Children's Hospital of Pittsburgh

Annual Progress Report: 2009 Formula Grant

Reporting Period

January 1, 2010 – June 30, 2010

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

Specific Aims:
Our goals during this initial six month period of funding were to provide proof of concept for the generation of fluorogen activating protein (FAP) tags and their use in the context of the cystic fibrosis transmembrane conductance regulator (CFTR). Accordingly, we wished to identify sites within CFTR where the fusion protein could be inserted without interfering with the cellular processing of CFTR protein or its function as a regulated anion channel. These initial goals have been largely achieved. Specifically, we sought to:

1. Fully characterize the biochemical and functional properties of FAP-fusions of WT and ΔF508 CFTRs using fluorescence assays and to compare their properties to those of WT CFTR.
2. Optimize fixed and live cell visualization of FAP-CFTRs using available fluorogens; evaluate WT and mutant CFTR cell surface and intracellular compartment expression; develop assays for CFTR endocytic fate and recycling; introduce FAP into CFTR's 4th extracellular loop (ECL4).
3. Create stable cell lines expressing WT and ΔF508 FAP-CFTR in Flp-HEK and airway cells.

Progress to Date:
During the first period, we achieved several milestones directed at the generation of FAP-CFTR and its 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. 1a, FAP-CFTR was generated as an N-terminal fusion with CFTR. Labeling was readily detected only in the plasma membrane by the impermeant fluorogen, malachite green. This discrete cell surface labeling pattern contrasts with the broad cellular distribution obtained with GFP-CFTR (also N-terminal fusion; Fig. 1b). These data demonstrate that live-cell labeling is condition- and location-dependent, thus overcoming one of the limitations of currently available technologies that report generalized protein location or composition. Western blot analysis of expressed FAP-CFTR revealed that the expression level of FAP-CFTR was similar to that of WT CFTR (Fig. 2d). The immature and mature forms of the protein were expressed in a similar ratio, and they migrated more slowly, as expected for the 17 kDa FAP fusion protein plus the additional trans-membrane segment from the PDGF receptor, which was added to display the FAP extracellularly (Fig. 1c).

Functional studies of FAP-CFTR were performed using a halide-sensitive fluorophore (SPQ), whose fluorescence is sensitive to halide concentration. Anion efflux was stimulated by CFTR activation (addition of 10 μM forskolin), producing an upward deflection in blue trace vs. control (red trace), indicating dequenching of the fluorophore as a result of stimulated halide efflux from the cell (Fig. 2). These cAMP/PKA-dependent changes in anion conductance are similar to those observed for WT CFTR in other studies. These data indicate that the FAP fusion did not alter CFTR's basic protein expression pattern, its trafficking to the plasma membrane, or its regulated anion transport properties.

In contrast to WT CFTR, when FAP was similarly fused to the common disease mutant, ΔF508 CFTR, no cell surface signal was detected, consistent with the complete degradation of the immature, mis-folded protein by ER quality control pathways (Fig. 3B). However, ΔF508 is a temperature sensitive mutant, and pre-incubation of cells at 30°C results in the trafficking of a small fraction of mutant CFTR to the cell surface. Consistent with this finding, FAP-ΔF508 was readily detected by MG in the plasma membrane following overnight exposure to reduced temperature (Fig. 3A). Cell labeling by permeant fluorogen detects a widespread ΔF508 distribution, consistent with its ER localization (Fig. 3C). These findings indicate that the rescue of FAP-ΔF508 CFTR to the plasma membrane can be detected by cell impermeant fluorogen, which will be significant for further basic and translational studies. We have also begun to explore the molecular cloning requirements for insertion of the FAP fusion protein at other sites of CFTR topology, such as an extracellular loop, which would avoid the use of an extra transmembrane for display purposes. We have experience with the introduction of epitope tags into the fourth extracellular loop, for example, where they retained excellent functional properties. This tagging procedure would be preferred, since it leaves the intracellular N-terminal domain of CFTR free to interact with binding partners that may modulate its activity. Finally, we have begun to generate stable cell lines expressing the N-terminal fusion of FAP with CFTR, and these will be characterized in a manner similar to that for transiently expressing cells discussed here.

