Children's Hospital of Pittsburgh

Annual Progress Report: 2009 Formula Grant

Reporting Period

July 1, 2010 – June 30, 2011

Formula Grant Overview

The Children's Hospital of Pittsburgh received $1,039,905 in formula funds for the grant award period January 1, 2010 through December 31, 2013. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

New Reporters of Protein Conformation and Compartment Composition – The purpose of this project is to develop new methods for the assessment of protein location and compartment composition that are necessary to determine the mechanism of human diseases and to develop therapeutic options for their treatment. These methods will be developed initially for the common genetic disease, cystic fibrosis (CF). These expressible reporters permit the detection of expressed proteins at exceptional signal-to-noise. Expressed tags that recognize a variety of fluorogens will permit also the determination of compartment composition, permitting functional anomalies attributed to CF can be assessed in primary airway cell cultures. These methods will be valuable for mechanistic evaluation and drug discovery opportunities for CF and a variety of other human diseases that affect cellular protein handling or localization.

Anticipated Duration of Project

1/1/2010 - 12/31/2013

Project Overview

New reporters of protein conformation and compartment composition are needed for a mechanistic understanding of human diseases and for the discovery of therapeutics. The development of fluorogenic probes and genetically encoded fluorogen-activated protein (FAP) tags, whose interaction generates intense fluorescence signals with minimal background will permit evaluation of membrane trafficking mechanisms, interactions between proteins, and evaluation of the compositional properties of near-membrane compartments. Our goal is to develop and optimize this approach for the low abundance integral membrane protein, CFTR. Mutations in the CFTR gene cause cystic fibrosis, a common, life-limiting genetic disease. The gene encodes an anion channel that regulates the volume and composition of the thin film of airway surface liquid (ASL) in the lungs. The most common CFTR mutant omits phenylalanine at position 508 (ΔF508), inducing protein misfolding and ER associated degradation (ERAD). Experimental conditions can rescue a small fraction of ΔF508 CFTR to the plasma membrane

(PM) where it is partly functional, but mutant protein rescued in this manner remains unstable and is rapidly cleared by peripheral quality control mechanisms. To date, tools have not been available to quantify CFTR density at the cell surface, or to define protein folding in cellular compartments. The development of these tools is vital to our understanding of the mechanisms that retain and degrade CFTR at the ER and PM, and to provide methods for implementing high throughput/high content screening for therapeutics. Furthermore, a key factor in CF lung disease is the buildup of thick mucins that fail to hydrate into appropriate mucin-gel networks due to the acidic ASL in CF airways. Therefore, defining the microenvironment pH, in conjunction with assessment of CFTR delivery and residence time at the PM, is also an issue of critical significance in assessing the efficacy of therapeutics. This proposal will build truly novel fluorescent probes that address each of these fundamental questions, by defining a) protein trafficking to the cell surface, b) protein interactions and colocalization, c) local calcium concentrations, and d) pH within the cell compartments and at the plasma membrane. These new, genetically encoded imaging probes will be of utility for a wide array of other relevant scientific questions in biomedicine beyond the focus area of cystic fibrosis in this proposal.

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Expected Research Outcomes and Benefits

This project will provide new probes of protein localization and sensors of the properties of localized compartments to examine physiological functions at the level of specific proteins. These tools will provide quantitative probes for protein expression, conformation and compartment composition in a broad range of biological applications. They are needed to provide information of basic disease mechanisms and they will also provide assays that can be used for the development of drug screens and the evaluation of new therapeutics. Among these applications are the screening of small molecules for their ability to promote the plasma membrane trafficking of the protein that is defective in the genetic disease, cystic fibrosis. Success in this endeavor will set the stage for the use of this technology in other diseases of protein folding, the list of which grows each year and includes a variety of neurodegenerative diseases. These fluorgenic biosensors will be produced in cell permeant and cell-impermeant forms, which will permit the assessment of cell surface and total protein in the same experiment on the same cells. CF has served as a paradigm for a variety of human diseases of protein conformation that impair folding or result in protein mis-localization or accumulation or

degradation. This new technology therefore has the potential to have a major impact on human health.

