WELCOME TO TOPIM 2013 – THE 7TH "HOT TOPIC IN MOLECULAR IMAGING" CONFERENCE OF THE ESMI

by Bertrand Tavitian – Chair of the TOPIM 2013 committee

Imaging the Hallmarks of Cancer

Welcome!

TOPIM (hot **TOP**ics in molecular **IM**aging) is the annual Winter Conference of the European Society for Molecular Imaging. Every year, TOPIM focuses on one hot topic chosen for its pertinence and timeliness, which is an exciting aspect, application or technique at the forefront of Molecular Imaging.

TOPIM takes place at the foot of the Mont Blanc, the highest mountain in Europe, in the village of Les Houches in the Chamonix valley with deep-rooted mountain traditions of hospitality and solidarity. The grandiose surroundings are a unique source of inspiration for visionary ideas, while the warmth and comfort of the remote chalet settings serve inter-disciplinary cross connections and informal discussions between participants. By combining expert descriptions of the most up-to-date imaging technologies and applications, TOPIM contributes in collectively describing and categorizing imaging approaches, and draws the landscape of in vivo imaging approaches for a given scientific issue.

A glance at past hot topics in previous years

The first TOPIM took place in 2007 on **Imaging in Neurodegeneration and Neuroinflammation**. What was pretty much a bench test for a new style of conference turned out into a tremendous success. For many of the participants it was a revelation that much added value and drive could stem from warm, friendly and informal discussions. Collaborations were started, future programmes were elaborated and TOPIM was on its way!

TOPIM 2008 followed with an ambitious programme on the **Imaging of Nano Objects**, groundbreaking food-for-thought at a time when the field of nanomedicine was beginning to bloom. TOPIM contributed in installing the complex but fruitful dialogue between material scientists, chemists and imaging scientists.

In 2009, **Dual and Innovative Imaging Modalities** continued the tradition of TOPIM to focus on subjects on the verge of swinging from bench to bedside. Again, this was an eye-opener towards the fantastic inventiveness of imaging physicists and their capacity to translate it into in vivo (pre)clinical applications.

TOPIM 2010 was special in two respects: it took place on the Italian side of the Alps in Bardonecchia, and it dealt with **Imaging and Systems Biology**, a beautiful demonstration of how interdisciplinarity can sparkle new modes of thinking and new avenues.

TOPIM 2011 returned to Les Houches and to a translational topic, Emerging Imaging Methods in Medicine. This edition showed how sophisticated modern imaging techniques fertilize the medical arts, demonstrating that today, more than ever, the eye guides the hand.

Last year, TOPIM 2012 **Processing Biomedical Images – Visualization, Modelling, Segmentation, Quantification, Registration** discussed how one extracts the relevant information from biomedical in vivo images. The provocative – or surrealistic - question was : "if images speak for themselves, exactly what language are they speaking?" and we attempted to answer it by discussing the state of the art and the future in modern processing of biomedical images.

Imaging the Hallmarks of Cancer

is the hot topic for TOPIM 2013

"Neither the sun nor death can be looked at with a steady eye" wrote Francois de La Rochefoucauld (1613-1680). In vivo imaging demonstrates that cancer is no longer synonym of death and can be looked at with steady eyes. Still, our vision lingers somewhere in between blindness and clarity, and there is an urgent need for methods that can produce in vivo images of the biological hallmarks of cancer. How can we acquire "better eyes" for a better vision of oncological disorders ?

Robert N Wagner Jr., who liked prophecies, wrote this sentence in 1995: *"If medicine is molecular, then imaging has to become molecular"*[1]. Cancer is a molecular disease and imaging cancer aims to address its molecular determinants for detection, staging, progression and followup of therapy. The success of 2-[^{18}F]fluoro-2-D-deoxyglucose (FDG) resides in its capacity to image a most prominent molecular hallmark of cancer, i.e. the maintenance of lactate glycolysis under aerobic conditions described by Otto Warburg. But not all cancer cells depict the Warburg effect and not all tumors take up FDG. What alternatives do we have? How can we image the other hallmarks of cancer defined by Douglas Hanahan and Robert A. Weinberg[2]: sustained proliferative signaling, evasion from growth suppressors and immune destruction, enabling of replicative immortality, tumor-promoting inflammation, activation of invasion and metastasis, induction of angiogenesis, genomic instability and mutation, resistance to cell death and deregulation of cellular energetics? What are the methods/tracers presently available and what are those of the future? Which targets are most relevant to staging, recurrence, and therapeutic response? How can we increase the pipeline leading to validation of imaging techniques in clinical settings? What is / what will be / the contribution of molecular imaging to the assessment of chemotherapy, radiotherapy, surgery?