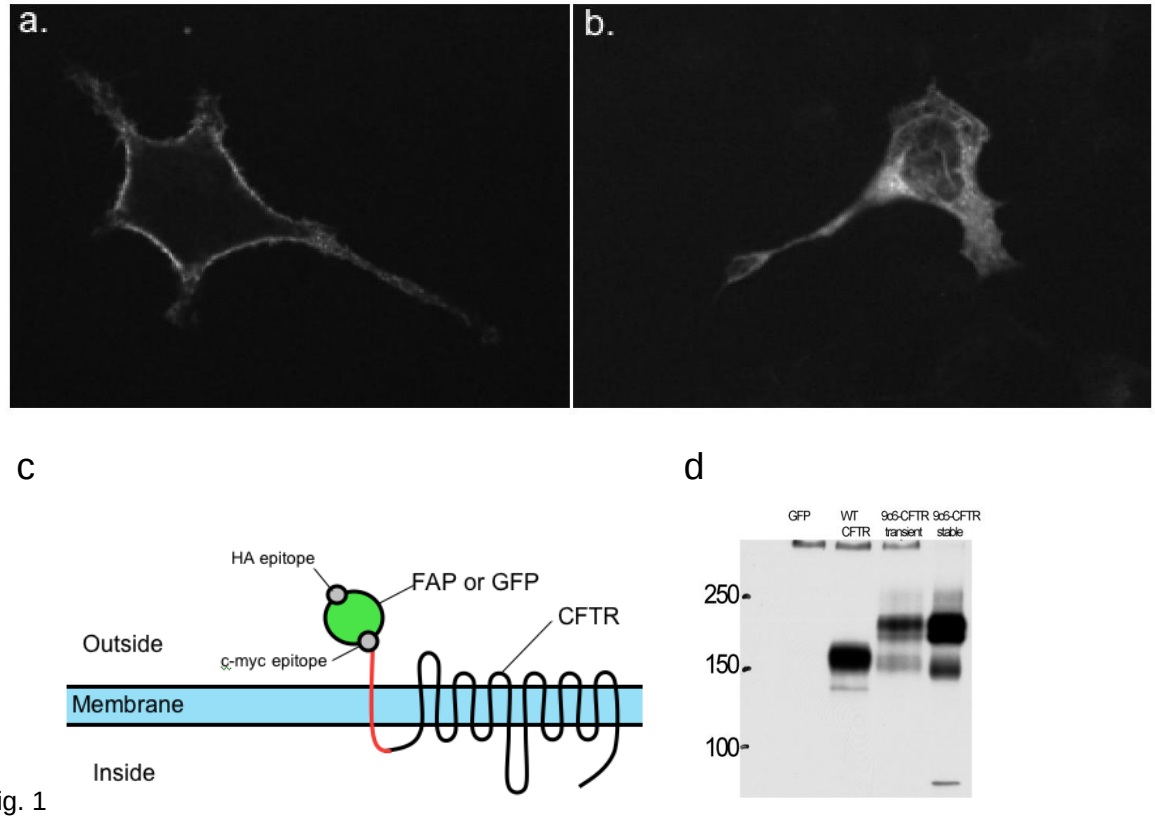

Fig. 1

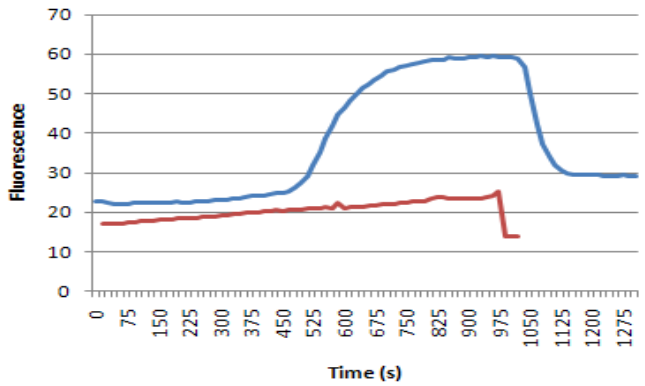

Fig. 2. Functional validation of FAP-CFTR. Blue: Forskolin stimulation of transfected HEK cells. The increase in F/F0 is due to Dequenching of SPQ fluorescence during preloaded iodide efflux via activated CFTR. Red: empty vector control. (n=50 cells).