Summary of Research Completed

Our goals during this one-year funding period were to provide further proof of concept studies that would validate the generation of fluorogen activating protein (FAP) tags and their use in examining potential therapeutic approaches to correction of defects in the cystic fibrosis transmembrane conductance regulator (CFTR). The common mutation in this protein produces a defect in its folding that elicits its degradation by quality control mechanisms in the endoplasmic reticulum (ER). Accordingly, having identified sites within CFTR, during the first year of support, where the FAP fusion protein could be inserted without corrupting the cellular processing of CFTR protein or its function as a regulated anion channel, we turned toward measurements of the protein at the cell surface, in order to examine the influence of small molecules that may improve the folding of CFTR and permit its trafficking to the cell surface. Specifically, we sought to:

1. Determine whether cell impermeant mallichite green (MG) dyes and cell permeant esters of them could identify the expression of the FAP construct and correction of expression of the ΔF508 mutant CFTR on the surface membrane. Both the N-terminal FAP fusion and the extracellular loop 4 (ECL4) FAP insertion were examined in this regard.
2. The cell surface signals under control and corrector-treated conditions were quantified, and they were compared with the correction of transepithelial CFTR currents across homozygous ΔF508 human bronchial epithelial (HBE) primary cultures. This comparison would allow quantitative validation of the utility of FAP constructs in CFTR corrector discovery efforts.
3. Stable cell lines expressing WT and ΔF508 FAP-CFTRs were generated in HEK293 cells and the generation of stable airway cell lines expressing these fusions is a goal for the coming year.

During the current period, we achieved several milestones directed at validation of the FAP-CFTR constructs and their detection in live cells. We demonstrated expression of FAP-CFTR signals at the cell surface using impermeant fluorogens. Using cell permeant probes, the distribution of CFTR throughout the protein secretory and endocytic pathways was revealed. As shown in *Fig. 1,* CFTR cannot be detected by the cell impermeant MG-11p in stable cells expressing the ΔF508 FAP-CFTR (FAP at the N-terminus); however, use of the permeant MG-ester dye shows that the cells are expressing the protein, and that it is localized in a peri-nuclear distribution characteristic of the ER. ΔF508 CFTR is known to be retained in this compartment by quality control mechanisms. In the corrector (C4 + C951) treated cells, MG-11p now recognizes the mutant protein at the cell surface membrane following corrector action. MG-11p shows a clear outline of the cell perimeter in this confocal section obtained at the depth of brightest fluorescence. Note the very low background staining achieved with these fluorophores, whose fluorescence efficiency increases ~15,000 fold upon FAP binding. In other studies, we have shown that a combination of correctors can give larger responses than use of the individual compounds. Our previous progress report provided evidence that the FAP fusion could detect the trafficking of mutant CFTR to the cell surface in response to low temperature incubation.

Our next goal was to determine the relation of the detection of cell surface FAP-CFTR with corrector action to the recovery of CFTR-mediated currents across HBE monolayers derived from patients homozygous for ΔF508 CFTR. Among CF patients, the ΔF508 mutation is the most common, being present on at least one allele in more than 90% of the patient population. Accordingly, our efforts are directed at the correction of this CFTR variant. For this analysis, we utilized a larger panel of corrector compounds (http://www.cftrfolding.org/CFFTReagents.htm) and combinations of them. The results are shown in *Fig. 2*. It is evident from the overall pattern of the data that the effects of correctors are in register (i.e. similar pattern of activity) whether the measure of corrector action is anion transport across human airway cells or is cell surface labeling of the mutant FAP-CFTR. These findings are very encouraging, and they suggest that the cell labeling assay can be a useful tool to search for more efficacious correctors, a significant need in CF therapeutics development at the present time. In addition, unlike the functional assay, the cell surface assay would detect correction of CFTR trafficking even if the effective compound were also an inhibitor of CFTR current. This is a significant issue, since compounds that correct the trafficking of mis-folded proteins, such as the cardiac hERG potassium channel, were originally found due to their ability to function as channel blockers. Obviously, such compounds would not be detectable if the screening assay relied on CFTR function as a measure of correction. Our next goal is to attempt to scale up the FAP labeling assay to a larger format that would permit the screening of a compound library of significant size.

