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Gadd45-α and Metastatic Hepatocellular Carcinoma Cell Migration

A Major Qualifying Project Report

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ABSTRACT

Gadd45-α is a tumor suppressor protein identified by microarray analysis with a reduced expression in hepatocellular carcinoma (HCC) cell lines with high migration ability. The role of Gadd45-α in HCC migration was tested by stable shRNA knockdowns in a non-metastasizing murine cell line (BL185). The migration levels of the Gadd45-α knockdowns were significantly increased relative to the parental non-metastasizing line, indicating that decreased Gadd45-α expression is sufficient to promote increased cell migration.

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BACKGROUND

Liver Cancer

Hepatocellular carcinoma (HCC) is a leading cause of cancer deaths worldwide, as 7th leading cause in Males and 9th leading cause in females (Leong and Leong, 2008). This type of carcinoma is particularly concerning due to the fairly low survival rates (only 11% survival at 5 years post diagnosis) and few treatment options (ACS, 2008). The incidence of HCC in the developed world has increased in the past two decades. The occurrence in the United States has seen a 70% increase since 1988, with half of all cases in the United States relating to hepatitis C disease (Leong and Leong, 2008). Hepatitis C and Hepatitis B (the latter for which a vaccine exists) are more likely to affect HCC rates in developing countries where vaccines or prevention may not be available. Hepatitis and liver cirrhosis are often implicated as causing, or as a comorbidity with, HCC, likely due to the damage in cells resulting in mutations. According to Leong, cirrhosis underlies 80-90% of liver cancers. Cirrhosis can be caused by prolonged alcohol abuse, hepatitis infection, or other diseases resulting in damage to the liver. Hepatitis B infection causes a 200-fold increase in risk for HCC (Leong and Leong, 2008). These comorbidities often are central to the low survival rate.

In addition to hepatitis and cirrhosis, there are not many other known causes of HCC (Leong and Leong, 2008). An increase in the incidence of HCC in males makes it 4-8 times more likely that a male will have HCC than a female. Exposure to chronic high levels of aflatoxins produced by *A. flavis* and *A. parasitans* can also increase the incidence of HCC (Leong and Leong, 2008). Beyond these known risk factors, causation is not well understood. The incidence of metastasis to sites outside the liver in HCC has been reported in greater than 50% of autopsies (Kummar and Shafi, 2003). This is of great concern because the increase in

metastasis parallels a decrease in survival. HCC commonly metastasizes to lungs, lymph nodes, adrenal glands, and bones.

The primary treatment of liver cancer is surgery to partially resect the liver, removing the tumor and leaving healthy liver to continue important body functions or performing a complete liver transplant. In cases where the liver is also otherwise damaged (i.e. by cirrhosis) or metastasis is present, resection or transplantation is not possible, greatly reducing survival rates. These patients have limited treatment options, including chemotherapy which has a generally poor response.

Tumor Cell Migration

Metastasis is the colonization of tumor cells at a site other than that of the primary tumor. Metastasis causes the majority of cancer-related deaths, and is therefore of great interest in research (Cancerquest, 2008). The ability of a tumor cell to migrate to a new part of the body, survive in that new location, and subsequently proliferate is very rare in individual tumor cells. However, at the tissue level the reason metastases are not unusual in cancer progression is that many cells will acquire the ability to move out of the tumor. Even if only a small fraction of these can go on to survive and grow new tumors, the vast numbers initially migrating and circulating in the blood make metastasis much more likely on the whole. In order for a cell to migrate it must be able to shift its cytoskeleton and have alterations in adhesion molecules on the cell surface. The cell must be able to crawl along other cells by attaching to new ones while simultaneously letting go of old ones. Once a cell can do this, it can move within the affected tissue, however in order to leave the tissue it first must be able to secrete enzymes that digest basement membranes made primarily of proteins and glycoproteins. The enzymes that digest the

basement membrane are called matrix metalloproteases (MMP). If a cell is able to secrete MMPs, then it has the opportunity to enter the blood stream or lymph system by squeezing between the cells that form the walls of the small vessels. Once in the blood or lymph, the cell circulates through the body and will often exit the circulatory system into a new location. If the cell can adapt to live and thrive in the new location, it can multiply and grow a metastatic tumor (Cancerquest, 2008). Metastatic tumors decrease survival because it is often difficult or impossible to remove all metastases by surgery or chemotherapy, leaving cancer cells to repopulate as new tumors. Metastatic tumors can also inhibit body function depending on location. For example, a large metastatic tumor in the lung would interfere with respiratory system functions in addition to any effects the original tumor has on function.

Many known biological pathways play a role in tumor cell migration and invasion phenotypes, which are important properties of metastatic cells. Of particular interest are the pathways that play a role in the Epithelial-Mesenchymal Transition (EMT). EMT is a transition in which epithelial cells lose the gene profile and phenotype of epithelial cells and gain that of mesenchymal cells. EMT is a normal process in wound healing and development, however in the context of tumor progression EMT is indicative of metastatic potential (Weinberg, 2007). Some of the signaling pathways involved in EMT are shown in **Figure 1**. The main pathways include the FGF/Ras pathway and Wnt/GSK-3β pathway for which the initiator molecules are potentially secreted by fibroblast cells located in the stroma, the EGF/Ras pathway for which the initiator molecule is potentially secreted by macrophages located in the stroma, the TGF-β pathway for which TGF-β is possibly secreted by myofibroblasts located in the stroma, and the TNF-α/NF-κB pathway for which the molecules are probably secreted by inflammatory cells in the stroma and all of which act on the epithelia tumor cells (Weinberg, 2007).