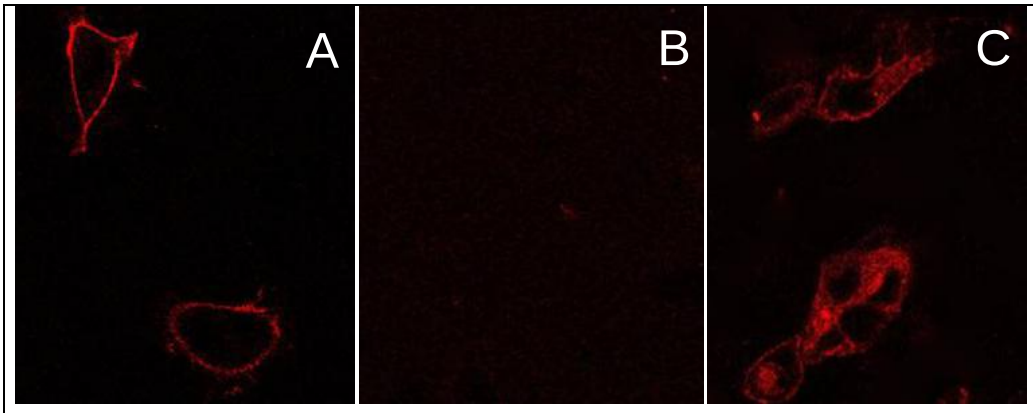

Fig. 3. FAP-ΔF508 CFTR rescue to the cell surface at reduced temperature. **A**: Impermeant fluorogen labeling of cells preincubated at 30ºC overnight shows rescue of FAP-mutant to the cell surface. **B**: Impermeant fluorogen labeling of cells preincubated at 37º yields no surface signal (cells are present by DIC). **C**: Cell permeant fluorogen labeling of cells preincubated at 30º showing ER retained FAP-CFTR.

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 points to the existence of so-called "cancer stem cells" (CSCs) in many malignant tumors. CSCs have been defined as generally rare subsets of tumor cells, which, in contrast to the non-CSC population, are highly efficient at initiating new tumors. By analogy to hematopoietic stem cells, CSCs possess the property of unlimited self-renewal, which allows them to retain their CSC identity and thus replenish the CSC pool. Alternatively, they can also differentiate irreversibly into highly proliferative "transient amplifying cells" (TACs). Unlike CSCs, TACs have a limited life span but a high proliferative index and thus account for the majority of tumor mass. The CSC hypothesis has spawned the idea that therapy failure is due to inherent CSC chemo-resistance. CSCs are also believed to be more resistant to acidotic and hypoxic conditions found within tumors. Thus novel approaches are needed to specifically target and eliminate CSCs. Unfortunately, their rarity and tendency to rapidly differentiate into TACs has limited the development of such therapeutics. We have recently shown in 4 different breast cancer cell lines that stable transfection of the promoter region for the Oct3/4 gene inhibits the differentiation of breast cancer CSC-like cells, thus allowing for their unlimited and stable in vitro expansion. We have shown these "frozen" CSC-like cells to be generally not only more resistant to standard chemotherapeutic agents but to hypoxia and acidosis as well. The stability of these CSCs suggests that they can be used to identify novel small molecules that are selectively CSC toxic and to uncover unique pathways needed to maintain CSC viability. Indeed, a preliminary screen of a library of pharmaceutically active compounds has identified four that selectively inhibit the growth of the CSC-like population. A number of CSC-specific siRNAs have been identified as well. Thus, in Specific Aim 1, we will generalize our findings by continuing to characterize CSC-like cells derived from other breast cancer cell lines as well as primary breast CSCs. In Specific Aim 2, we propose to screen larger libraries of LMW compounds to identify those which possess selective toxicity towards CSCs. In Specific Aim 3, we will continue to conduct a genome-wide siRNA screen to identify pathways involved in the proliferation and/or maintenance of CSCs versus TACs.

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Expected Research Outcomes and Benefits

It is currently believed that cancers may recur because current therapies largely target the non-CSC population, which comprises the bulk of the malignant cells. While constituting only a minority population, CSC are nonetheless believed to be resistant to many standard chemotherapeutic agents, survive initial chemotherapeutic treatment, and initiate the recurrence. Specifically targeting the CSC population, possibly in conjunction with traditional agents, would ensure that these cells are unable to contribute to the relapse.

