell.

FIGURE 5: MODEL PREDICTING CAUSES OF FGP/K8-C'S FRAGMENTATION SIGNAL

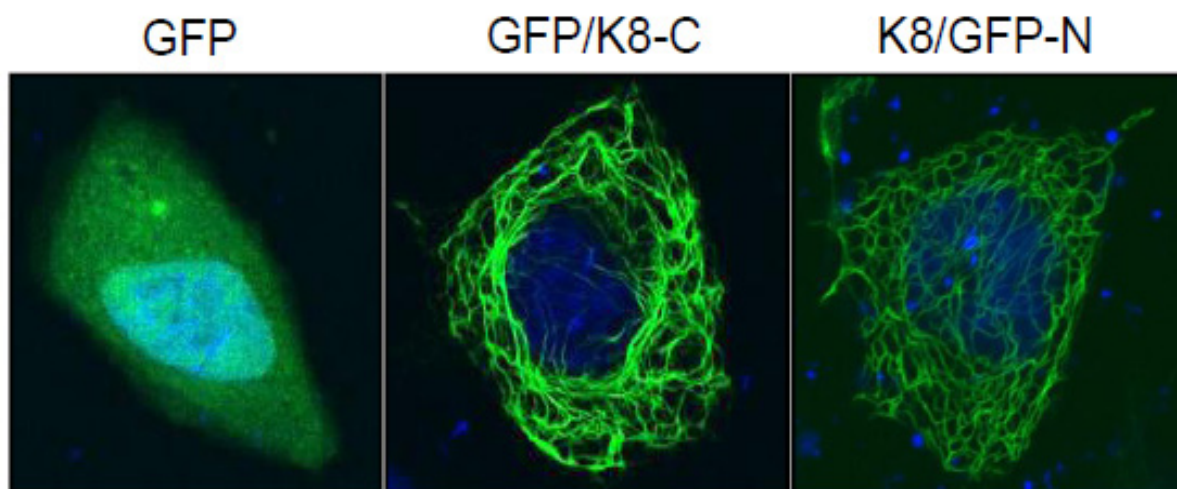


The above model shows predicted destinations of cleaved GFP/K8 fragments and detectability of the fragments using α -GFP. Those fragments that remain within the cytosol and have intact GFP should theoretically be present on a western blot of a cytosolic fraction (Figures 4 and 5).

Confocal imaging was carried out utilizing the HeLa cell line due to its relatively increased expression levels compared to the MDA-MB-231 line. These images showed distinct IF formations in HeLa cells transfected with either GFP/K8-C or K8/GFP-N, while those transfected with the original GFP plasmid showed a ubiquitous signal throughout the cell without any well-defined pattern (*Figure 6*). The ability of the fusion construct to form IF networks demonstrates that the construct is a fully functional K8 protein. Additionally, these images showed an IF structure formation within the cells transfected with the fusion protein construct, while lacking the distinct signal at the plasma

membrane one would expect from the Gires study (Gires et al. 2005). In Gires's study, anti-K8 antibodies were used to probe for the protein's presence at the plasma membrane of non-permeabilized carcinoma cells. Images produced in that study showed K8 localization universally across the entire membrane which created a shell outline of the cell. The lack of a well-defined signal at the plasma membrane by the K8/GFP protein, while exhibiting a distinct cytoskeleton structure, suggested that the extracellular projection of K8 reported by Gires et al. is an artifact of their immunohistochemical procedure (Gires et al. 2005). Since K8 is bound to GFP in our system, it can be imaged directly, thereby overcoming the limitations of the previous study.

FIGURE 6: K8/GFP DISPLAYS INTERMEDIATE FILAMENT FORMATIONS



HeLa cells were transfected with the above mentioned plasmids and their DNA was stained using To-Pro 3 Iodide. Those transfected with only GFP showed localization throughout the cell and no structural formation, while cells transfected with either GFP/K8-C K8/GFP-N showed cytosolic localization and intermediate filament formation.