This 7th edition of TOPIM is a unique opportunity to present and discuss the state of the art of in vivo imaging of the hallmarks of cancer. The organizers have tried to make the programme as exhaustive as possible, while keeping balance between submitted and invited presentations, and wish to express their deepest gratitude to the keynote speakers Douglas Hanahan from Lausanne, Switzerland, John Condeelis from New York, and Robert Gillies from Tampa, USA. We are most privileged that have kindly accepted to speak at TOPIM.

The scientific committee would like to thank the scientific board and the President of the Ecole de Physique des Houches for having made TOPIM possible, once again for the seventh time this year. It is a very great honour for TOPIM to be hosted in one of the highest places, in every sense of the word, of scientific Education. Our warmest thanks also to the administrative and support staff of the Ecole and to the governing board and the staff of the European Society for Molecular Imaging for supporting TOPIM.

[1] Wagner, H.N Jr. in "Principles of Nuclear Medicine", Wagner, Szabo, Buchanan, editors, Saunders, Philadelphia, 1995.; [2] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011;144(5):646-74.

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TOPIM 2013 PROGRAMME OVERVIEW

	Monday, 21 January	Tuesday, 22 January	Wednesday, 23 January
08:00 - 08:45		breakfast	
08:45 - 08:50	Welcome - Bertrand Tavitian, Paris	**invasion and metastasis** Live in vivo imaging of the impact of hypoxia on cancer cell migration and invasion - Anne Herrmann, Liverpool	**reprogramming of energy metabolism and genome instability** Image-guided development of metabolic interventions targeting DNA replication in cancer - Caius Radu, L.A.
08:50 - 08:55			
08:55 - 09:00			
09:00 - 09:05	**Introductory Keynote Lecture** Imaging Metabolic Hallmarks and Their Sequelae - **Robert Gillies, Tampa**	**invasion and metastasis** Efficient quantification of cell migration and invasion in three-dimensional time-lapse video sequences - Carlos Ortiz de Solorzano, Pamplona	
09:05 - 09:10			
09:10 - 09:15			
09:15 - 09:20			
09:20 - 09:25			
09:25 - 09:30			
09:30 - 09:35		**invasion and metastasis** Live imaging of metastatic cell interactions with components of tumor matrix - Witold Kilarski, Lausanne	**genome instability** Visualizing the DNA damage response in cells and tumors Dik van Gent, Rotterdam
09:35 - 09:40			
09:40 - 09:45			
09:45 - 09:50			
09:50 - 09:55			
09:55 - 10:00			
10:00 - 10:05		**invasion and metastasis** Visualizing Src targeting using intra-vital FLIM-FRET - Kurt Anderson, Glasgow	
10:05 - 10:10			
10:10 - 10:15			
10:15 - 10:20		coffee break	
10:20 - 10:25			
10:25 - 10:30			
10:30 - 10:35			
10:35 - 10:40			
10:40 - 10:45			
10:45 - 10:50	**Introductory Keynote Lecture** Imaging metabolic hallmarks and their sequelae - **Robert Gillies, Tampa**	**invasion and metastasis** Non-invasive bioluminescence imaging animal models to study tumor growth and metastases progression of human colorectal cancer - Yolanda Fernandez, Barcelona	**inducing angiogenesis, and activating** Imaging the hallmarks of cancer: tumor angiogenesis - Fabian Kiessling, Aachen
10:50 - 10:55			
10:55 - 11:00			
11:00 - 11:05			
11:05 - 11:10			
11:10 - 11:15			
11:15 - 11:20			
11:20 - 11:25		**reprogramming of energy metabolism** Hallmarks of cancer: reprogramming of energy metabolism John Griffiths, Cambridge	**inducing angiogenesis, and activating** VEGFR-2 expression changes during anti-angiogenic therapy:assessment by immunohistochemistry and ultrasound molecular imaging - Mathew von Wronski, Plan-les