In addition, we have verified results from the first period showing that the N-terminus FAP fusion is more robustly expressed at both the protein biochemistry and the cell surface labeling levels. Thus, our future focus will be on the former fusion protein. Among the assays we plan to interrogate during the next funding period is the use of FACS to obtain a cell population assessment of corrector activity and to determine whether this approach can be used in novel corrector identification

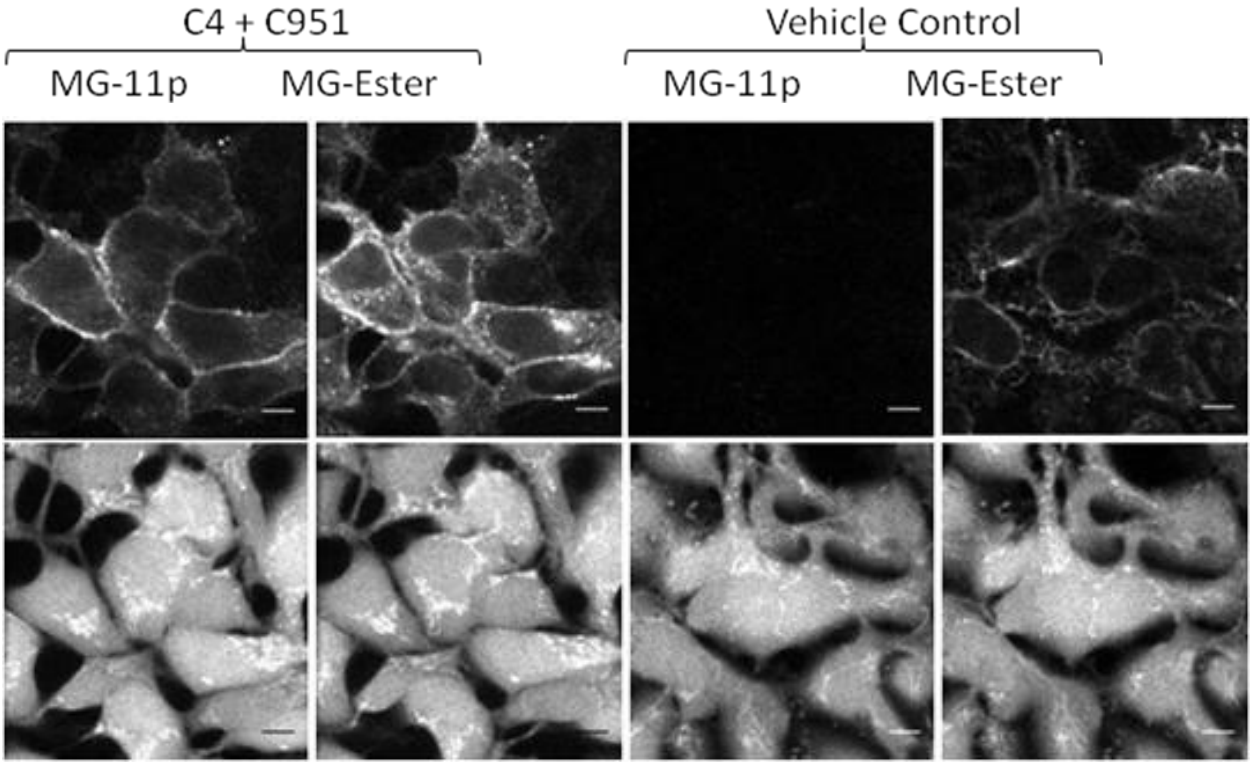

Figure 1: Stable HEK293 cells expressing ΔF508 CFTR were stained with either the cell impermeant MG-11p or the cell permeant MG-ester. Cells shown in the right panels were DMSO treated vehicle controls, while those in the left panel were treated overnight with the CFTR corrector compounds, C4 and C951 (obtained from CFFT, www.cftrfolding.org). The lower panels are stained with cell tracker to identify their location on the coverslip.