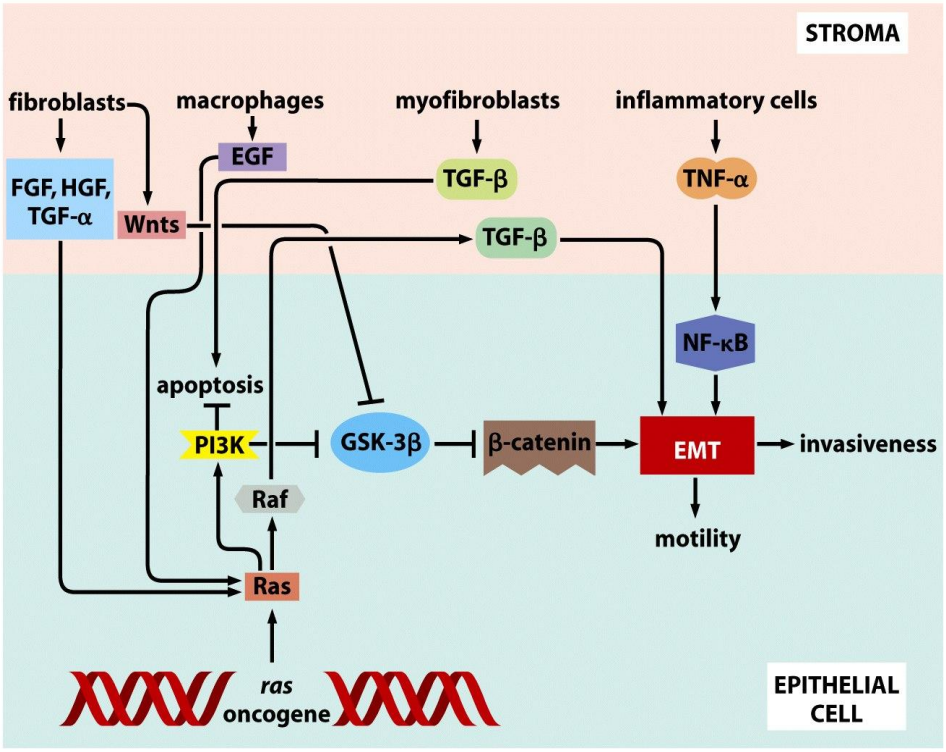

Figure 1: Signaling Pathways that Trigger EMT. The main pathways include the FGF/Ras pathway and Wnts/GSK-3β pathway which are potentially induced by fibroblast cells in the stroma, the EGF/Ras pathway which is potentially induced by macrophages in the stroma, the TGF-β pathway which is possibly induced by myofibroblasts in the stroma, and the TNF-α/NF-κB pathway which is possibly induced by inflammatory cells in the stroma (Weinberg, 2007).

Protein Gadd45-α

A protein termed Growth Arrest and DNA-Damage 45-Alpha (Gadd45-α) is a nuclear protein important to genomic stability, DNA repair, and suppression of cell growth (Gramantieri et al., 2005). The gene is located on chromosome 1 in humans, and on chromosome 6 in mice (NCBI, 2010). The gene includes 4 exons, and encodes the Gadd45-α protein which has 165 amino acid residues and is 18 kDa (GeneCards, 2009). The 3D structure of the Gadd45-α protein is shown in **Figure 2** (Jmol, 2009), and includes five alpha helices and a central beta sheet.

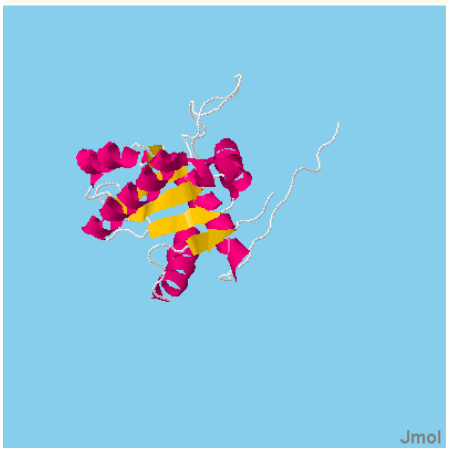

Figure 2: The Proposed 3D Structure of Protein Gadd45-α. Red coils represent alpha helix structures and yellow arrows denote the beta sheet area. (Jmol, 2009)

Gadd45-α expression is mediated by p53-independent and –dependent pathways (GeneCards, 2009). Gadd45-α is often activated in response to DNA-damage and plays a role in cell cycle regulation. If DNA damage has occurred, Gadd45-α can inhibit a cell's entry into S-phase preventing the cell from completing mitosis (Gramantieri et al., 2005). Gadd45-α also plays a role in activating the G2 check point (Gramantieri et al., 2005), which indicates further roles in controlling cell cycle progression. These known cell cycle regulation properties of Gadd45-α implicate the protein in the induction of cell cycle arrest and the reduction of cell growth (Gramantieri et al., 2005). These functions point to Gadd45-α as a known important player in tumor suppression (Hildesheim et al., 2004).