11:25 - 11:30			
11:30 - 11:35			
11:35 - 11:40			
11:40 - 11:45			
11:45 - 11:50			
11:50 - 11:55		**reprogramming of energy metabolism** Targeting Amino Acid Transporters LAT1, ATB0,+ and Dopa Decarboxylase DDC for Tumor Characterization by PET - Stefanie Krämer, Zurich	**inducing angiogenesis, and activating** Evaluation of preclinical, multimodal therapy monitoring based on multiparametric MR using a hNIS- expressing tumour xenograft mouse model - Eva Jolanthe Koziolek, Hamburg
11:55 - 12:00			
12:00 - 12:05			
12:05 - 12:10			
12:10 - 12:15			
12:15 - 12:20			
12:20 - 12:25			
12:25 - 12:30			
12:30 - 13:00		lunch	
13:00 - 15:45		break	
15:45 - 15:50			
15:50 - 15:55			
15:55 - 16:00			
16:00 - 16:05	**invasion and metastasis** The MET oncogene: a functional marker for stem and cancer stem cell - Paolo Comoglio, Candiolo/Torino	**Keynote Lecture** Hallmarks of cancer: From concepts to applications **Douglas Hanahan, Lausanne**	**Keynote lecture** The behavior of cells in breast tumors during invasion and metastasis - **John Condeelis, New York**
16:05 - 16:10			
16:10 - 16:15			
16:15 - 16:20			
16:20 - 16:25			
16:25 - 16:30			
16:30 - 16:35			
16:35 - 16:40			
16:40 - 16:45			
16:45 - 16:50	**invasion and metastasis** Non-Invasive bioluminescent imaging of protease activity in living mice - Elena Dubikovskaya, Lausanne	Coffee break & **Poster session 1** poster# 1-12	Coffee break & **Poster session 2** poster# 13-25
16:50 - 16:55			
16:55 - 17:00			
17:00 - 17:05			
17:05 - 17:10	**invasion and metastasis** Hypoxia induced plasticity of cancer cell invasion: collective to amoeboid-transition - Steffi Lehmann, Nijmegen		
17:10 - 17:15			
17:15 - 17:20			
17:20 - 17:25			
17:25 - 17:30	break		
17:30 - 17:35			
17:35 - 17:40			
17:40 - 17:45			
17:45 - 17:50			
17:50 - 17:55			
17:55 - 18:00	**sustaining proliferative signaling** Imaging signal transduction in cancer - Eyal Mishani, Jerusalem	**enabling replicative immortality** Intravital lineage tracing to study replicative immortality of tumor cells - Jacco van Rheenen, Utrecht	**inducing angiogenesis, and activating** Imaging the Hallmarks of Cancer - Inducing Angiogenesis - Klaas Nicolay, Eindhoven
18:00 - 18:05			
18:05 - 18:10			
18:10 - 18:15			
18:15 - 18:20			
18:20 - 18:25			
18:25 - 18:30			
18:30 - 18:35			
18:35 - 18:40	**sustaining proliferative signaling** Intravital imaging of cancer stem cell plasticity in mammary tumors - Anoek Zomer, Utrecht	**open discussion on** invasion and metastasis sustaining proliferative signalling enabling replicative immortality	**inducing angiogenesis, and activating** Multi-modal PET/MR imaging of angiogenesis induction and inhibition in an orthotopic rat model of glioblastoma - Thomas Viel Muenster
18:40 - 18:45			
18:45 - 18:50			
18:50 - 18:55			
18:55 - 19:00	**Champagne Reception by BiospaceLab**		**inducing angiogenesis, and activating** Multiparametric Imaging of Patient Derived Human Cervix Cancer Xenografts. Shawn Stapleton, Toronto
19:00 - 19:05			
19:05 - 19:10			
19:10 - 19:15			
19:15 - 19:20			
19:20 - 19:25			
19:25 - 19:30			
19:30 - 19:45			dinner
19:35 - 19:40			
19:40 - 19:45			
19:45 - 20:15	dinner		
20:15 - end	come together	come together	**open discussion on** reprogramming of energy metabolism inducing angiogenesis, and activating genome instability