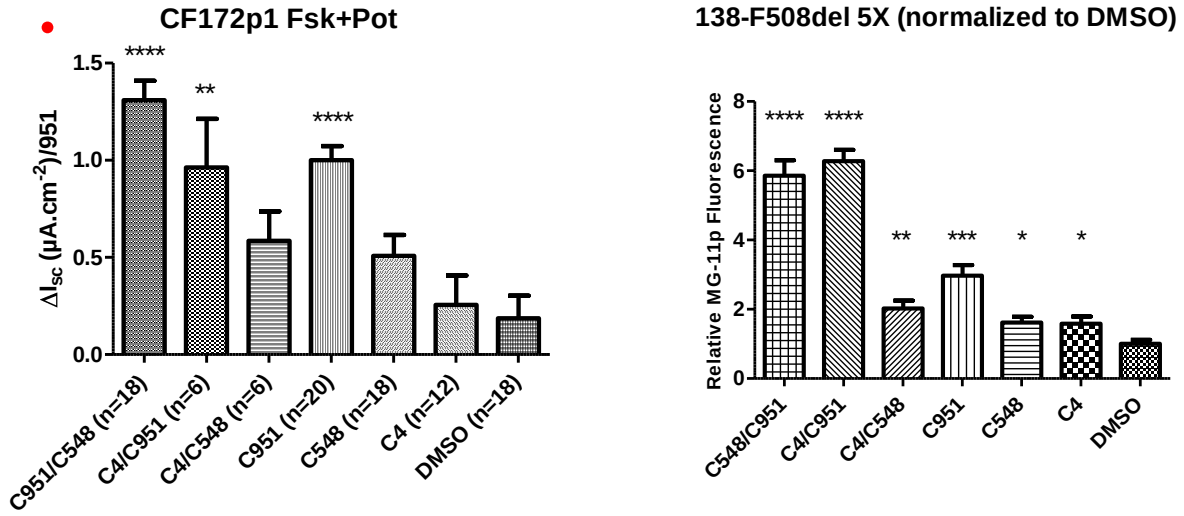

Figure 2: CFTR corrector actions on transepithelial currents across primary HBE (ΔF508/ΔF508, CF patient 172) airway cultures treated overnight with the corrector compounds shown (left panel; values are normalized to those obtained with C951). These data are compared with the relative intensity of FAP-ΔF508 CFTR staining in HEK cells by the cell impermeant dye, MG-11p (right panel, normalized to the fluorescence level obtained from the vehicle control). The asterisks (numbering 1 to 4) indicate relative levels of significance of corrector values from DMSO value, from <0.05 to <0.0001, respectively.

Research Project 2: Project Title and Purpose

Therapeutic Targeting of Breast Cancer Stem Cells - We have identified a means of inhibiting the differentiation of breast cancer stem cells (CSCs), thus allowing the expansion of this otherwise exceedingly rare and difficult to study population. Characterization of CSCs from 4 different breast cancer cell lines shows them to possess common properties, including a >100-fold increased tumor initiating capacity relative to non-CSCs and resistance to traditional chemotherapeutic drugs. We have identified a number of novel small molecules and siRNAs which selectively inhibit CSC proliferation. We propose to expand these studies to CSCs from primary tumors, to screen for additional small molecule inhibitors, and to better characterize the siRNA-targeted pathways that are necessary for CSC viability. These studies could point the way to new rationally designed strategies for improving breast cancer treatments.

Anticipated Duration of Project

1/1/2010 - 12/31/2013

Project Overview

Recent work indicates the presence of so-called "cancer stem cells" (CSCs) in many malignant tumors. CSCs are generally rare subsets of tumor cells, which, in contrast to their non-CSC counterparts, are highly efficient at initiating new tumors. Similar to hematopoietic stem cells (HSCs), CSCs divide slowly but show unlimited self-renewal, which allows them to replenish the CSC pool and provides for an essentially CSC population. Through a type of binary choice which remains incompletely understood, CSCs can also irreversibly differentiate into highly proliferative "transient amplifying cells" (TACs) with a finite replicative capacity. Thus, TACs, either in the proliferative or quiescent state, account for the bulk of most tumors. The CSC hypothesis has spawned the idea that therapy failure is due to inherent CSC chemo-resistance and this has indeed proven to be the cases in several small studies. CSCs are also believed to be more resistant to acidotic and hypoxic conditions found within tumors. Together, these properties of CSCs suggest that their elimination is not only difficult but essentially for complete control over tumor progression to be attained and that novel approaches are needed to eradicate CSCs.

We have recently shown that the Oct3/4 gene promoter region inhibits the differentiation of breast cancer CSC-like cells. As a result, these cells can be expanded indefinitely in vitro. Their inoculation into immuno-compromised mice leads to faster and more efficient tumor growth and the malignant cells recovered from such tumors consist almost exclusively of CSCs. The blocked CSC populations tend to be more resistant to standard chemotherapeutic agents and to hypoxia and acidosis. This has suggested that blocked CSCs can be used to identify novel small molecules or siRNAs that selectively block CSC proliferation and/or survival. We have performed such screens and have identified several small molecules and siRNAs that fulfill this role.

