


# Era of Hope

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# PROCEEDINGS

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## P16-5: A NOVEL COMBINATION OF THERMAL ABLATION AND HEAT-INDUCIBLE GENE THERAPY FOR BREAST CANCER TREATMENT

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High-intensity focused ultrasound (HIFU) can convert focused acoustic energy into heat and thus produce well-defined focal lesions in vivo. In recent years, HIFU has emerged as a promising treatment modality for breast cancers. However, the current HIFU therapy cannot eradicate 100% of the tumor volume or treat the metastatic cancer cells outside of the primary tumor site. We hypotheses that HIFU can not only destroy primary tumor tissue but also induce therapeutic effects in sublethally injured tumor cells. This investigation is to explore the potential synergistically combining HIFU thermal ablation and heat-induced gene therapy to improve the efficiency and overall quality of breast cancer treatment.

The HIFU exposure system was constructed by integrating a single element air-back annual HIFU transducer with a phased array ultrasound probe. Cells were transfected with heat-sensitive adeno-virus vectors. Inducible marker gene expression in vitro was evaluated in a thermal dosage-dependent manner by monitoring marker gene green fluorescent protein (GFP). An optimized HIFU exposure condition was thereafter resulted to guide an animal experiment on a tumor-bearing murine model. An in vivo bioluminescence imaging system was used to record luciferase distribution in the hind limbs of Balb/C mice subcutaneously inoculated with 4T1-hsp70B-Luc cancer cells. Furthermore, a 3D cell-embedded tissue mimicking phantom was developed. With this phantom model, the observed gene expression pattern was correlated with in situ delivered thermal dosage from HIFU exposure.

The HIFU system was applied to generate different exposure pattern and dosage while the acoustical and thermal fields were systematically determined. HIFU-induced gene expression was first investigated in the HeLa-hsp70B-GFP cell suspension, and the maximum HIFU-induced gene expression was achieved at 60° in 5s. The maximum luciferase gene expression in the tumor-bearing murine model was then achieved at a peak temperature of 65–75° within 10–20s. Thermal stress, in both in vitro and in vivo studies, was identified as the primary physical mechanism for HIFU-induced gene expression. In the 3D phantom study, GFP positive cells were primarily found within a circumferential region sounding the primary site of lesion formation, which also coincides with the thermal necrosis boundary, indicating that gene expression was primarily induced in the sublethally injured cell population along the HIFU lesion boundary.

In conclusion, an image-guided computer-controlled experimental HIFU system was developed and characterized for both in vitro and in vivo studies. The efficiency of HIFU-induced gene expression under the control of hsp70B promoter was separately examined in cell suspension and tumor model. To elucidate the underlying physical mechanism, a 3D cell-embedded tissue mimicking phantom was developed with similar acoustic and thermal properties to breast tissue. Based on these results, we will focus on completion of the spatial correlation between HIFU-induced thermal dose and gene expression in the tissue mimicking phantom and combination of HIFU thermal ablation with heat-regulated IL-12 gene therapy to be tested in a 4T1 murine tumor model. If successful, this synergistic combination of HIFU thermal therapy and heat-inducible gene therapy can lead to a superior tumor suppression effect due to simultaneous reduction of primary tumor mass and significantly boosted therapeutic effects.

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## P16-6: HYPERTHERMIA-INDUCED TUMOR METABOLIC CHANGE AND TUMOR REOXYGENATION

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Tumor hypoxia, which is caused by imbalance between $O_2$ supply and consumption, is related to poor prognosis and therapy resistance. Hyperthermia (HT) is a strong adjuvant cancer treatment because of its cell killing and radiosensitizing effects on both normoxic and hypoxic cells. HT, especially at mild temperatures between 39 and 43 degrees, induces tumor reoxygenation, which results in improved response to radiotherapy. It has been suggested that tumor reoxygenation is due to increased blood flow and decreased oxygen consumption. However, the underlying mechanism is not well understood yet.

Under hypoxia, cancer cells exhibit an adaptive mechanism via hypoxia-inducible factor-1 (HIF-1), a transcription factor that activates a variety of genes involved in tumor progression. In prior studies, our group has demonstrated that radiation-induced reoxygenation activates HIF-1 through reactive oxygen species (ROS), and inhibition of HIF-1 leads to the enhanced antitumor effects. Since HIF-1 is known to regulate tumor metabolism by activating glycolytic enzymes and inhibiting mitochondrial functions, we hypothesized that HT-induced tumor reoxygenation is caused by the alteration of tumor metabolism, and HIF-1α plays a role in it.