Potential Role of Gadd45 in HCC

The role of Gadd45-α in HCC has been investigated in regard to the relationship between cirrhosis and HCC, and DNA repair and cell proliferation (Gramantieri et al., 2005). The investigation reported that DNA repair using Gadd45-α is likely ongoing in HCC, and that 76.9% of HCCs show a down-regulation of Gadd45-α mRNA compared with cirrhotic tissue

(Gramantieri et al., 2005). Unfortunately, a correlation between mRNA levels and protein levels was not made, due to what the authors state is an inability of Gadd45-α protein to be detected when it is actively participating in DNA repair (Gramantieri et al., 2005). According to the GeneCards entry (GeneCards, 2009) on Gadd45-α, its levels in normal liver in comparison to liver cancer are similar, illuminating the mostly unknown role of Gadd45-α in HCC (GeneCards, 2009). However, there may be in fact an up-regulation of Gadd45-α in liver cirrhosis when compared to normal liver, leading to what Gramantieri et al. report as a down-regulation of Gadd45-α in HCC when compared to cirrhosis, while GeneCards reports no change between normal liver and HCC, but the distinction is not yet clear.

Potential Role of Gadd45 in Cell Migration

In addition to its role as a tumor suppressor blocking the cell cycle and cell growth, Gadd45-α has also been implicated in inhibiting cell migration and the invasion of keratinocytes (Hildesheim et al.,2004). It was shown that murine Gadd45-α-null keratinocytes migrate 1.7-fold faster *in vitro* than wild type keratinocytes, indicating Gadd45-α may play an important role in a cell migration pathway. This is further supported by the inhibition in migration when Gadd45-α is over-expressed in the Gadd45-α-null keratinocytes. Additionally, Hildesheim et al. demonstrate that Gadd45-α-null keratinocytes express higher levels and activities of some types of MMPs (2004). Thus, Gadd45-α may play a role in the suppression of MMPs (to inhibit EMT as previously mentioned). This paper also implicates Gadd45-α in a cell migration pathway which regulates β-catenin (as seen in the brown box in **Figure 1**) and therefore EMT. The proposed cell migration pathway by Hildesheim et al. is shown in **Figure 3**, and proposes that Gadd45-α activates GSK3β of the wnt pathway to block β-catenin, preventing β-catenin from

promoting MMP expression (2004). Although Gadd45 may play a role in inhibiting the invasion of keratinocytes, its potential role in inhibiting HCC migration is currently unknown.

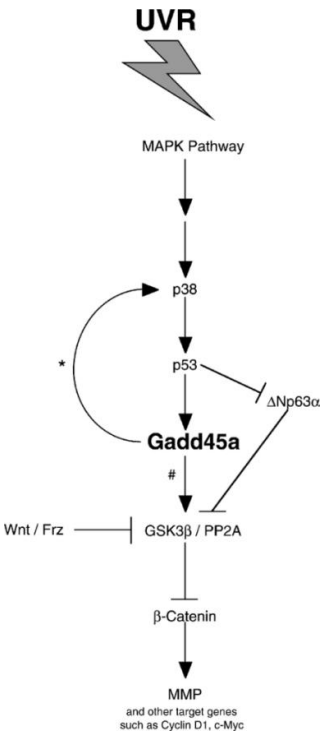

Figure 3: Proposed Role of Gadd45-α in a Cell Migration Pathway. Gadd45-α antagonizes the wnt pathway to block GSK3β, increasing the blockage of β-catenin, to decrease the activity of metastasis promoting MMPs (Hildesheim et al., 2004).

Lewis Lab HCC Research

The study of HCC tumor cell migration is important to enhance HCC survival rates. Previously, members of Dr. Brian Lewis' lab at the University of Massachusetts Medical School (Worcester) described a cell line derived from HCC, induced by PyMT in the liver of a p53-null mouse. This BL185 cell line is derived from a non-metastatic tumor, and was selected for two subpopulations of cells that had significantly higher (10-fold) *in vitro* migration and invasion activities. The higher migrating and invading subpopulations are called BL185-M1, and BL185-

I1. A gene expression microarray assay was performed on these cell lines which showed expression differences (greater than a 2-fold change) in 313 genes between the original BL185 line and either BL185-M1 or BL185-I1 (Lewis, unpublished data). One gene identified by the microarray analysis as having a 20.8-fold down-regulation ($p=0.006$) in the BL185-M1 subpopulation, and a 12.4-fold down regulation ($p=0.0135$) in the BL185-I1 subpopulation is Gadd45-α. Due to its previously described roles in keratinocyte cell migration in other carcinomas (discussed previously), this gene is of interest to the Lewis lab as a potential regulator of HCC cell migration and metastasis.

PROJECT PURPOSE

Gadd45-α has been shown to inhibit keratinocyte cell migration, but its potential role in inhibiting HCC cell migration has not been demonstrated. The expression of Gadd45-α is down-regulated in about 76.9% of HCCs compared with cirrhotic tissue (Gramantieri et al., 2005), but that study did not address cell migration. The hypothesis of this project is that Gadd45-α is important to cell migration in HCC. This will be investigated by creating stable shRNA knockdown of Gadd45-α in the non-metastatic BL185 cell line to discover whether a lack of Gadd45-α is sufficient to induce cell migration.