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Expected Research Outcomes and Benefits

One of the major reasons cancers recur is because of failure to eradicate the CSC population. Supporting this idea are studies showing that tumors that recur following chemotherapy commonly contain higher fractions of CSCs than the original tumor from the same individual. Thus, while constituting only a minority cell population, CSCs are believed to be resistant to many standard chemotherapeutic agents and thus to be instrumental in initiating relapse. Moreover, the unlimited proliferative potential of CSCs implies that even extremely small numbers of surviving cells will be sufficient to initiate tumor re-growth. In contrast, small numbers of TACs would be expected to be lost to attrition due to their limited proliferative abilities. Identifying small molecules and siRNAs that specifically targeted the CSC population might be expected to provide several benefits. First, new drugs that specifically eradicate the CSC population might be identified. The efficacy of such drugs might not have been previously appreciated as they would not have led to appreciable shrinkage of tumors, whose CSC population is generally small. Second, the combined used of CSC-specific drugs and more traditional agents, which are quite good at attacking TAC populations, might be expected to be synergistic. Finally, siRNAs might identify novel, CSC-specific pathways for which new agents could be developed in much the same way as the recognition of the bcr-abl pathway in chronic myelogenous leukemia led to the development of Gleevec.

Specific Aim 1: The generation of frozen CSCs from other established cells and primary tumors will allow direct comparisons of their different properties and susceptibilities to the CSC-specific agents that we propose to identify in Specific Aims 2 and 3.

Specific Aim 2: Novel agents that selectively target breast CSCs will serve as index compounds for the optimization of therapeutic and pharmacologic properties.

Specific Aim 3: Identifying specific genes whose expression is needed for CSC survival serves to immediately identify the most important pathways upon which CSCs proliferation and expansion depend. This could lead to the design and testing of novel small molecules, specifically and rationally designed to target these pathways.

Summary of Research Completed

We report below the progress we have made toward these aims as well as several new findings. Most notably, we report that certain of our results in the above breast cancer cell lines appear to apply to primary glioblastoma, a type of highly aggressive brain tumor that responds poorly to all therapeutically modalities and is uniformly fatal within two years.

Specific Aim 1: We have begun to obtain primary breast cancer stem cells and to separate them into CSC and non-CSC populations utilizing cell surface immuno-staining for the $CD44^{hi}/CD24^{lo}$ phenotype. The Oct3/4 promoter driving the expression of GFP has been stably introduced. We are currently attempting to distinguish GFP+ CSC and GFP+ non-CSC populations and to separate these populations.

Specific Aim 2. We showed in our previous Progress Report that small molecule inhibitors of Topoisomerase I could selectively impair the proliferation/survival of breast cancer CSCs and that these cells contained higher levels of the enzyme than their non-CSC counterparts, thus potentially explaining this differential sensitivity. In the course of performing these studies, we tested these inhibitors against the CSC and non-CSC populations isolated from seven primary human glioblastomas (GBMs). As seen in *Fig. 1*, all three of the tested CSC/non-CSC pairs showed greater sensitivity of the CSC populations to the TopoI inhibitor β-lapachone.

We next tested these same cells against structurally unrelated TopoI inhibitors to verify that the initial results with β-lapachone were specifically attributable to TopoI inhibition. As shown in *Fig. 2*, both inhibitors selectively impaired the survival of the CSC populations. Taken together, these results strongly suggested that the observed effects of the three small moleculaes were due to a specific inhibition of TopoI.

We next hypothesized that the selective inhibition of GBM CSCs by the three compounds might be due to differences in the actual TopoI levels in the two different populations. Because cellular sensitivity to TopoI inhibitors has been previously shown to be directly related to TopoI levels, we further hypothesized that the GBM CSCs would contain higher levels of the enzyme than their paired non-CSC counterparts. Indeed, as shown in *Fig. 3*, 6 of 7 primary tumor samples showed much higher levels of TopoI in the CSC population. A seventh tumor (XO-3) also showed higher levels in the CSC vs. non-CSC populations, although overall levels of the enzyme were lower than in any of the other tumors.

In order to verify the above results, we have begun an assessment of TopoI levels in *in situ* primary surgical specimens. For these studies, we have obtained primary frozen tumor tissue sections following the diagnosis of GBM by a board-certified neuropathologist . These samples were then stained for TopoI and for the GBM CSC marker CD133. As shown in *Fig. 4*, there was generally good agreement between the staining for these two markers. Additional tumors are currently being evaluated and preliminary results suggest a similar co-localization of TopoI and CD133 in at least some of the samples.