To determine the effect of heat on HIF-1, we first heat-treated a mouse mammary carcinoma cell line (4T1) at a range of temperatures for 1 hour and measured HIF-1 levels using the luciferase reporter. This luciferase reporter contains the oxygen-dependent degradation (ODD) domain of HIF-1α, enabling us to directly detect HIF-1 stability. Interestingly, at mild hyperthermic temperatures, HIF-1 levels were significantly increased and were the highest at 42°C. Increased HIF-1 activity after heat treatment was also detected using HIF-1 ELISA and measuring VEGF secretion, a downstream target of HIF-1. The same experiments at different time points (0, 1, 2, 3, 6, 12, and 24 hours) showed that HIF-1 activity increased immediately and peaked 6 hours after heat shock. Our in vivo study growing the same reporter cell line (4T1) on the flank of nude mice confirmed our in vitro data. Plasma levels of PAI-1, another HIF-1 target, were significantly increased after HT. To determine the possible role of ROS in HIF-1 activation, we treated 4T1 cells with an SOD mimetic. Treatment with the SOD mimetic abolished the upregulation of HIF-1, indicating that ROS play a role in HIF-1 regulation after HT. Since mitochondrial ROS regulates HIF-1, the link between mitochondrial function and HIF-1 activity was next examined by measuring mitochondrial membrane potential with JC-1. JC-1 aggregates decreased 24 hours after HT, indicating the disruption of membrane potential.

In conclusion, our study showed that mild HT increased HIF-1 activity is related to mitochondrial ROS production. Thus, alteration of tumor metabolism by HIF-1 causes reduced oxygen consumption, and as a result, the tumor is reoxygenated. Future studies will include measurement of ROS production and glycolytic enzymes, as well as inhibition of HIF-1 using an HIF-1 knockdown cell line.

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## P16-7: MAGNETICALLY LABELED TRANSGENIC ENDOTHELIAL PROGENITOR CELLS AND DENDRITIC CELLS AS PROBES FOR CELLULAR MRI AND GENE DELIVERY VEHICLES

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Cancer gene therapy is at a point where the need for an optimal gene delivery vector has become the rate-limiting step. Several factors, including lack of an efficient vector and delivery system itself, limit the effectiveness of systemically delivered genes. Very recently, stem/progenitor cells have been considered as delivery vehicles for transferring exogenous genes to the cancer cells. The purpose of this study was to determine whether endothelial progenitor (EPCs) and/or dendritic cells (DCs) can be used as gene delivery vehicles and as cellular probes for magnetic resonance imaging (MRI). In this study, we used superparamagnetic iron oxide (SPIO)-labeled EPCs and DCs to carry human sodium iodide symporter (hNIS) gene to the sites of implanted breast cancer. In vivo real-time tracking of these cells was performed by MRI and expression of hNIS was determined by Tc-99m pertechnetate (Tc-99m) scan.

Three million human breast cancer (MDA-MB-231) cells in 50 μL of serum free media were subcutaneously implanted in the right flank of nude mice. EPCs (CD34+/AC133+) were isolated from fresh human cord blood. DCs were made either from the cord blood CD34+ or CD14+ cells using established protocols. Both EPCs and DCs were genetically transformed to carry hNIS gene using adenoviral vectors. Genetically transformed EPCs and DCs were magnetically labeled with ferumoxides-protamine sulfate (FePro) complexes as previously published. Magnetically labeled genetically transformed cells were administered intravenously in tumor bearing mice. MRIs were acquired 3 or 7 days after cell injection and SPECT images were acquired within the 24 hours of performing the MRI. T2, T2,* and 3D GRE images were obtained using a 7 Tesla, 20 cm bore superconducting magnet interfaced to a BRUKER console. SPECT images were acquired with custom built micro-SPECT using Tc-99m. After SPECT, animals were euthanized, perfused, and whole tumors were collected for histochemistry.

Both cell types were efficiently magnetically labeled (>90%). MRI images clearly showed the presence of low signal intensity areas around the tumors in mice that received iron-labeled cells, indicating the accumulation of administered cells. The presence of iron-labeled cells was also confirmed by Prussian blue staining. In addition, SPECT images showed significantly higher radioactivity in tumors in animals that received transfected cells ($p$-value$<0.01$). The activity of Tc-99m in the tumors in 3 and 7 days post-injection transfected groups were significantly higher than that of control groups that received non-transfected cells ($p$-value$<0.01$). However, there were no significant differences between 3 and 7 day post-injection transfected groups. Immunohistochemistry proved the presence of hNIS positive cells in the tumors.