METHODS

Migration Assay

5 x 10^4 cells/ml in 500 µl DMEM was added to BD BioCoat Control inserts. These inserts were in individual wells and are part of a 24-well plate. 750 µl of DMEM supplemented with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (p/s) was added to each well. After 20-24 hour incubation, the media was removed from the inserts, and wells and the cells were fixed with methanol, stained with Gimesa reagent, and the inner side of the inserts was swabbed to remove non-migrating cells. The membranes of the inserts were removed and slides were created for counting under 100X magnification. The samples were performed in duplicate or triplicate for each assay repetition.

Cell Culture

All cells were incubated at 37°C and 5% CO_2 in DMEM supplemented with 10% FBS and 1% p/s. Additionally, the BL185-clone (1,2,3,4,5,GFP) cells had 8 µg/ml puromycin added to the media.

Isolation of Cell Lysates for Western Blot

After pelleting the cells, the pellet was re-suspended in 100-300 µl of RIPA buffer, and incubated on ice for 30 minutes. After the incubation, all samples were centrifuged to pellet the lipids leaving the protein in the supernatant, which was saved. Total protein concentration was determined using the Bradford assay.

Western Blot

Cell lysate was added to 4x sample buffer and incubated at 100°C for 10 minutes. These samples were then run on a 12% acrylamide gel. The protein was then transferred to a PVDF transfer membrane from GE Healthcare using wet transfer for 1 hour at 100 Volts and 4°C. The membrane was blocked using 5% dry non-fat milk in 20ml TBS-T at 4°C overnight with shaking. Primary antibody (**Table 1**) was then added at a concentration of 1:500 for Gadd45-α and 1:1000 for Actin in 5% dry non-fat milk in 10 ml of TBS-T. This was incubated for 1 hour at room temperature with shaking. The membrane was then washed 3 times for 10 minutes each with TBS-T. Secondary antibody (Anti-rabbit IgG antibody from Cell Signaling) was then added to the membrane at a concentration of 1:5000 in 10 ml of 5% dry non-fat milk in TBS-T. This was incubated for 1 hour at room temperature with shaking. The membrane was then washed 3 times for 20 minutes each with TBS-T at room temperature with shaking. SuperSignal West Femto Maximum Sensitivity Substrate from Thermo Scientific was then added to the membrane for 30 seconds. The membrane was then developed in a dark room using Kodak Scientific Imaging Film. The developing time was about 1 minute.

Protein Probed for	Primary Antibody
Gadd45-α	GADD 45α (H-165) Rabbit polyclonal IgG from Santa Cruz Biotechnology
Actin	Actin (C-11)-R Rabbit polyclonal IgG from Santa Cruz Biotechnology

Table 1: Primary Antibodies used for Western Blot

Proliferation Assay

The proliferation assay was performed by adding 1000cells/well in 100 µl DMEM with 10% FBS and 1% p/s to the wells of a collagen-coated 96-well plate. For each sample at each

time point triplicate wells were assayed. For each time point the media was removed and 50 µl DMEM supplemented with 10% FBS and 1% p/s was added along with 10 µl MTS reagent, then samples were incubated for 30 minutes at 37°C and 5% CO_2. The absorbance of the wells of interest was then obtained using a plate reading spectrophotometer set to 490 nm.

Soft Agar Assay

A hard agar base was established by adding 7 ml of 1.4% agar with 2x DMEM to a 10-cm plate. Upon hardening a 3 ml soft agar layer was added containing 0.8% agar, 2x DMEM, and $1X10^5$ cells to each plate. After this layer hardened 8 ml 1x DMEM was added to the plate and incubated for 3 weeks at 37°C and 5% CO_2. After incubation, colonies in 10 fields per plate were counted at 10X magnification using size exclusion. For each sample two plates were prepared and counted.

shRNA Infection

Five shRNA constructs obtained from Open Biosystems were used in addition to a GFP plasmid. The shRNA constructs correspond to the identification numbers as outlined in **Table 2**.

Construct (Clone) number	Identification number
1	TRCN0000054688
2	TRCN0000054689
3	TRCN0000054690
4	TRCN0000054691
5	TRCN0000054691

Table 2: Open Biosystems Identification Numbers for shRNA constructs

Once the plasmids containing the shRNAs were isolated from bacteria they were each added to a mix of pMPG, pCMV, effectene transfection reagent and enhancer. These mixes were then added to 293T cells, and incubated at 37°C and 5% CO_2 overnight. The media was then changed, and the cells incubated for 48-72 hours, after which the retroviral supernatant was harvested and sterilized. The sterilized supernatant with polybrene was added to the target (BL185) cells and incubated at 37°C and 5% CO_2 for 5 hours. After this incubation period, the supernatant was removed and normal media was added and incubated as usual. Puromycin was added to the media after the first day incubation. The cells were thereafter referred to as BL185-Clone 1, BL185-Clone 2, BL185-Clone 3, BL185-Clone 4, BL185-Clone 5, BL185-Clone GFP.

Quantitative Real Time-Polymerase Chain Reaction

Total cellular RNA was isolated from cultured cells using triozol, chloroform, and finally isopropanol. The RNA was purified using RNeasy Qiagen kit and then the TURBO DNA-free protocol. The RNA was then converted into first strand cDNA using superscript III First-Strand Synthesis System for RT-PCR. This cDNA was then used for Quantitative Real Time-PCR using TaqMan Universal PCR Master Mix from Applied Biosystems, Mouse ACTB primer from Applied Biosystems (Actin) or the qRT-PCR kit for Gadd45-α from Applied Biosytems (Gadd45-α) and 10 ng/µl of cDNA. The prepared samples (in triplicate) were then analyzed on a 7300 Real Time PCR System machine with matching software from Applied Biosystems. The PCR cycle was as indicated in **Table 3**. Data collection occurred at stage 3, step 2.