TOPIM 2013 PROGRAMME OVERVIEW

	Thursday, 24 January	Friday, 25 January
08:00 - 08:45		
08:45 - 08:50		**evading immune destruction**
08:50 - 08:55	best poster presentation	Dynamic imaging reveals serial killing ofcCancer cells by CTL: New
08:55 - 09:00	poster session 1	strategies to overcome tumor resistance niches -
09:00 - 09:05		Bettina Weigelin, Nijmeger
09:05 - 09:10		
09:10 - 09:15		
09:15 - 09:20		
09:20 - 09:25	**resisting cell death**	**evading immune destruction**
09:25 - 09:30	Imaging tumour metabolism with hyperpolarized MRI -	Human gliomas and immune system:
09:30 - 09:35	Kevin Brindle, Cambridge	A complex interplay -
09:35 - 09:40		Federico Roncaroli, London
09:40 - 09:45		
09:45 - 09:50		
09:50 - 09:55	**resisting cell death**	
09:55 - 10:00	Use of hyperpolarized 13C-MRS to monitor tumor response to	best poster presentation
10:00 - 10:05	Sorafenib treatment, in comparison with DW-MRI - Benedicte	poster session 2
10:05 - 10:10	Jordan, Brussels	
10:10 - 10:15		
10:15 - 10:20		
10:20 - 10:25		
10:25 - 10:30		
10:30 - 10:35		
10:35 - 10:40		
10:40 - 10:45		
10:45 - 10:50		**evading growth suppressors**
10:50 - 10:55		Dynamic intravital imaging highlights the main role of
10:55 - 11:00	**evading growth suppressors**	microenvironment for glioblastoma progression -
11:00 - 11:05	The Role of p53 in the life of stem cells: p53 counteracts	Franck Debarbieux, Marseille
11:05 - 11:10	reprogramming by inhibiting	Comparison of 177Lu-DOTA,Tyr3-octreotate (177Lu-octreotate
11:10 - 11:15	mesenchymal-to-epithelial transition -	versus RAD001 in rat and mouse tumour models; increase of
11:15 - 11:20	Varda Rotter, Rehovot	metastatic tumour behavior by mTOR inhibition - Marion de Jong,
11:20 - 11:25		Rotterdam
11:25 - 11:30		
11:30 - 11:35	**inflammation**	**open discussion**
11:35 - 11:40	Imaging brain metastasis: SPECT imaging of tumour	evading growth suppressors
11:40 - 11:45	induced gliosis - Emma O'Brien, Oxford	
11:45 - 11:50		
11:50 - 11:55	**inflammation**	
11:55 - 12:00	MRI of reporter gene expressing cancer associated fibroblasts	conclusion -
12:00 - 12:05	enables in vivo measurement of transgenic cell fraction and peri-	Bertrand Tavitian
12:05 - 12:10	vascular localization - Moriel Vandsburger, Rehov(	
12:10 - 12:15		
12:15 - 12:20		
12:20 - 12:25		
12:25 - 12:30		lunch and departure
12:30 - 13:00		
13:00 - 15:45		
15:45 - 15:50		
15:50 - 15:55		
15:55 - 16:00		
16:00 - 16:05		
16:05 - 16:10		
16:10 - 16:15		
16:15 - 16:20	**inflammation**	
16:20 - 16:25	Tracking immune cells in action -	
16:25 - 16:30	Erik Aarntzen, Nijmegen	
16:30 - 16:35		
16:35 - 16:40		
16:40 - 16:45		
16:45 - 16:50		
16:50 - 16:55		
16:55 - 17:00	**evading immune destruction**	
17:00 - 17:05	Immune-mediated cytotoxic activity in tumors: lessons from	
17:05 - 17:10	in vivo imaging -	
17:10 - 17:15	Philippe Bousso, Paris	
17:15 - 17:20		
17:20 - 17:25		
17:25 - 17:30		
17:30 - 17:35		
17:35 - 17:40	break	
17:40 - 17:45		
17:45 - 17:50		
17:50 - 17:55		
17:55 - 18:00		
18:00 - 18:05		
18:05 - 18:10	**evading immune destruction**	
18:10 - 18:15	"All you can eat"; targeted induction of tumor cell	
18:15 - 18:20	phagocytosis -	
18:20 - 18:25	Wijnand Helfrich, Groningen	
18:25 - 18:30		
18:30 - 18:35		
18:35 - 18:40		
18:40 - 18:45		
18:45 - 18:50	**open discussion**	
18:50 - 18:55	inflammation	
18:55 - 19:00	resisting cell death	
19:00 - 19:05	evading immune destruction	
19:05 - 19:10		
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19:40 - 19:45		
19:45 - 20:15		
20:15 - end	come together	

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INMiND - Imaging of Neuroinflammation in Neurodegenerative Diseases.

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committee & support

day one: monday january 21, 2013

educational keynote lecture

IMAGING METABOLIC HALLMARKS AND THEIR SEQUELAE

Gillies, R.

H. Lee Moffitt Cancer Center Cancer Imaging and Metabolism, Tampa, USA

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Evolutionary Dynamics of Carcinogenesis

Malignant cancers, whether inherited or sporadic, can be characterized by genetic instability within highly selective local microenvironments. This combination promotes somatic evolution and the emergences of clades of cells in spatially explicit micro-habitats. The rate of this evolution is predicted by Evolutionary Game Theory, and is dramatically affected by both Phenotypic (Genotypic) Diversity and Selection Pressures. From a practical standpoint, malignancy can be defined by these habitats, which increase the probability that cancers will develop therapy resistant phenotypes. The concept of somatic evolution in cancers is not new, being articulated by Nowell in 1976 (1). However, it has been gaining wider acceptance (2-5), likely based on two related observations. First, malignant cancers have a high degree of mutational heterogeneity that can be traced to common ancestors (6, 7). Indeed, histological nuclear heterogeneity across cancers has been known for years (8) and is a strong predictor of poor prognosis (9). Second, therapies that are exquisitely targeted to driver oncogene mutations usually result in benefits measured in months, not years, (10). For most advanced cancers and most patients, response to therapy is fleeting, owing to the inevitable evolution and proliferation of a resistant population (11). Because of these large scale genomic alterations and consequent diversity, the emergence of resistance is a predictable and fundamental property of carcinogenesis itself. This is commonly ignored in the design of treatment strategies(12).