Both, MRI and SPECT images showed accumulation of administered EPCs and DCs in implanted breast cancer and expression of hNIS gene, respectively. Our study indicates that both EPCs and DCs can be used to deliver genes by systemic administration. Genetically transformed, magnetically labeled DCs or EPCs can be used both as delivery vehicles as well as cellular probes for detecting in vivo migration and homing of cells by MRI. This method can be used in the future development of gene therapy approaches where genetically modified cells can be tracked by real time in vivo MR scanning in different disease processes.

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## P16-8: NOVEL BREAST CANCER THERAPEUTICS BASED ON BACTERIAL CUPREDOXIN

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**Background and Objectives:** Reports of regression of cancer in humans infected with microbial pathogens date back more than 100 years. However, live bacteria produce significant toxicity, limiting their use. The unprecedented observation that the small bacterial protein *Pseudomonas aeruginosa* azurin forms a complex with the well-known tumor suppressor protein p53 and triggers cell death provides a new avenue for cancer research. Despite being a novel concept to target cancer, there are no thermodynamic details known for the proposed azurin-p53 complex. This project aims to fill this gap as outlined in four specific aims.

**Aims and Methodologies:** We will reveal (1) which p53 domain interacts with azurin and probe affinity and stoichiometry, (2) the molecular mechanism by which azurin increases cellular levels of p53, (3) the region on azurin that interacts with p53 and (4) use the acquired information to propose smaller molecules that retain properties of azurin. For this, we use a battery of biophysical, spectroscopic and biochemical techniques in conjunction with purified proteins and strategic variants for in vitro experiments.

**Results to Date:** To this date, several discoveries have been made: most importantly, azurin is found to bind to the unstructured N-terminal domain of p53 and a small 13-residue peptide is able to reproduce part of the azurin interaction. Also, properties of two human copper-metabolism proteins have been identified; these proteins are important as they may cross-react with azurin-based drugs.

**Conclusions and Impact:** Our biophysical project provides key physical, chemical, and structural understanding of azurin's interaction with p53 in vitro. We propose that the results of our studies may be used to develop small peptide constructs that bind and stabilize p53 like full-length azurin. If these molecules turn out to work in vivo, it may be the gateway to an innovative class of new cancer therapeutics.

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## P16-9: THE ROLE OF VITAMIN D IN AROMATASE INHIBITOR-INDUCED BONE LOSS

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Breast cancer (BCa) is the most common fatal cancer in women. Emerging evidence suggests that vitamin D is inversely related to BCa: low vitamin D levels are associated with increased BCa risk and vitamin D insufficiency is prevalent among women with BCa. Potent aromatase-inhibitors (AI) effectively suppress BCa growth by blocking local estrogen synthesis in the breast tissue, but they also cause severe side effects including hot flashes, muscle and joint pains, and bone loss leading to a substantially increased risk of fractures. These side effects are likely related to profound suppression of local estrogen production in bone and muscle tissues. Accumulating evidence indicates that higher than currently recommended doses of vitamin D are effective in the prevention and treatment of several cancers, including BCa. Preliminary data also suggest that vitamin D is potentially capable of reversing the AI-induced deleterious effect on bone and muscle. Human and animal experiments are currently under way to determine whether co-administration of vitamin D along with AIs is safe and effective in protecting patients with BCa from AI-induced increases in the rate of bone turnover that lead to bone loss and fractures, as well as preventing or ameliorating AI-induced muscle and joint pains. If vitamin D, this inexpensive and widely available therapeutic agent, is proven safe and effective in women with BCa, it will potentially open new avenues to investigate the role of vitamin D in prevention or treatment of BCa in the future.

