Cancer Angiogenesis and Metastasis; molecular, cellular and *in vivo* imaging studies

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Introduction

Our research is focused on investigating cancer angiogenesis and metastasis, specifically hormonal regulation of these processes in breast cancer. In addition, we search for metabolic markers of breast malignant transformation. The studies are performed on breast and lung cancer cells of human origin and on tumors developed from these cells growing in rodent animal models. We also develop research methods to monitor cancer progression, emphasizing non invasive imaging techniques using magnetic resonance imaging (MRI) and spectroscopy (MRS), as well as optical imaging. The new MRI techniques are being translated clinically to detect and diagnose cancerous tumors through their unique vascular function.

Estrogen Regulation of VEGF in Breast Cancer cells *in vitro* and tumors *in vivo*: the role of c-Myc

Estrogen regulation of vascular endothelial growth factor (VEGF) is a key process in breast cancer angiogenesis. However, the molecular mechanisms underlying this regulation and the physiological consequences in tumors are not fully known. We have investigated estrogen regulation of VEGF and vascular permeability in breast cancer, and determined the role of c-Myc in mediating this regulation, using MCF7 cells stably transfected with an inducible c-myc gene. Our results *in vitro* and *in vivo* clearly demonstrate two regulation modes of estrogen: a transient, short-term effect and a chronic, long-term effect. We found that estrogen transient induction of c-Myc *in vitro* is necessary for the transient stimulation of VEGF transcription. Furthermore, both c-Myc and the activated estrogen receptor were found to co-bind the VEGF promoter, indicating a novel mechanism for estrogen regulation of VEGF (FIGURE 1). *In vitro* chronic estrogen treatment and long-term overexpression of c-Myc alone, maintained a moderate and constant increase in the expression levels of VEGF. However, hypoxic conditions dominated VEGF expression and substantially elevated VEGF levels. Similarly, *in vivo* chronic estrogen treatment of tumors and continued c-Myc activation sustained stable levels of VEGF and a functional vascular permeability, whereas, estrogen withdrawal eliminated estrogen regulation of VEGF and increased VEGF level and vascular permeability, presumably as a result of hypoxic conditions. In conclusion, we have discovered a novel role for c-Myc in mediating estrogen regulation of VEGF. The results emphasize the correlation between *in vitro* and *in vivo* estrogen regulation by stressing the importance of the mode of hormonal treatment and the effects of microenvironmental conditions.

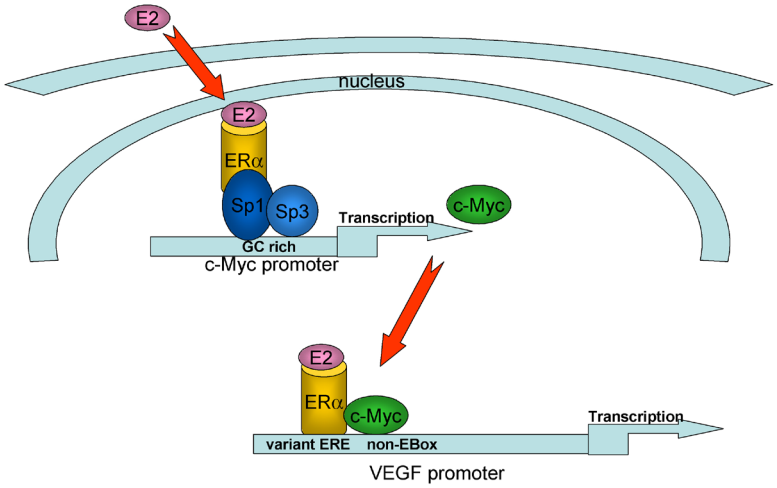

Fig. 1 *Schematic model illustrating the mechanism by which c-Myc mediates estrogen induction of VEGF transcription. Estrogen stimulates c-Myc transcription through recruitment of Sp proteins to the c-Myc promoter. The activated estrogen receptor and c-Myc co-bind to the VEGF promoter at 1.5 kb upstream of the transcription start site inducing VEGF transcription.*

Novel Selective Estrogen Receptor Modulators for Non–Invasive Molecular Imaging (in collaboration with Prof. D. Milstein, Department of Organic Chemistry and Prof. Joel Sussman, Department of Structural Biology)