Preliminary immunoprecipitation (IP) studies of media taken from HeLa cells transfected with both GFP/K8-C and K8/GFP-N utilizing α -GFP antibodies were performed over a 72 hr period, collecting media and harvesting cells every 24 hrs (data not shown). Samples were run through Western blot procedures after the IP, probing with an α -GFP antibody. A protein band immunoreactive with α -GFP antibody was observed at the anticipated 80.5 kDa mobility of K8/GFP, but no noticeable lower molecular mass fragments were observed. It is possible secreted K8/GFP fragment bands may have been obscured by the robust signal produced from the heavy chain of α -GFP IgG utilized to pull the fusion protein out of the media.

These results provide a strong indication that the protein produced by these plasmids was in fact K8 fused to GFP in a functional conformation. Within the transfected HeLa cells, a well-defined IF network incorporated products from both of the GFP/K8-C and K8/GFP-N plasmids. Also, phenotypes such as the secretion of the fusion product into the media and the aggregation of the product into K8 containing inclusion bodies (data not shown) were observed. These findings would lead us to conclude that the developed model system correlates well with what would be expected from the current K8 literature, and that K8 may not ubiquitously localize at the plasma membrane as once hypothesized (Gires et al. 2005).

FUTURE DIRECTIONS FOR THE PROJECT

Future experiments, such as secretion and localization studies, will help to provide answers to K8's role in precancerous cell transformation. In order to evaluate the model presented in *Figure 5*, secretory studies could be performed on K8/GFP-N expressing HeLa cells. First, samples of media taken from these cell cultures would be subjected to immunoprecipitation utilizing anti-GFP antibodies. Western blot analysis would then help to show if partially degraded K8 bound to GFP was being secreted or released preferentially from the K8/GFP-N expressing cells.

Other secretory studies utilizing GFP and Red Fluorescent Protein (RFP) molecular tags could be carried out by transfecting a culture of cells with K8/GFP and mixing them with cells transfected with K8/RFP. If K8/RFP protein is observed on a cell expressing K8/GFP, or K8/GFP protein on a K8/RFP expressing cell, the co-localization of the two fluorophores will