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

At the time of this Progress report, the application had only been active for slightly over five months. Despite this short time-frame, we have made significant progress on Specific Aims 2 and 3. We also have had one manuscript accepted for publication:

Sajithlal GB, Rothermund K, Zhang F, Dabbs DJ, Latimer JJ, Grant SG, and Prochownik EV Permanently blocked stem cells derived from breast cancer cell lines. *Stem Cells* (in press)

Progress on Specific Aim 2. We showed in our original application that we could identify several small molecules that were selective inhibitors of the blocked cancer stem cell (CSC) population derived from MCF7 cells. Among the small molecules we identified was β–lapachone, a well-known inhibitor of Topoisomerase I (Topo I). If CSCs are selectively sensitive to Topo I inhibition, then differential levels of the enzyme might be expected to be present in CSCs and non-CSCs. We therefore analyzed Topo I levels by immunoblotting. As seen in Fig. 1 below, MCF7, MDA-MB231 and MDA-MB453 CSCs contained 3-5-fold elevated levels of Topo I relative to their non-CSC counterparts whereas BTL12 CSCs and non-CSCs contained equivalent Topo I levels.

Taken together, these results strongly imply that β–lapachone works to selectively target Topo I, which is commonly expressed at higher levels by CSCs.

In addition to using small molecule analogs to confirm the mechanisms of action of CSC-specific "hits," we can also employ genetic methods and provide one such example here using shRNA-mediated Topo I knockdown. Because long-term inhibition of Topo I was expected to be deleterious, we used a conditional knockdown approach in MCF7 and MDA-MB453 CSCs and non-CSCs. Cells were infected with the puromycin-selectable pTRIPZ lentiviral vector encoding a TopoI shRNA (www.openbiosystems.com). The shRNA is expressed as a doxycycline (Dox)-inducible miRNA-30 primary transcript, which adds a Drosha processing site to the hairpin transcript to increase knockdown efficiency. The tet-response element of this vector also drives red fluorescent protein (RFP), which allows a visual, albeit indirect, confirmation of shRNA induction. RFP expression in the puromycin-resistant transduced population also allows a way for cells with the highest levels of shRNA (and presumably the highest degree of target knockdown) to be sorted away from the remaining population simply by treating the pooled population overnight with Dox, isolating the brightest fraction by flow cytometry, and then propagating these in the absence of Dox. Such purified populations from both groups ("sh cells") were pooled and characterized in parallel. Dox induced the expression of RFP in both cell types and also reduced Topo I protein by ca. 80-90% (Fig. 2A).

We next simultaneously assessed the effect(s) of Topo 1 knockdown on growth and cloning efficiency. Equivalent numbers of sh-CSCs and non-CSCs were seeded into 6 well plates with or without Dox and assessed for colony formation 12-14 days later. Although Topo 1 knockdown did not noticeably affect cloning efficiency, the average size of colonies was significantly reduced (Fig. 2B). In these studies, stem cells were only slightly more sensitive to Topo I knockdown than were non-stem cells. The difference between this outcome and that obtained with β–lapachone may reflect the fact that the small molecule affects the activity of the enzyme rather than its levels whereas shRNA-mediated inhibition has the opposite effect. Nevertheless, these studies provide independent genetic confirmation that Topo I is a viable molecular target. We are currently examining the remaining CSC lines by this method and are beginning *in vivo* studies as well.

Progress on Specific Aim 3. In our original application, we described the identification of over 50 siRNAs which selectively inhibited the proliferation/survival of MCF7 CSCs relative to that of their non-CSC counterparts. One such target, whose knockdown significantly affects MCF7 CSCs, is TIF1 (transcriptional intermediary factor 1/Trm24), which has been reported to affect tumorigenesis and aging and, at the molecular level, to interact with the ligand-inducible activation functions of numerous nuclear receptors, including those for estrogen, vitamin D3 and retinoic acid. Moreover, our preliminary analyses documented elevated TIF1 transcripts in all four CSC populations (not shown).