In an aim to investigate the spatial distribution and temporal changes of estrogen receptor α (ERα) in breast cancer we embarked on developing non-invasive molecular imaging methods, synthesizing novel selective estrogen receptor modulators (SERMs) tagged to a magnetic resonance or fluorescence contrast agent (i.e., chelated lanthanides). ERα plays an important role in the development of female reproductive organs, in bone homeostasis, and in neoplastic progression of breast cancers. It is the primary target for hormonal therapy of breast cancer and its level serves as a marker to predict response to endocrine therapy. The level of ERα and its response to estrogen is tightly controlled *in vivo*. The dynamic fluctuations of ERα are primarily mediated through the ubiquitin-proteasome pathway in response to the changing cellular environments. Different SERMs affect ERα degradation mechanism in a different manner; estrogen-induced ERα protein turn-over is concomitant to estrogen-induced transcriptional activation and coactivator recruitment whereas tamoxifen stabilizes ERα and does not enhance its degradation. Thus,

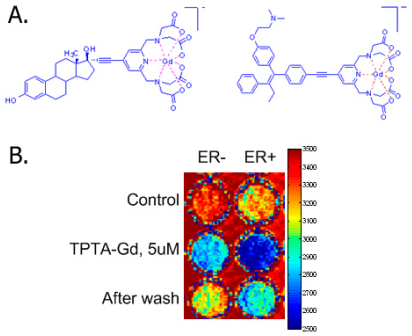

Fig. 2 *A. Structure of EPTA-Gd (left) and TPTA-Gd (right).*
B. T1 maps of ER+ and ER- MDA-MB-231 cells cultivated on Biosilon beads using standard medium, in the presence of 5 μM TPTA-Gd, and after washout of TPTA-Gd with standard medium.

proteasome-mediated degradation may play an important role in ERα transcriptional function and its antagonistic mechanism.

We have investigated the structural, functional, and imaging properties of two novel SERMs composed of 17β-estradiol or tamoxifen conjugated to a lanthanide (Gd(III) or Eu(III)) pyridiniumtetraacetic acid: EPTA-Gd and TPTA-Eu, respectively; (Figure 2A). Binding affinities of ERα to these new SERMs, measured by competitive radiometric and by Eu-fluorescence assays, were found to be in the micromolar range. Structural information was obtained by x-ray diffraction studies of the ligand binding domain bound to the novel SERMs. Various cellular hormonal-induced activities (proliferation and specific upregulation of induced genes), as well as degradation were tested in ER-positive MCF7 and T47D human breast cancer cells, and in MDA-MB-231 human breast cancer cells, engineered to express ERα under tetracycline, in comparison to ER-negative MDA-MB-231 cells. EPTA derivatives are agonistic and enhance ER degradation in ER positive cells. TPTA derivatives show a mild agonistic activity but do not enhance ER degradation. Both EPTA-Gd and TPTA-Gd enhance the magnetic resonance T1 and T2 relaxation rates in solution and show increased enhancement in ER-positive living human breast cancer cells cultured on beads, indicating binding to ERα (Figure 2B).

In conclusion, the first estrogen and tamoxifen conjugated Gd(III) and Eu(III) contrast agents have been designed, synthesized and their structure and function in cell free and in human breast cancer cells have been studied. The results indicate that these agents open the possibility for *in vivo* mapping of the level of ERα in a quantitative manner. *In vivo* studies in the rat uterus and in breast cancer xenografts are currently underway.

Vascular perfusion and Barriers to Drug Delivery in Tumors

Drugs are transferred to tissues according to concentration gradients and pressure gradients. The latter gradients are largely determined by the interstitial fluid pressure (IFP). Solid tumors often develop high interstitial fluid pressure due to water accumulation as a result of increased water leakage from the capillaries and impaired lymphatic drainage. In addition, increased collagen production and interaction with the fibroblasts surrounding the tumors' cells induce pressure in the tissue. The high interstitial fluid pressure modulates the physiological positive pressure gradients from the capillaries outwards and induces outward convection forming a barrier to drug delivery. We have developed a novel, non invasive method based on MRI, which maps throughout the entire tumor the transfer rates determined by pressure gradients and concentration gradients. This method can, therefore, be used to predict barriers to drug delivery. Furthermore, we demonstrated its application in monitoring reduction of IFP induced by treatment with collagenase, an enzyme that degrades the collagen fibers and reduces interstitial hypertension (Figure 3). The method and its application to monitor collagenase effects were tested in H460 ectopic human non-small-cell lung cancer xenografts implanted in immunodeficient mice. It is based on sequential recording of images during slow infusion of a Gd-based contrast agent followed by analysis with a novel physiological model of tumor perfusion. The tumors exhibited positive pressure dependent transfer constants at the boundaries and negative pressure dependent transfer constants in internal region. These negative transfer constants reflected increased interstitial fluid pressure as was confirmed by using the "wick in needle" method.