The origins of cancer heterogeneity and the accumulation of metabolic hallmarks occur early during carcinogenesis. All carcinomas develop within ducts, which are avascular environments. Consequently, the peri-luminal aspects of developing cancers are poorly perfused. These perfusion deficits lead directly to a physical microenvironment that is pooly oxygenated, substrate-limited, and acidic. This niche is genotoxic and highly selective for cancer cells that are hyperglycolytic, resistant to apoptosis, chronically autophagic and resistant to acidosis. The acidic microenvironment induces local invasion, which can be inhibited with systemic buffer therapy. Once cancers locally invade, if they can recapitulate the acidic-hypoxic-limited environment, they will have an evolutionary selective advantage over the stromal cells into which they invade. It can be shown by evolutionary theory that this environment will also generate distinct "clades" of tumor cells in spatially explicit micro-habitats. From a practical standpoint, malignancy can be defined by these habitats, which increase the probability that cancers will develop therapy-resistant phenotypes.

Imaging Cancer Physiology

Because the microenvironmental factors of hypoxia, acidity and glucose limitation are present in growing solid tumors and because these factors select for malignancy, there is a compelling need to develop non-invasive methods to measure them and their spatial distribution. Such information could have profound effects on our understanding of carcinogenesis and malignancy, and also provide important information for therapy decision support. Tumor oxygenation can be imaged in vivo using either magnetic resonance or positron imaging approaches, reviewed in (13, 14). For magnetic resonance, both nuclear (NMR) and electron (EPR) imaging approaches have been used. In MRI, both 19-F of exogenous hpoxia sensitive tracers, and 1-H of endogenous indicators of biological hypoxia have been used. EPRI commonly involves a stable free radical whose linewidths are oxygen dependent (15). In PET imaging most, but not all, tracers are based on a 2-nitroimidazole center, which becomes covalently trapped in the absence of oxygen. Optical methods are also available. The most used has been phosphorescence, but this use has generally been restricted to window chamber models. A new report uses fluorescently tagged antibodies against the biological hypoxia biomarker, CA-IX to identify hypoxic volumes in vivo. In almost all cases, important controls compare signal intensities to the immunohistochemical distribution of pimonidazole.

Tumor pH can also be imaged in vivo using either magnetic resonance or nuclear imaging approaches, reviewed in (16). In MRI, a number of approaches are available, including 31-P MRS of 2-aminopropylphosphonate, 1-H MRSI of imidazoles, MR relaxometry, and chemical exchange saturation transfer (CEST) with either hydroxyl or amide-containing tracers (diaCEST) or tracers containing paramagnetic rare earths (paraCEST). Radionuclide approaches are currently lmited to the use of a low pH insertion peptide, pHLIP, which can be labeled with 64-Cu or 18-F (for PET) or 99m-Tc for SPECT. Optical methods to measure pH are highly developed and can be used in vitro and in vivo. Until recently, the concentration of glucose in tumors had to be inferred from either invasive microperfusion systems, or through reaction-diffusion modeling. However, recently there are two independent reports where CEST MRI has been used to detect and measure glucose levels in tumors (17, 18). These approaches have great potential to illuminate metabolism of cancers.

Image 'omics

Because these microenvironmental factors are highly selective, they will amplify somatic evolution and the emergence of distinct genetically related sub-populations (clades) of cells within tumors. An emerging advance is to use profound image analysis ("radiomics") to identify these regions of heterogeneity (19). Heterogeneity can be viewed radiographically, wherein a non-uniform pattern of enhancement or attenuation ("texture") can be associated with poor outcome (20, 21). These radiographically visible sub-regions reflect underlying molecular and cellular alterations. In order to systematically address this issue, we have created a database structure that can be populated with images, as well as quantitative image feature data (e.g. Texture, Shape, Density features) that can be mined in combination with patient outcomes and genetic data from biopsies. This is allow real-time data analyses and association of features with prognostic, diagnostic and predictive models (22).

Current quantitative measurements are limited to dimensional measurements of tumor size via one (RECIST) or two (WHO) dimensional long axis measures (23). These measures do not reflect the complexity of tumor morphology or behavior, nor, in many cases, are changes in these measures predictive of therapeutic benefit (24). When additional quantitative measures are performed, they generally average values over an entire region of interest (ROI). In focused studies, texture features have been shown to provide significantly higher prognostic power than ROI-based methods (25-28). This is reflective of the fact that tumors are highly heterogeneous systems,