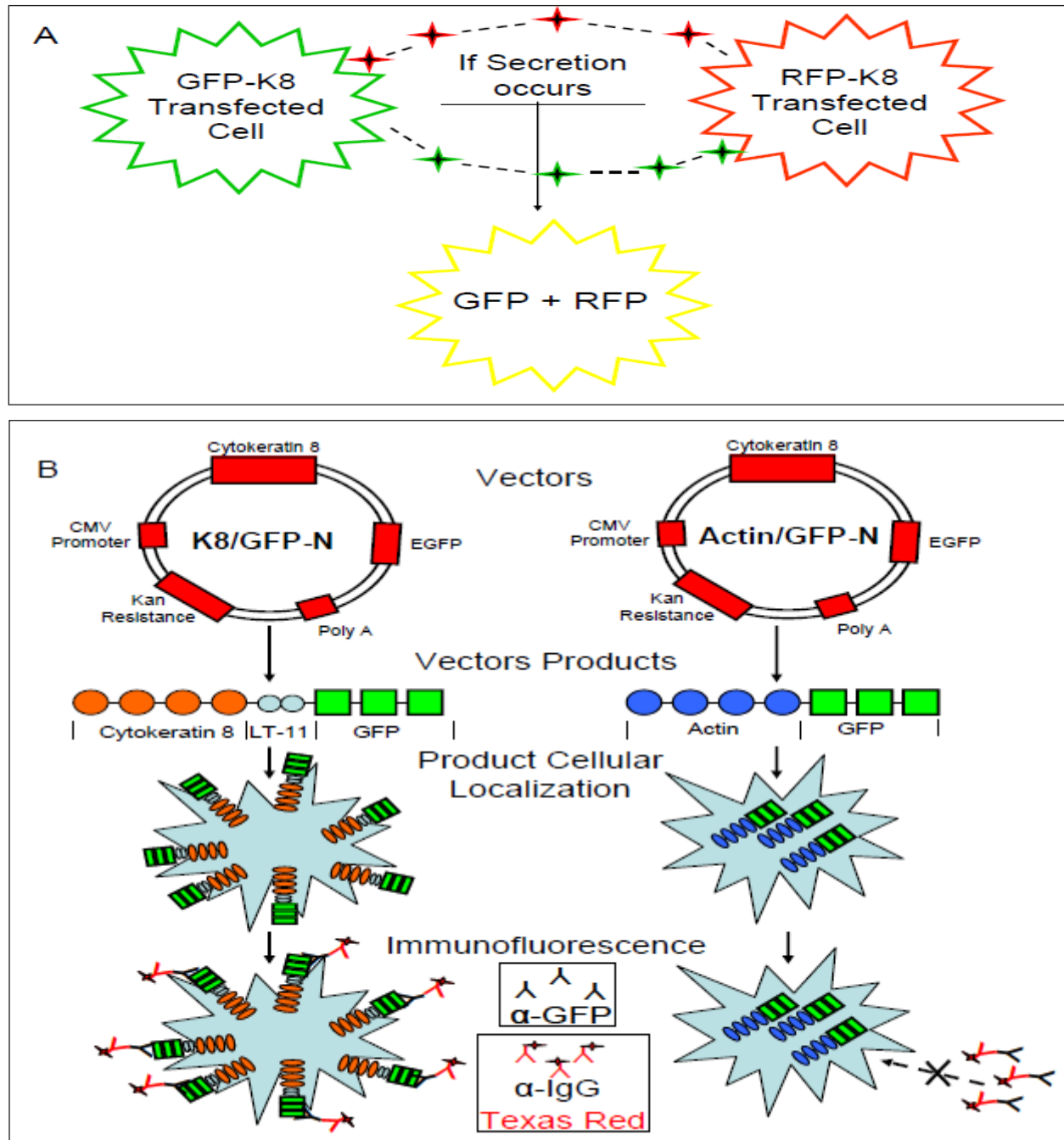
generate a yellow signal. If such a signal were observed, one could then conclude that cancer cells have the ability to secrete and attach K8 onto neighboring cells (*Figure 7A*). Similar evaluations of K8's potential to stick to the outer membrane surface could be studied by purifying the K8/GFP product and adding it at varying concentrations to non-transfected cell cultures. Those cultures could then be imaged under a confocal microscope to check for the presence of the fusion protein on the outside of non-permeabilized cells.

Localization studies could also be carried out utilizing K8/GFP transfected cells in direct comparison to Actin/GFP transfected cells, since actin is a known cytosolic protein. Non-permeabilized cells could then be probed with a primary α -GFP and a red fluorescent secondary antibody to better evaluate if the observed localization of K8 is an artifact or not (*Figure 7B*). These experiments could exploit the advantages of the immunofluorescence approach, while actively addressing criticisms to a "leaky membrane."

Finally, the current model could be utilized as an assay for the discovery of K8 filament formation inhibitors. Since K8's role is considered relatively more supportive than essential and the deconstruction of its filaments have been found to attenuate drug resistance (Lau & Chiu 2007; Wang et al. 2008; Liu et al. 2008), alleviate aggressive metastatic phenotypes (Raul et al., 2004; Gilbert, Loranger, Daigle, & Marceau 2001) and in some cases, promote proapoptotic receptors at the cell surface (Gilbert, Loranger, Daigle, & Marceau 2001), it may be considered as a potential target for cancer therapeutics. By adding various potential inhibitors and monitoring K8/K18 filament formation under a confocal microscope, the model has the potential to discover new drugs which may be useful alone or in combination with current chemotherapeutics against various forms of carcinoma.

By utilizing this model system and beginning preclinical trials of the CARE test (Thorntwaite et al. 2004), K8's true potential as a tumor marker can be elucidated. One day, such a diagnostic could be used to screen large numbers of patients for a variety of cancers during routine blood work in order to catch the disease when it is at a more manageable stage. Early cancer detection will enable old and new treatments alike to become far more effective, and allow such procedures to produce far more optimistic prognoses.

FIGURE 7: FUTURE SECRETION AND LOCALIZATION STUDIES



A K8/GFP (green) and K8/RFP (red) secretion onto neighboring cells and resulting potential signal (yellow).

B Immuno-fluorescent determination of K8's localization at the plasma membrane.

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