Stage/step	Stage 1	Stage 2	Stage 3/step 1	Stage 3/Step 2
Number of Repeats	1	1	40 (for stage 3)	(40 for all of stage 3)
Temperature	50°C	95°C	95°C	60°C
Length	2 minutes	10 minutes	15 Seconds	1 minute

Table 3: qRT-PCR Conditions

RESULTS

The purpose of this project was to determine if Gadd45-α levels in a hepatocellular carcinoma (HCC) cell line effect the migratory tendency of those cells. In order to explore this question, a HCC cell line lacking Gadd45-α needed to be created. This was done through stable shRNA knockdown using the shRNAs indicated in the methodology. The knockdown was verified using qRT-PCR in comparison to the GFP-infected BL185-Clone GFP cell line. **Figure 4** is a representative graph of the qRT-PCR for BL185-Clone 2 and BL185-Clone 3 compared to BL185-Clone GFP, showing reduced levels of the Gadd45-α mRNA in both shRNA-infected cell lines. The qRT-PCR was also run for BL185-M1 and BL185-I1 cell lines, providing confirmation of microarray data in **Table 4**.

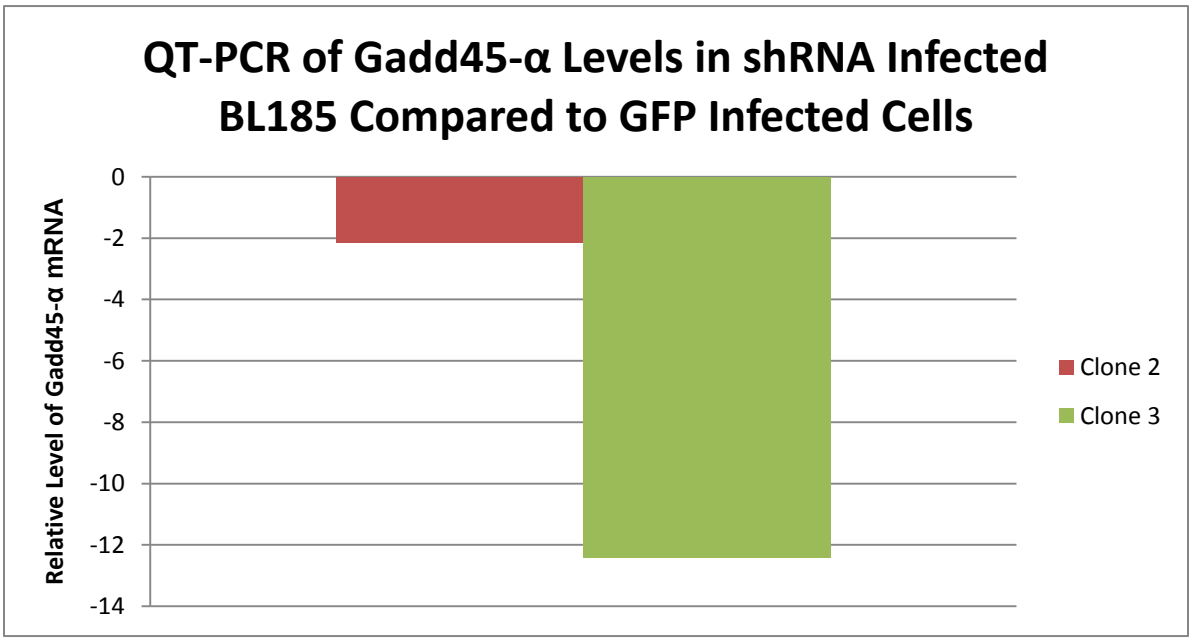

Figure 4: qRT-PCR of Gadd45-α Levels in shRNA Cells Compared with BL185-Clone GFP. Representative data of qRT-PCR of Gadd45-α mRNA levels for BL185-Clone 2 and BL185-Clone 3 shown as a fold change relative to BL185-GFP.

Cells compared to BL185	Micro Array (fold change)	P-Value for MicroArray	qRT-PCR (fold change)
BL185-M1	-20.8119	0.006	-1.587
BL185-I1	-12.4344	0.0135	-3.603

Table 4: Microarray and qRT-PCR Confirmation of microarray data for Gadd45-α mRNA levels for the high migrating cell lines BL185-M1 and BL185-I1.

In order to confirm knockdown of Gadd45-α protein, a western blot was performed. As seen in **Figure** 5, the level of Gadd45-α is greatly decreased in the shRNA knockdowns BL185-Clone 2 and –Clone 3 in comparison to the BL185-Clone GFP. The actin levels for these samples are fairly similar as well, ensuing equal amounts of sample were loaded in each lane. The western blot confirmation of knockdown is important because some shRNAs do not degrade mRNA, but rather bind to it preventing translation of the protein and effectively inhibiting the function. In this case, the qRT-PCR and western blot data together confirm that the targeted shRNAs reduce Gadd45-α levels.