and that such heterogeneity has high prognostic power (29). Profound analyses of such image features can improve prediction of clinical CT (30), MR (31) or PET (32) images. Although paradigm-shifting, these analyses have been performed manually and the studies were underpowered. In order to qualify as a clinically useful biomarker, such studies have to be performed with larger cohorts in prospective, multi-institutional trials. In the current iteration of radiomics, image features have to be extracted automatically and with high throughput, putting a high premium on novel machine learning algorithm developments. The goal of radiomics is to convert images to mineable data, with high fidelity and high throughput. The radiomics enterprise can be divided into five processes with definable inputs and outputs, each with its own challenges that need to be overcome: (i) image acquisition and reconstruction; (ii) image segmentation and rendering; (iii) feature extraction and feature qualification (iv) databases and data sharing; and (v) ad hoc informatics analyses (19). Each of these steps must be developed de novo and, as such, poses discrete challenges that have to be met. For example, optimum protocols for image acquisition and reconstruction have to be identified and harmonized. Segmentations have to be robust and involve minimal operator input. Features have to be generated that robustly reflect the complexity of the individual volumes, but cannot be overly complex or redundant. Informatics data bases that allow incorporation of image features and image annotations, along with medical and genetic data have to be generated. Finally, the statistical approaches to analyze these data have to be optimized, as radiomics is not a mature field of study. Variation in results may come from variations in any of these individual processes. Thus, after optimization, another level of challenge is to harmonize and standardize the entire process, while still allowing for improvement and process evolution.

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THE MET ONCOGENE: A FUNCTIONAL MARKER FOR STEM AND CANCER STEM CELL

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Metastasis follows the inappropriate activation of a genetic program termed invasive growth (or epithelial-mesenchymal transition), which is a physio-logical process that occurs during embryonic development and post-natal organ regeneration. Burgeoning evidence indicates that invasive growth is also executed by stem and progenitor cells, and is usurped by cancer stem cells. The MET proto-oncogene, which is expressed in both stem and cancer cells, is a 'master gene' in the control of invasive growth, acting upstream 'squire genes' such as RON and ROR. MET encodes the tyrosine-kinase receptor for "Scatter Factor" -also known as HGF- a sensor of adverse microenvironmental conditions (such as hypoxia or ionizing radiations) and drives cell invasion and metastasis through the transcriptional activation of a set of genes ('the invasive growth signature), that can be exploited as 'surrogate markers' for MET activation. In cancer cells the MET tyrosine kinase stimulates cell scattering, invasion, protection from apoptosis and angiogenesis, thereby acting as a powerful expedient for dissemination. In some cancers, MET has been genetically selected for the long-term maintenance of the primary transformed phenotype, and those cancers appear to be dependent on (or 'addicted' to) sustained MET activity for their growth and survival. Because of its dual role as an adjuvant, pro-metastatic gene for some tumour types and as a necessary oncogene for others, MET is a promising target for therapeutic intervention. By Zr labelled monoclonal antibodies it is possible to detect MET amplification by PET imaging.Recent progress in the development of molecules that inhibit MET function –both small molecules and antibodies- will be discussed. Their application in subsets of human tumours potentially responsive will be considered.

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NON-INVASIVE BIOLUMINESCENT IMAGING OF PROTEASE ACTIVITY IN LIVING MICE

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Introduction: Proteases play an important role in tumor progression, invasion, and metastasis. However, no high-trough output methods exists to study activity of tumor-associated proteases in real-time non-invasively in living animals. In addition, no tools are currently available for such studies in combination with other cancer-related biological processes such as inflammation. The discovery of bioorthogonal reactions has had a tremendous impact on chemical biology, allowing the study of numerous biological processes directly in complex systems. However, despite the fact that multiple bioorthogonal reactions have been developed in the past decade, only the Staudinger ligation works well in living mice.

Methods: Here we report that D-cysteine and 2-cyanobenzothiazoles can selectively react with each other *in vivo* to generate a luciferin substrate for firefly luciferase. The success of this "split luciferin" ligation reaction has important implications for both *in vivo* imaging of proteases and bioorthogonal labeling strategies. First, the production of a luciferin substrate can be visualized in a live mouse by bioluminescence imaging, and furthermore allows interrogation of targeted tissues using a "caged" luciferin approach.We therefore applied this reaction to the real-time non-invasive imaging of apoptosis associated with caspase 3/7.

Results: Caspase-dependent release of free D-cysteine from the caspase 3/7 peptide substrate DEVD-(D-Cys) allowed selective reaction with 6-amino-2-cyanobenzothiazole *in vivo* to form D- aminoluciferin with subsequent light emission from luciferase. Importantly, this strategy was found to be superior to the commercially-available DEVD-aminoluciferin for imaging caspase 3/7 activity.