Specific Aim 3. In our previous progress report, we demonstrated a selective effect on breast cancer CSC proliferation/survival when the TIF I transcription factor was knocked down using a novel lentiviral vector (pTRIPZ) encoding a doxycline-inducible short hairpin shRNA to achieve conditional silencing of the target. Since this initial finding, we have generated a series of additional CSC/non CSC pairs in which we have targeted transcripts whose initial transient knockdown in our genome-wide siRNA screen suggested a selective effect on CSCs. Thus far, the targeted transcripts include 5'lipoxygenase (ALOX5), PTEN-induced kinase 1 (PINK1), mitochondrial processing peptidase-β (PMPCB), lactate dehydrogenase C (LDHC), ferridoxin reductase (FDXR), and cAMP-dependent protein kinase (PRKAR1A). We are continuing to characterize these cell lines with regard to shRNA inducibility, sapecific target knockdown, and the phenotypic consequences of these specific knockdowns.

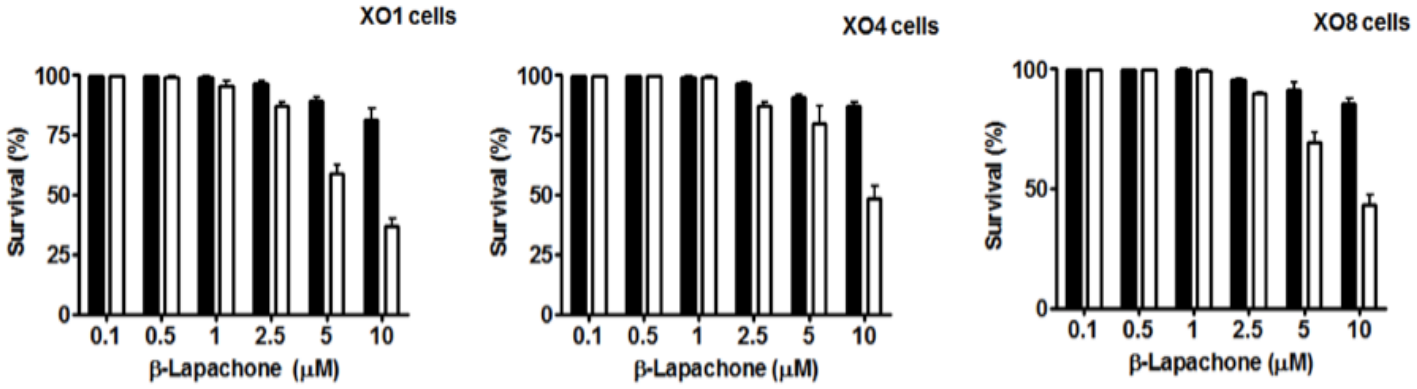

Fig. 1. Selective inhibition of GBM CSCs by the Topo I inhibitor, β-lapachone. GBM CSCs and non-CSCs were isolated from primary tumors XO1, XO4 and XO8 using CD133 selection. Equivalent numbers of CD133- non-CSCs and CD133+ CSCs were then plated and maintained in the indicated concentrations of β-lapachone for three days at which point the fraction of viable cells was determined. The surviving fraction of each population is expressed relative to that of untreated cells whose viability exceeded 95% in each case. CSCs and non-CSCs are indicated by black and white rectangles, respectively. Note the greater sensitivities of the CSC populations in each of the three cases tested.