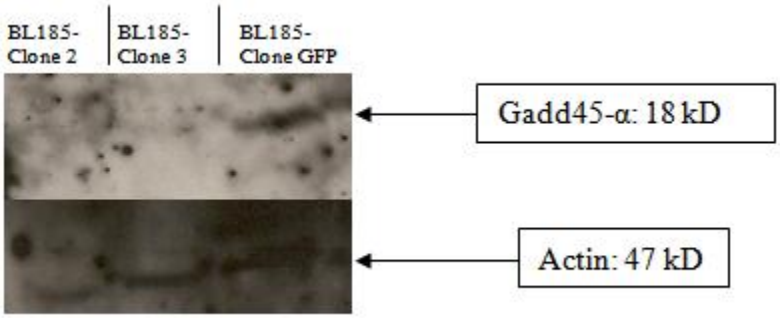

Figure 5: Gadd45-α Western Blot.

Upon confirmation of knockdown in the BL185-Clone 2 and BL185-Clone 3 cell lines, the characteristics of these cell lines in comparison to the parental BL185, BL185-M1 (high migrating), BL185-I1 (high migrating), and BL185-Clone GFP (control) cells was of interest. A soft agar assay was performed to determine the degree of transformation of the cells. Cancer

cells are typically considered to have a relatively high transformation rate, but additional transformation is possible. **Figure 6** shows that the level of transformation of the cell lines is similar. However, the BL185-I1 cell line (3rd histobar) has significantly ($p=0.01$) less colonies than the other cell lines. This assay was only preformed once, so an explanation of this anomaly is not available. Overall these data suggest that reduction of Gadd45-α levels does not have a significant effect on transformation in the established HCC cell lines.

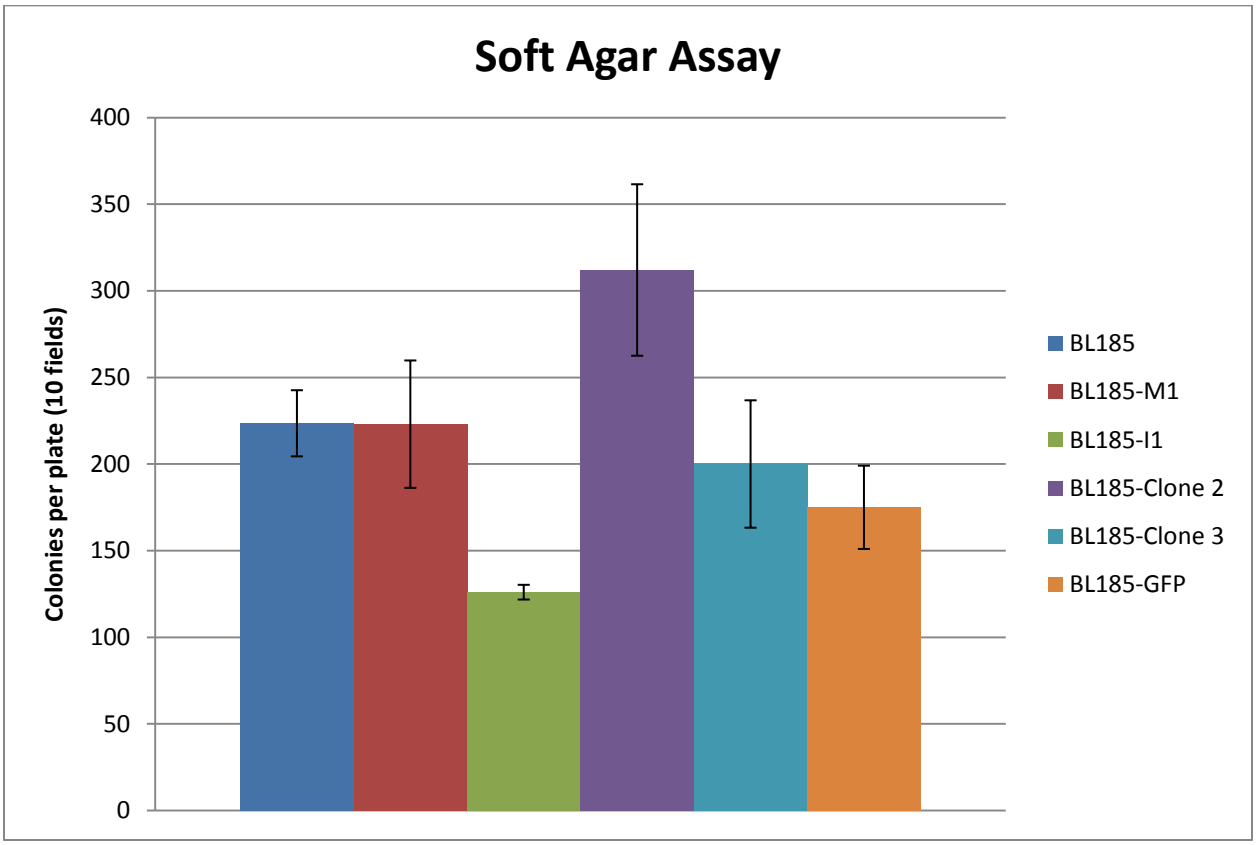

Figure 6: Soft Agar Assay. The number of transformed colonies (Y-axis) is fairly consistent between all cell lines for one trial of this assay

In order to compare the rate of growth for each cell line, a cell proliferation assay was performed (

Figure 7). Proliferation is important for better understanding the effect that reduction of Gadd45-α has on the cells ability to divide. This is of particular interest because of the role Gadd45-α is

known to play in the activation of cell cycle checkpoints and therefore prevention of proliferation. The rate of cell division was found to be similar at 24 hours for each cell line, but diverges after 48-120 hours. The proliferation assay revealed similar growth rates for BL185-Clone 2, BL185-Clone 3, and BL185-GFP after 24 hours (P=0.4). This is of relevance for the migration assay, which was incubated for 24 hours. The cell division differences between cell lines increases after 24 hours until becoming similar at about 5 days (upon confluence). The most striking difference is an increased proliferation observed for shRNA BL185-Clone 3, potentially relating to the additional role of Gadd45-α in preventing proliferation. This assay was performed twice for the 24, 48, and 72 hour time points.