Using the same conditional knockdown approach described above for Topo I, we similarly depleted MDA-MB231 CSCs and non-CSCs cells of TIF1 protein by ca. 80-90% (Fig. 3A). We further showed that the induction of TIF1 shRNA selectively reduced colony formation by CSCs. Together, these experiments confirm in another CSC cell line and using stable shRNA expression that the depletion of TIF1 selectively and negatively impacts the proliferation/survival of CSCs.

Fig. 1(below). Topo I levels in paired CSC/non-CSC breast cancer cell lines. Oct3/4-GFP-blocked CSCs (S) and their non-CSC (NS) counterparts were examined by immuno-blotting for Topo I levels. Note the ca. 3-5-fold higher Topo I levels in all CSC lines except those originating from BTL12 cells. β–tubulin served as a loading control.

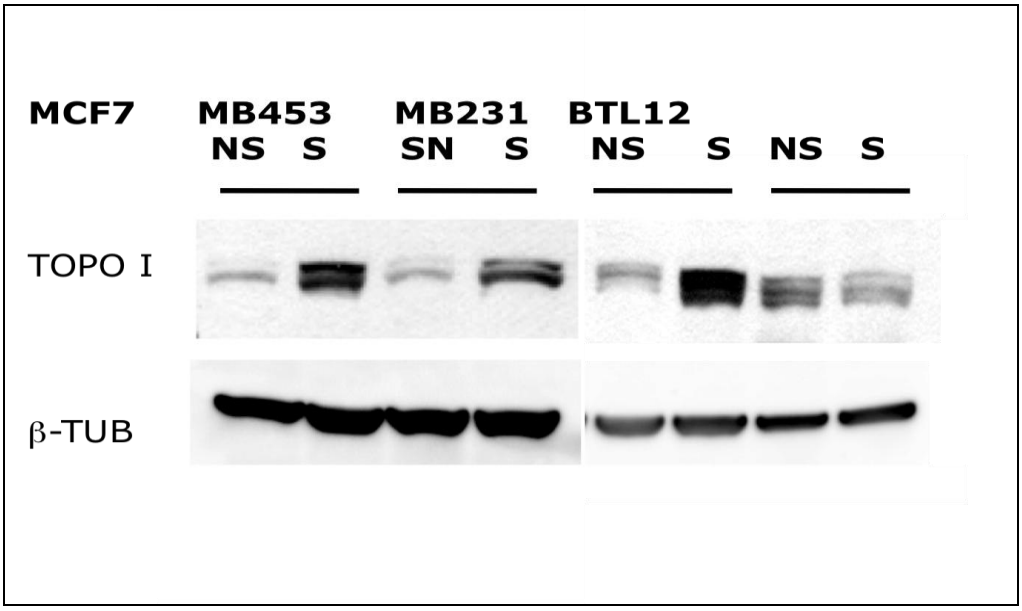

Fig. 2. (Below) Consequences of Topo I knockdown. MCF7 and MDA-MB453 CSCs and non-CSCs were transduced with the Dox-inducible pTRIPZ-shTIF1 vector. Puromycin-resistant cells were then selected and pooled. These cells were then exposed to Dox (2 μg/ml) overnight and the brightest 10% of each population was isolated by FACS. These cells were then expanded and used in all subsequent experiments. (A). Topo 1 western blots +/- Dox. Total cell lysates were analyzed following a 4 d. exposure to Dox. Note that, in the absence of Dox, higher levels of Topo I were again confirmed in CSCs (Fig. 1). (B). Selective inhibition of CSC-mediated colony formation. Equivalent numbers of the indicated CSCs or non-CSCs were seeded in triplicate 6 well plates and grown +/- Dox (2 μg/ml) for 14 days with the medium being changed every other day. Colonies were then stained with crystal violet.