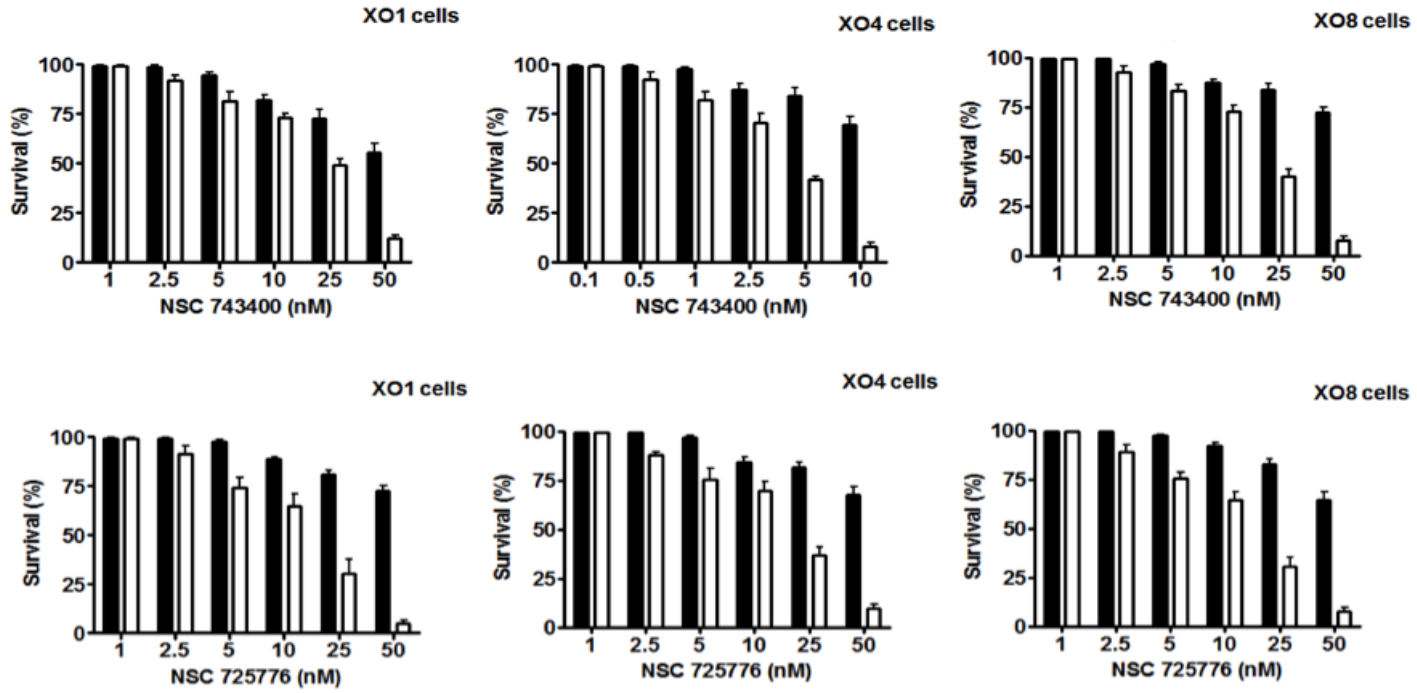

Fig. 2. Selective inhibition of GBM CSCs by the Topo I inhibitors NSC 743400 and 725776. CSC and non-CSC populations from the indicated tumors were isolated as described for Fig. 1. Equivalent numbers of these cells were then plated and maintained in the presence of the indicated concentrations of two TopoI inhibitors, which are structurally unrelated to β–lapachone. As before, the survival of CSCs was selectively inhibited relative to that of their homologous non-CSC counterparts.

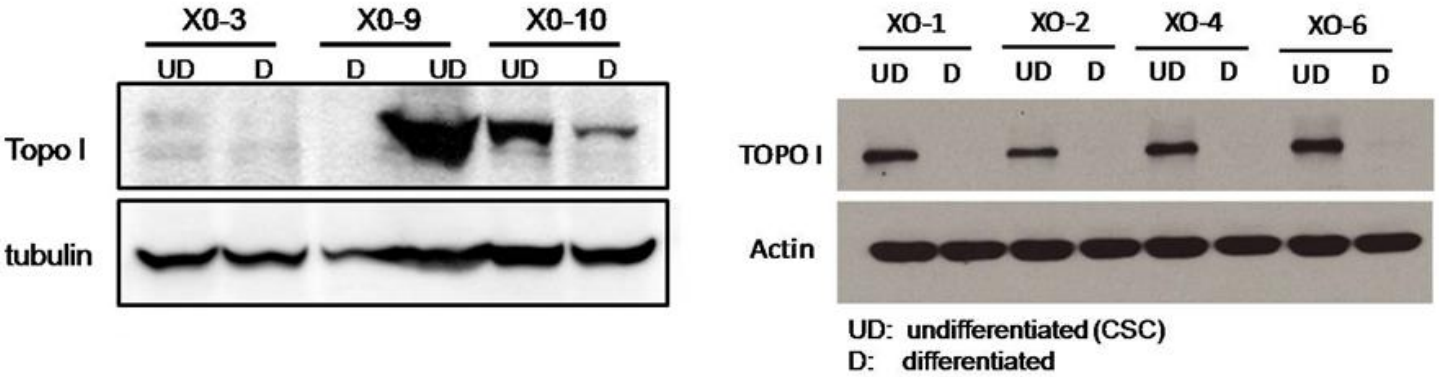

Fig. 3. GBM CSCs express higher TopoI levels than their paired non-CSC counterparts. The indicated CSC/non-CSC pairs were selected as described in Fig. 1, expanded in vitro for several days and then harvested for immunoblotting for TopoI. UD=undifferentiated (CSCs); D=differentiated (non-CSCs). Note the higher levels of TopoI in all seven of the primary pairs although the levels of TopoI in CSCs from tumor XO-3 lower than in the other tumors.