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## P16-10: TOPOISOMERASE IIβ AND DOXORUBICIN CARDIOTOXICITY

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**Background and Objective:** Doxorubicin (Adriamycin) is one of the most effective anticancer drugs used in the clinic for treatment of breast cancers. However, doxorubicin therapy can cause life-threatening cardiotoxicity. Despite the severity of this dose-limiting toxicity, the molecular mechanism underlying doxorubicin cardiotoxicity remains unclear. The free radical hypothesis is currently most favored due to the redox cycling ability of doxorubicin. However, this hypothesis is still controversial since many free radical scavengers fail to rescue doxorubicin cardiotoxicity. The only clinically used cardioprotective agent is dexrazoxane (also known as ICRF-187). It is well known that Top2 is the major cellular target of doxorubicin. The antitumor activity of doxorubicin is due to the formation of a Top2-doxorubicin-DNA ternary complex (the cleavage complex). However, there are two Top2 isozymes (Top2α and Top2β) in human cells. Both isozymes can be targeted by doxorubicin. Top2α is only expressed in proliferating cells such as tumors. By contrast, Top2β is expressed in terminally differentiated cells such as adult cardiomyocytes. We hypothesize that doxorubicin cardiotoxicity is primarily Top2β-mediated. There are at least three lines of evidence supporting our hypothesis. First, the cardioprotective agent dexrazoxane is a known derivative of bis(2,6-dioxopiperazines) compounds that are also Top2 catalytic inhibitors. Top2 catalytic inhibitors are known to antagonize the formation of Top2 cleavage complexes. Second, Top2β, but not Top2α, is highly expressed in cardiomyocytes of the adult heart. Third, a C-terminal truncated form of Top2β has been identified in mitochondria that are known to be abundant in cardiomyocytes.

The goal of this application is to rigorously test our hypothesis that the Top2β isozyme is primarily responsible for doxorubicin cardiotoxicity.

**Methodology:** The role of Top2β and proteasome in doxorubicin-induced DNA damage is investigated using primary *TOP2β*$^{+/+}$ and *top2β*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and H9C2 cardiomyocytes, respectively. Doxorubicin-induced DNA damage signal γ-H2AX (by western blotting) and DNA double-strand breaks (DSBs) (by neutral comet assay) are monitored. In addition, a mouse model will be developed to determine the role of Top2β in doxorubicin cardiotoxicity. Temporal-controlled heart-specific *top2β* knockout mice will be generated by crossing the floxed *top2β* mouse line with the transgenic mouse line expressing an α-MHC promoter-driven progesterone receptor/Cre fusion protein. Cre-mediated deletion of the *TOP2β* gene will be achieved by the intraperitoneal (i.p.) injection of RU486. Cardiotoxicity induced by a single injection of doxorubicin will be monitored by transthoracic echocardiography, histological analysis, and TUNEL assay.

**Results and Conclusion:** We show that doxorubicin-induced DNA damage is much reduced in *top2β*$^{-/-}$ MEFs, suggesting the involvement of Top2β in doxorubicin-mediated cell killing. Proteasome inhibitors also reduce doxorubicin-induced DNA damage, suggesting the involvement of the proteasome pathway in doxorubicin cytotoxicity. These findings will be further tested in a mouse model using a conditional heart-specific *top2β* knockout mouse line. Results from our study will have important implications in the clinic. If doxorubicin cardiotoxicity is indeed Top2β-mediated, Top2α-specific anticancer drugs should be developed to reduce side effects during chemotherapy.

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## P16-11: A CHEMOTHERAPY-ASSOCIATED SENESCENCE BYSTANDER EFFECT IN BREAST CANCER CELLS

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A bystander effect typically refers to the death, altered growth, or damage of cells that have not directly received chemotherapy or irradiation. Cancer cells derived from solid tumors readily undergo senescence in response to chemotherapeutic agents, prompting us to test for the existence of a senescence bystander effect. MCF-7 breast cancer cells were acutely exposed to adriamycin to trigger senescence. Naïve MCF-7 cells, when cultured in conditioned media from senescent breast cancer cells, growth arrested despite mitogenic stimulation and exhibited SA -β-galactosidase activity, an enlarged cell size, and stable upregulation of $p21^{WAF1}$ protein, collectively indicating a senescent state. In contrast, HCT-116 colon cancer cells, which also undergo p53-mediated senescence in response to acute AdR, did not undergo growth inhibition or senescence when cultured with conditioned media from senescent HCT-116 cells. Reciprocal experiments indicated that naïve HCT-116 cells are susceptible to the growth inhibitory effects of a breast cancer-derived mediator, which is independent of drug in conditioned media. Our study reveals a novel action of adriamycin, which may contribute to its potent anti-breast cancer activity and lead to the discovery of additional therapeutic targets for the exploitation of a senescence bystander effect.

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