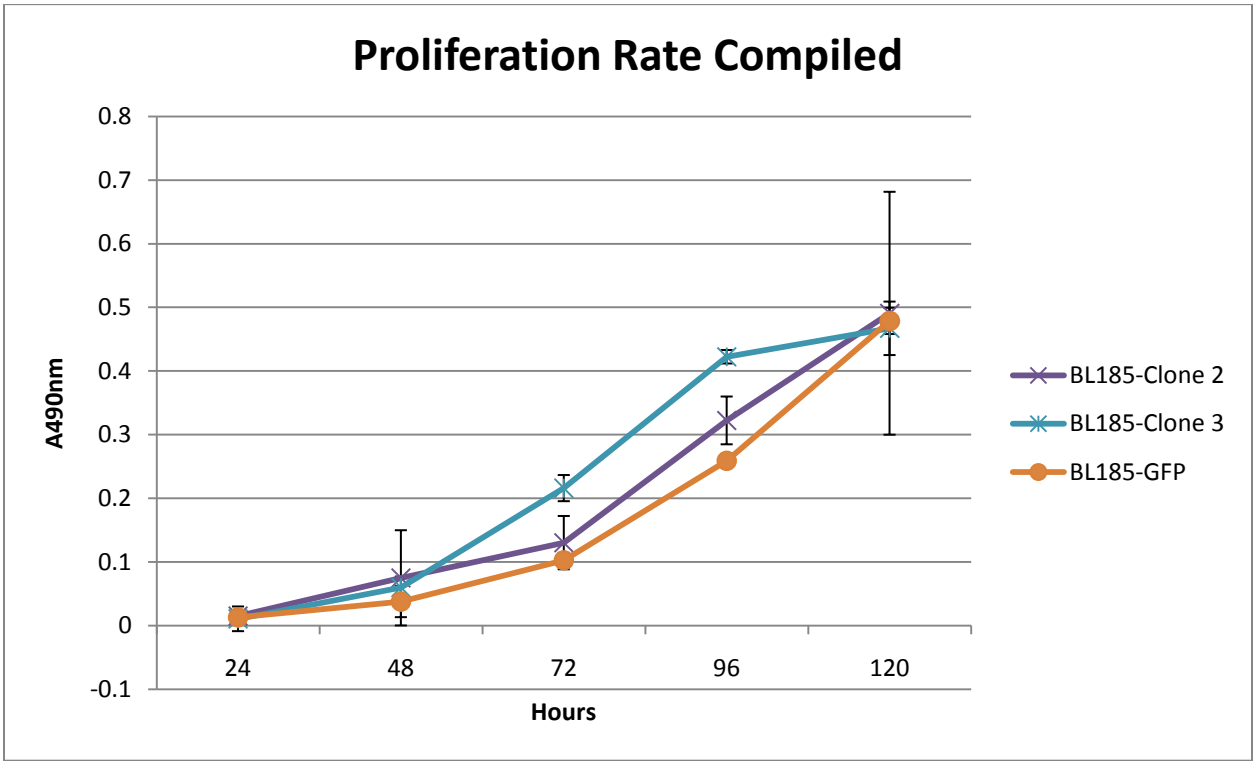

Figure 7: Proliferation Assay. The cell lines at 24 hours have the same growth rate, but diverge after 24 hours before converging again after 120 hours when confluence occurs. The assay was performed twice at the 24, 48, and 72 hr time points. Error bars denote one standard deviation.

Finally, with the characterization of the knockdown cell lines complete, migration assays were performed to assess the effect of Gadd45-α knockdown on migration. Migration is an important characteristic of metastasis of tumor cells, and is therefore often used as an indicator for metastasis *in vitro*.

Figure 8 shows a graph of a single assay which is representative of the overall trend. This figure shows that both shRNA clones (BL185-Clone 2 and BL185-Clone 3) have increased migration over control BL185-GFP. When all repetitions of this assay were compiled, the results show a p-value of 0.05 for BL185-Clone 2, and 0.025 for BL185-Clone 3. This indicates that the increase in migration of the Gadd45-α knockdowns over the GFP infection is significant.