Conclusions: Similar to imaging of caspase 3/7 activity, the amine group on the D-cysteine moiety can be caged as a substrate for various proteases responsible for apoptosis or inflammation, prostate specific antigen, and many other protease specific sequences. It is noteworthy, that the synthesis of short peptide sequences with N-terminal D-cysteine can be easily performed with the help of automated peptide synthesis, which is a widely available and versatile technique. Therefore, this new tool represents a powerful approach for *in vivo* non-invasive real-time studies of the activity of many proteases, for which a protease-specific peptide sequence is known, as well as presenting a method for screening of new peptide sequences associated with proteases of interest directly in living animals. Moreover, the split luciferin approach enables the modular construction of bioluminogenic sensors, where either or both reaction partners could be caged to report on multiple biological events.

HYPOXIA INDUCED PLASTICITY OF CANCER CELL INVASION: COLLECTIVE TO AMOEBOID-TRANSITION

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Introduction: Tumor hypoxia, a clinically established micro-environmental parameter, strongly contributes to overall cancer progression to a metastatic disease phenotype and increased resistance to therapy. Through the stabilization of a family of oxygen-sensitive transcription factors, so-called hypoxia inducible factors (HIFs), reduced oxygen concentrations initiate an adaptive program which promotes angiogenesis, anaerobic metabolism, cell survival and cell migration. *In vitro* studies using cells grown in 2D monolayers link hypoxia and HIF activation to the induction of epithelial-to-mesenchymal transition (EMT), including increased motility and invasiveness. However, the relevance of these findings in more complex 3D environments and *in vivo* remains unclear.

Methods: To assess the impact of low oxygen concentrations on the mode and efficiency of cancer cell invasion, we used spheroid models of breast (4t1) and squamous carcinoma (SCC38) cells, embedded into extracellular collagen lattices. These 3D carcinoma cultures were incubated under normoxic or hypoxic (0.2% oxygen) conditions and their invasion behavior was monitored by live cell imaging or analyzed after 72-96 hours by immune-fluorescence staining combined with confocal microscopy. Imaging data were further confirmed by *in vitro* western blot. The role of hypoxia inducible factor (HIF) in the phenotypic changes observed under hypoxia, was further studied by pharmacologically inducing HIF activity using dimethyloxalylglycine (DMOG) under normoxic conditions and by stable, shRNA mediated knock-down of HIF-1α. To examine the functional consequences of hypoxic pre-incubation on the metastatic seeding potential *in vivo*, hypoxia-conditioned and normoxic control 4t1 cells, stably expressing firefly luciferase, were tail-vein injected into syngeneic mice (BALB/C) and their metastatic seeding behavior studied by bioluminescence *in vivo* imaging as well as *ex vivo* histological analysis.

Results: Within 72-96 hours, hypoxia led to a drastic EMT-like switch from collective to single cell dissemination, with down-regulation of E-cadherin and up-regulation of Snail and Oct-4. In line with these results, hypoxia produced individually migrating cells, moving in a mesenchmyal, protease-dependent manner. Beyond the classical concept of EMT, however, a subset of singly disseminated cells utilized an amoeboid, protease-independent migration mode with increased nuclear deformation and reduced pericellular collagen degradation as well as proteolytic trail formation. Hypoxia-induced plasticity of invasion was pharmacologically induced by DMOG, confirming a HIF-dependent process, which was reverted by stable knock-down of HIF-1α. Upon tail-vein injection of hypoxia-conditioned cells into syngeneic animals, significantly more metastatic lesions were observed in lungs from animals injected with the normoxic cells. However, the hypoxic seeds showed faster growth rates as determined by the relative increase in bioluminescence photon counts.

Conclusions: Our results suggest hypoxia to form a specific, micro-environmental niche, which induces pronounced plasticity of cancer cell invasion and dissemination via HIF-1α. While first *in vivo* experiments indicate a higher metastatic seeding potential of normoxic cells, metastatic seeds from hypoxia-conditioned cells showed faster growth rates.

IMAGING SIGNAL TRANSDUCTION IN CANCER

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wwProtein tyrosine kinases (PTKs) have a crucial role in signal transduction pathways that regulate both intracellular signaling and multicellular communication and their regulated signaling is pivotal to the development and survival of normal and cancer cells. PTKs catalyze the transfer of phosphate in ATP to specific tyrosine residues within proteins, thereby altering their structure and function. They are involved in multiple processes such as metabolism, cell movement, cell proliferation, angiogenesis and inhibition of apoptosis. Malfunction in PTK's is the hallmark of various human cancers. Hence, PTKs, whether receptors or cellular proteins, have become valid targets to combat cancer and the EGF and VEGF families of membrane receptors are the most relevant markers in this class. The concept of targeted therapies by specifically inhibiting PTK's in cancer is very attractive. Two approaches were attempted: The first was based on small organic molecule drugs targeting the internal TK domain and inhibiting autophosphorylation; the second, using antibodies targeting the extracellular ligand binding site. In the latter approach inhibition of cancer cell growth is achieved by either several direct processes including interruption of the receptor signaling by blockage of their ligands binding leading to inhibition of cell cycle progression or DNA repair, decelerating angiogenesis and/or inducing apoptosis, or via indirect processes mediated by the immune system. However, most of drugs that have either been approved by the FDA or have entered clinical trials have yielded suboptimal results. Enhancement of PTK's targeted therapy hinges on the development of a reliable in vivo quantitative molecular imaging method. This will enable monitoring receptor drug binding and receptor occupancy in vivo, determining duration of PTK's inhibition in vivo and potentially, identifying the existence of a primary or secondary mutation in PTK's leading to either drug interaction or loss of PTK's recognition by the drug. The presentation will describe the most recent developments and accomplishments in visualization and quantification of PTK's in cancer by nuclear medicine imaging modalities and will focus on small organic labelled compounds targeting the internal EGFR and VEGFR binding domains.