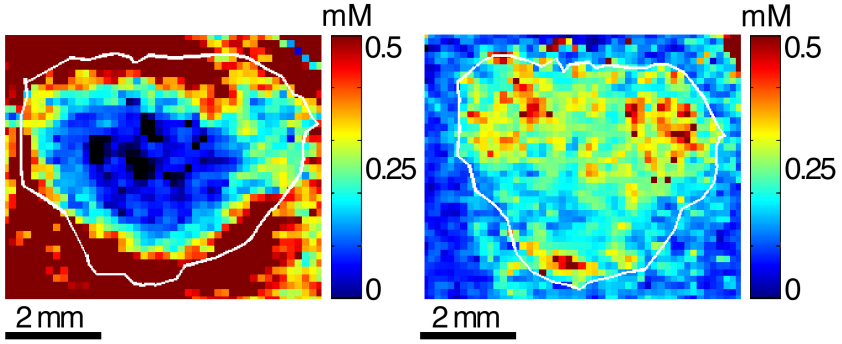

Fig. 3 *Collagenase induced changes in IFP of human lung cancer tumor in mice.*
Map of the steady state tissue GdDTPA concentration 24 hours before (left) and 5 h after administration of collagenase (right), calculated from T1 relaxation rates.

In summary, our contrast enhanced MRI methods using slow and steady state infusion protocols provide new imaging means for mapping the in vivo physiological parameters that determine the barriers to successful drug delivery to tumors, as well as a quantitative measurement for testing new drugs that eliminate these barriers.

Vascular Perfusion of Lung Cancer

Tumors in the lung can obtain their blood supply from two different systems: the pulmonary and bronchial circulations. Consequently, angiogenesis and perfusion of nodules in the lung, particularly lung carcinoma,

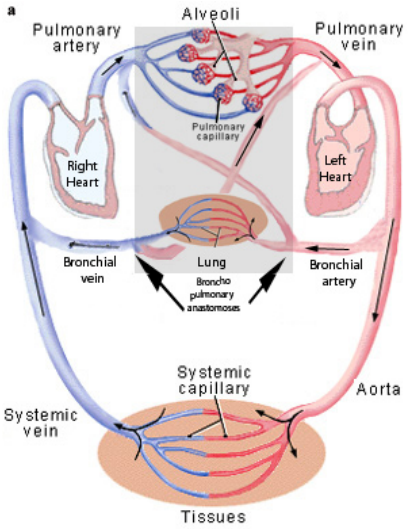

Fig. 4 *A scheme of the two blood circulation systems (pulmonary and bronchial) in the lung and the body systemic capillary system.*

may exhibit unique features. Overall, there are only few studies on lung cancer neovascularization and little knowledge is available on the involvement of the bronchial and/or pulmonary systems in lung cancer angiogenesis. The unique dual blood supply in the lung underscores the importance of utilizing orthotopic animal models in the study of lung cancer vasculature and the effects of therapy on the progression of this disease; hence, we are concentrating on establishing such rodent lung cancer models, and combining optical imaging and MRI means to monitor angiogenesis and microvascular perfusion.

Anatomical and dynamic contrast enhanced MRI of human lung cancer in a rat model were acquired at high spatial resolution, registered, and analyzed, pixel by pixel and globally, by means of a model-based algorithm. The MRI output yielded color coded parametric images of the influx and efflux transcapillary transfer constants which indicated rapid microvascular perfusion. The transfer constants were about one order of magnitude higher than those found in other tumors or in non orthotopic lung cancer, with the influx constant median value of 0.42 min-1 and the efflux constant median value of 1.61 min-1. The rapid perfusion was in accord with the immunostaining of the capillaries that suggested tumor exploitation of the existing alveolar vessels. In addition, using lung cancer cells transfected with red fluorescence protein, we were able to detect lung cancer in a mouse model employing optical *in vivo* imaging. The angiogenic processes and neovascularization of these tumors are now being investigated.

As lung cancer is responsible for most deaths due to cancer around the world, we hope that this research will potentially improve the MRI detection and diagnostic capability of this malignancy and also help developing specific lung cancer antiangiogenic therapies.