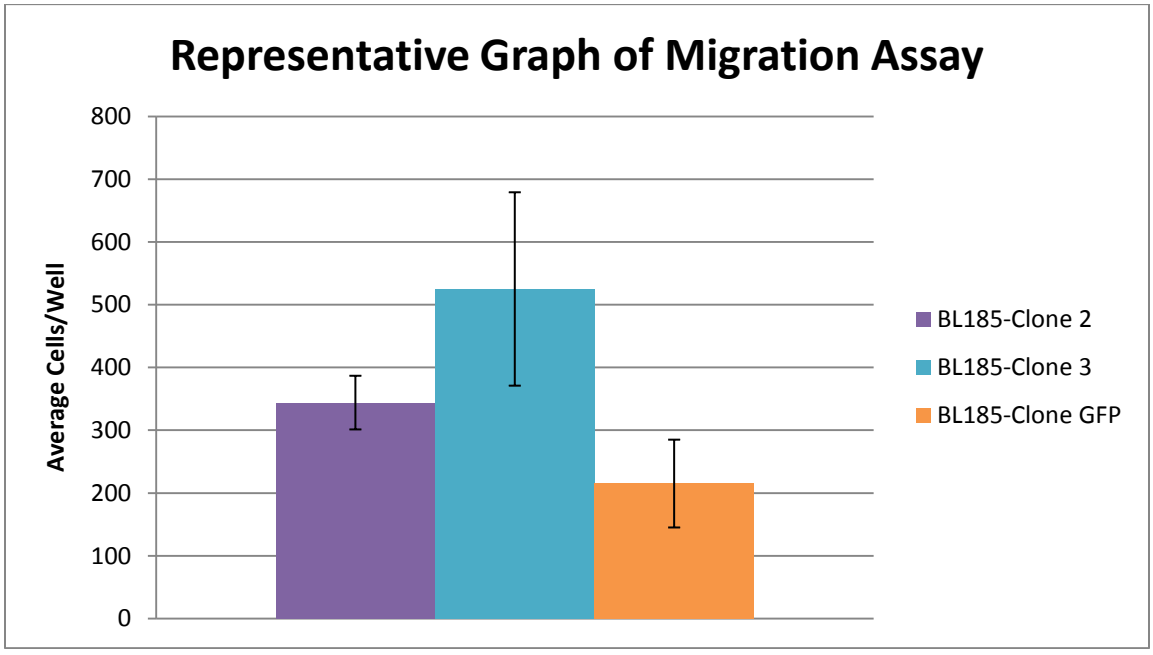

Figure 8: Representative Graph of the Cell Migration Assay. Representative data showing the trend observed in which Gadd45-α knockdown clones BL185-Clone 2 and BL185-Clone 3 have a significant increase in cell migration compared to control BL185-Clone GFP. Error bars denote one standard deviation.

DISCUSSION

This project investigated whether a reduction of Gadd45-α levels is sufficient to cause an increase in cell migration in HCC cells. Gadd45-α was chosen as target for knockdown after an analysis of the previously mentioned microarray data indicated a significant down-expression of Gadd45-α in migrating HCC cells. Gadd45-α was also of interest because of its known role in regulating cell cycle checkpoints and DNA damage. The project results revealed that decreasing Gadd45-α is sufficient to increase cell migration in HCC murine cell lines. The cell lines with Gadd45-α shRNA stable knockdown showed migration levels similar to the high migrating cell lines BL185-M1 and BL185-I1, and showed significantly more migration than control BL185-Clone GFP. The knockdown cell lines had the same level of proliferation at 24 hours as the BL185-Clone GFP cell line, which shows that increased migration is not an artifact caused by an increase in cell division over the 24 hour incubation period of the migration assay. This helps to confirm the significance of the higher migration in the knockdown cell lines, which had p values of 0.05 (BL185-Clone 2) and 0.025 (BL185-Clone 3) when compared with the control clone expressing GFP. These data provide similar results to the paper by Hildesheim et al. (2004) which suggested the potential cell migration pathway shown in the Background in **Figure 3**. In this pathway, Gadd45-α antagonizes wnts preventing the blockage of GSK3β, which in turn blocks β-catenin from activating MMPs and other migration-promoting genes. The data from this project suggest that decreasing Gadd45-α is sufficient to increase migration in HCC cells. The potentially important role in migration suggested by the data poses the possibility that Gadd45-α also plays an important role in metastasis of HCC. This is especially important because resection or transplantation of the liver to cure HCC is prevented when metastases are present. Increasing

the understanding of the mechanisms underlying metastasis in HCC is essential for the future prevention of metastasis.

The data in this project also reveal the important role that Gadd45-α plays in cell proliferation. After 48 hours, one of the knockdown cell lines (BL185-Clone 3) had increased proliferation in comparison to the GFP control. This shows that Gadd45-α is an important negative regulator of the cell cycle. Because this increase in proliferation does not occur until after 24 hours (the length of a migration assay) it seems unlikely that the regulation of migration by Gadd45-α is due to its known role in cell cycle regulation. More likely Gadd45-α is participating in another pathway such as the one suggested in **Figure 3**.

An interesting future investigation may be to over-express Gadd45-α in the migrating cell lines (BL185-M1, BL185-I1) to discover if an increased cellular level of Gadd45-α is sufficient to prevent cell migration. This approach has been started with attempts to clone the Gadd45-α gene and insert it into an appropriate plasmid vector (pBABE-puro) for future infection. Unfortunately this process has been difficult and has yet to be completed.

An additional assay which might provide interesting information would be an invasion assay for the knockdown cell lines. The pathway proposed by Hildesheim et al. (2004) (**Figure 3**) results in promotion of Matrix Metalloproteinases (MMPs) which are known to degrade extracellular matrix proteins. Degradation of extracellular matrix proteins is a recognized property important in invasion ability, so the invasion assay may provide additional insight regarding the potential mechanism through which a reduction of HCC may facilitate metastasis of HCC cells. Additional assays may be performed to determine the levels of other proteins in the proposed pathways in the knockdown or to create over-expression cell lines to provide insight into the validity of the proposed pathway.

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