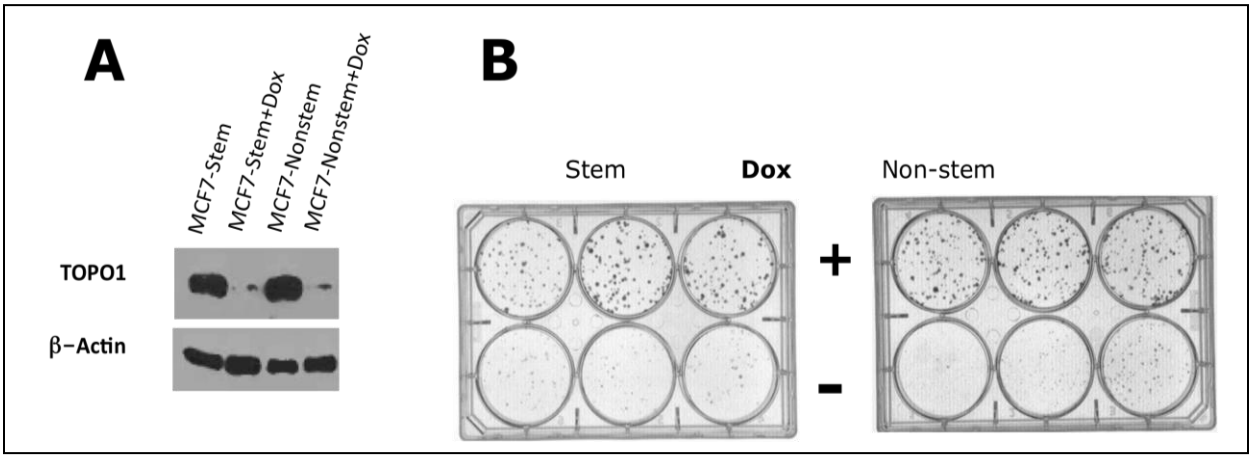
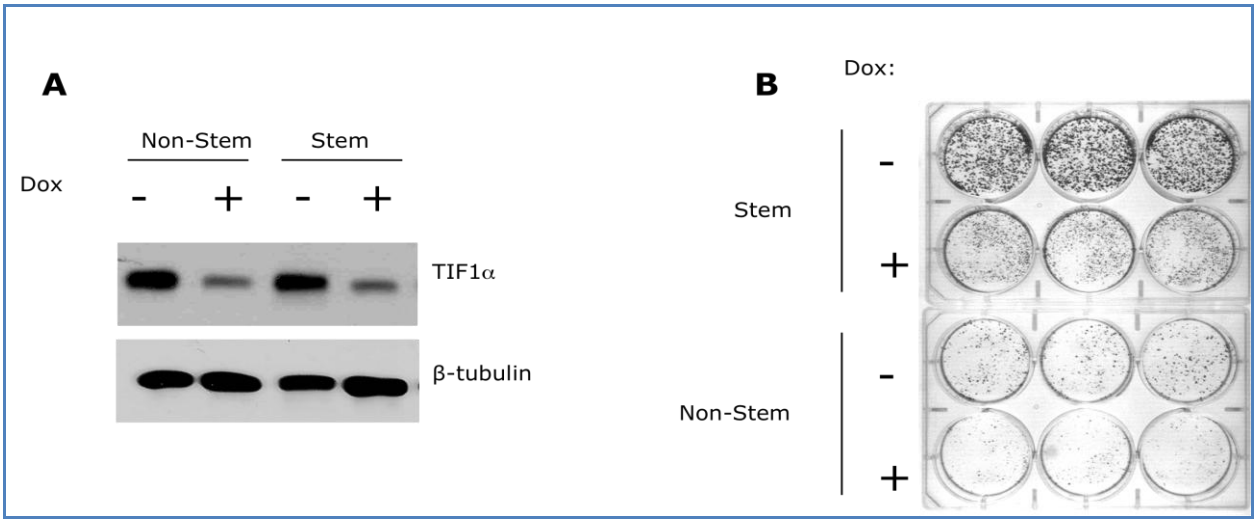
Fig. 3. (below)Consequences of TIF 1 knockdown. MDA-MB231 CSCs and non-CSCs were transduced with the Dox-inducible pTRIPZ-shTIF1 vector and puromycin-resistant cells were selected and pooled. The cells were then treated overnight with Dox (2 μg/ml) and purified by FACS to obtain the brightest population of RFP+ cells. (A). TIF 1 western blots +/- Dox (2 μg/ml for 4 days). (B). Selective inhibition of CSC-mediated colony formation. Equivalent numbers of MDA-MB231 CSCs or non-CSCs were seeded in triplicate into 6 well plates and grown +/- Dox (2 μg/ml) for 12-14 days. Colonies were then stained with crystal violet.