The effects of the Microenvironment on Choline Metabolism in Human Breast Cancer cells and Orthotopic tumors in Mice

Magnetic Resonance Spectroscopy (MRS) of normal and malignant breast cells and tissues have indicated that choline metabolites, predominantly phosphocholine (PCho) and glycerophosphocholine (GPCho), are associated with breast malignant transformation. Thus, the presence of elevated levels of these metabolites is used as a diagnostic marker of breast cancer. We have previously explored the molecular basis and biochemical pathways responsible for the augmentation of PCho in various human breast cancer cell-lines. However, studies of choline metabolites in orthotopic breast cancer of the same cell origin implanted in mice have indicated differences between the levels found *in vivo versus in vitro.* Specifically, PCho remained high but GPCho was substantially elevated in part of the tumors as compared to its level in the cells. To find out how tumor microenvirnomental conditions affect choline metabolism we monitored by 31P MRS perfused breast cancer cells subjected to standard, acidosis and hypoxic conditions, as well as extracts of cells subjected to these conditions.

Changing from standard to acidic medium (pH ~6) indicated a reduction in the external pH, and to a lower extent in the intracellular pH. Acidosis induced a 50% reduction in PCho level whereas hypoxia induced a 30% increase in PCho. The reduction upon acidosis was reversible after returning to standard conditions. GPCho levels increased under both acidosis and hypoxia. Thus, it appears that acidosis and hypoxia differentially affected the synthesis (of PCho) and breakdown (to GPCho) of phosphatidylcholine suggesting independent mechanisms of action for these processes in response to various stress conditions.

In conclusion, the dynamic changes and high heterogeneity of the microenvironmental conditions in breast cancer would affect the level and distribution of these metabolites. Avergage values over the entire tumor of the combined PCho and GPCho signal should therefore be cautiously interpreted, particularly during monitoring of response to therapy.

Kinetic Study of Hyperpolarized Pyruvate Metabolism in Living Breast Cancer Cell Cultures (In collaboration with Prof Lucio Frydman, Department of Chemical Physics)

Many metabolic processes are altered in the course of malignant transformation. For example, it has been well established since the early work of Warburg that cancer cells usually have a higher rate of aerobic glycolysis and hence lactate production than normal cells. Monitoring 13C labeled substrates and products by 13C MRS is a suitable method for determining non-invasively the kinetics of numerous metabolic processes. However, previous attempts to study metabolic processes in living cells have been limited to relatively slow kinetics due to the low signal-to-noise ratio of 13C enriched metabolites at physiological concentrations. Recently, a new technique has been developed that enhances the magnetic resonance signal of 13C more than 10,000 in the liquid state (Ardenkjaer-Larsen et al., 2003, PNAS, 100:10158-63). We currently use pyruvate hyperpolarized by this method to characterize at high temporal resolution the kinetics of its conversion to lactate in living breast cancer cell cultures.

MDA-MB-231 and T47D breast cancer cells cultured on beads showed a proportional increase in lactate production as cell number increased, with an average rate of 15.02±1.92 nmol/s/10^8 cells. Studies are underway integrating a perfusion system with the injection of the hyperpolarized pyruvate and investigating a range of metabolic processes ocurring in breast cancer cells.

Breast cancer detection and diagnosis using a hybrid Model-Free and Model-Based Analysis of Dynamic Contrast Enhanced MRI

Breast cancer is the most common malignancy among women and a major health burden worldwide. The mortality rate from breast cancer has been fairly constant in western countries, and since 1990 a decrease has been detected where screening has been introduced. Dynamic contrast enhanced (DCE) breast MRI emerged to become an important adjunct tool for detecting and diagnosing breast lesions, as well as monitoring response to breast cancer treatment. Analysis methods of DCE-MRI can be divided to physiological based models that take into account the vascular and tissue specific features that influence tracer perfusion, and to model free algorithms that decompose enhancement patterns in order to segment and classify different tissue types. We developed a general hybrid method for analyzing DCE images integrating a mathematical, model-free technique, principal component analysis (PCA), with a model approach using the Three Time Point (3TP) method that characterizes tissue microvasculature function. We demonstrated the application of the method for breast cancer diagnosis. The PCA method yielded n eigenvectors, where n is the number of time points in a dynamic data set, of which only two were relevant for characterizing breast malignancy. The physiological interpretation of these two eigenvectors was revealed by a quantitative correlation with the 3TP method leading to a specific rotation of the eigenvectors so that they reflected wash-in and wash-out rates of the contrast agent. This hybrid method is fast, standardized, and can be used to improve breast cancer early detection and diagnosis.

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