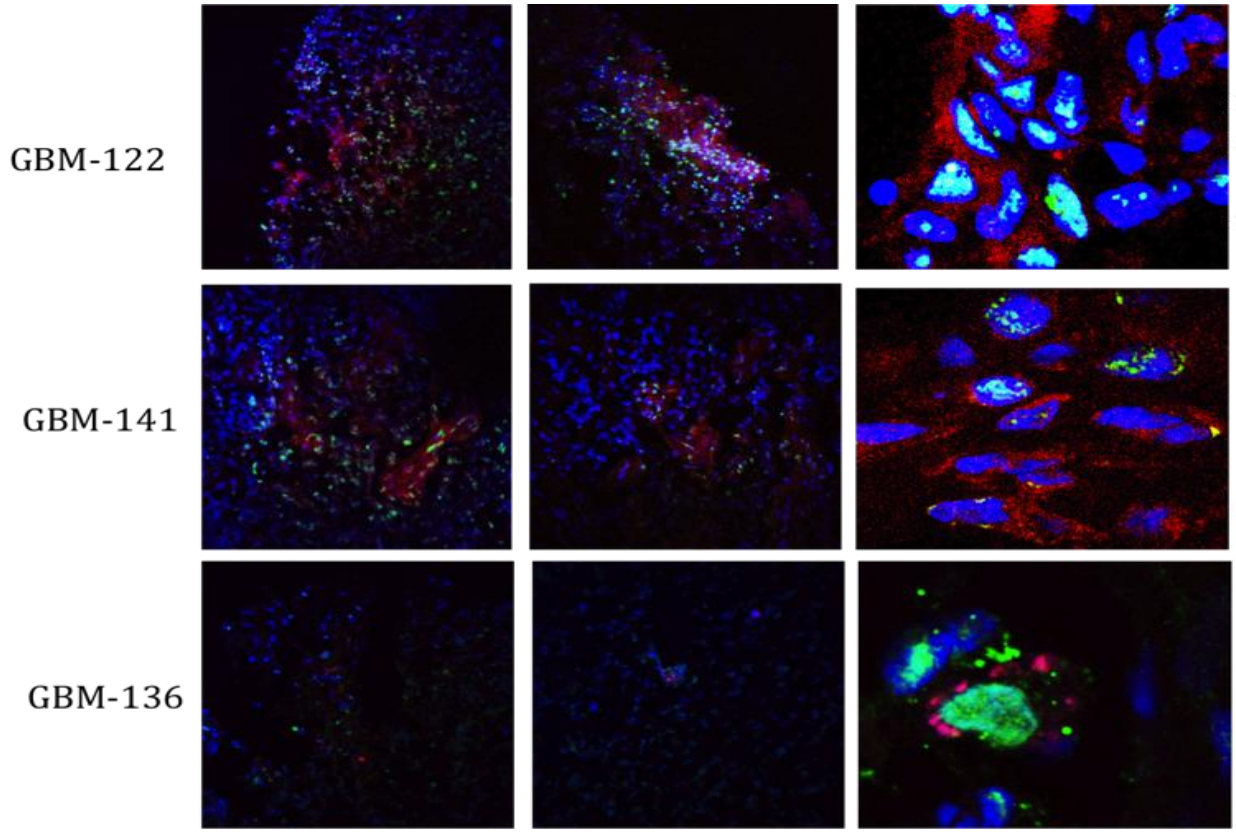

Fig. 4. Co-localization of TopoI and CD133 in surgical sections of primary GBM. Frozen sections from three primary GBMs were immuno-stained for CD133 (red) and TopoI (green) and the counter-stained with DAPI to identify nuclei. The first two columns show typical 20X magnifications of the merged images of typical fields. The third column shows 100X magnifications to identify areas of TopoI-positive nuclear staining and cytoplasmic CD133 staining. Note the tendency of CD133 CSCs to cluster in small areas as has been previously described for GBM CSCs.