Notes

INTRAVITAL IMAGING OF CANCER STEM CELL PLASTICITY IN MAMMARY TUMORS

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Introduction: Tumors are heterogeneous and contain multiple cell types. It is widely debated whether all tumor cells in mammary tumors have the same potential to propagate and maintain tumor growth, or whether there is a hierarchical organization[1-3]. Studies using lineage tracing recently demonstrated the existence of a small population of cells with stem-like properties, referred to as cancer stem cells (CSCs), that maintains and provides growth in squamous skin tumors[4] and intestinal adenomas[5]. However, the tracing techniques used in these studies provide static images and lack the ability to study the gain or loss of stem-like properties, a process referred to as CSC plasticity, including non-CSCs that (temporally) become CSCs, quiescent CSCs that become active, CSCs that lose stemness and CSCs that become quiescent. Here we investigate whether the growth of mammary tumors is hierarchically organized and whether this organization is dynamic.

Methods: To study whether mammary tumor growth is hierarchically organized, we developed intravital lineage tracing tools to trace individual tumor cells and their progeny in the same animal over periods of up to three weeks. Through our mammary imaging window[6], we lineage traced the growth of genetic PyMT mammary tumors that express a Creinducible confetti construct. In these mice, expression of one of four confetti colors (CFP, GFP, YFP, RFP) is stochastically induced in Cre-expressing cells and therefore, these cells and their progeny will be genetically marked which allows intravital lineage tracing[7].

Results: By combining transgenic confetti reporter mice that spontaneously develop breast tumors with the latest intravital microscopy techniques we show the presence of mammary CSCs in non-manipulated primary mammary tumors, an observation which is consistent with the recent identification of CSCs in squamous skin tumors and intestinal tumors. Moreover, we observed CSC-plasticity: cells gain and lose stemness and therefore the potential to provide lineage tracing.

Conclusions: We report for the first time the existence of CSCs in unperturbed mammary tumors and demonstrate CSC plasticity. Our data indicate that existing CSCs disappear and new CSCs form during mammary tumor growth, illustrating the dynamic nature of these cells.

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LIVE *IN VIVO* IMAGING OF THE IMPACT OF HYPOXIA ON CANCER CELL MIGRATION AND INVASION

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Introduction: I*n vivo* studies of cancer cells have often focused on classical histological methods, end point measurements or fixed tissue samples thus failing to address the dynamic spatio-temporal behaviour of tumour cells in patients. We herein report a live imaging system using an avian model to study cancer cell migration and invasion permitting real-time visualisation of cellular dynamic events in the intact living organism that are crucially linked to the progression of cancer metastases and lethality. Oxygen deprivation (hypoxia) occurs in solid tumours such as neuroblastoma and has been suggested to promote reprogramming of neuroblastoma cells leading to the emergence of an aggressive stem cell phenotype able to evade chemotherapy and resulting in poor clinical prognosis. We use a versatile chick embryo model to investigate the role of the oxygen microenvironment on the dedifferentiation and aggressiveness of neuroblastoma cells *in vivo*.

Methods: To monitor neuroblastoma cells and their ability to invade tissues and form tumours, neuroblastoma cells were labelled with fluorescent proteins and grown under several levels of oxygen. Single-cell suspensions were injected intravenously into chick embryos at embryonic day 3 (E3). Cells were either (i) imaged *in ovo* and followed for up to E10 using a standard fluorescent stereo microscope prior to dissection or (ii) the embryo was retrieved from the egg immediately after injection, transferred onto a glass bottom dish and imaged with a custom-built high-resolution fluorescent microscope for up to 30 h. Neuroblastoma cells submitted to varied culture conditions such as adherent cells, 3D tumour spheres or under low oxygen tensions to mimic intratumoural conditions were injected in the chick embryo and their behaviour in terms of migration velocity and tissue invasion was imaged and measured.

Results: Neuroblastoma cells subjected to hypoxia (1% O2) showed a decreased cell mobility in the blood vessel with an increased aggregation when compared to co-injected cells grown in normoxia. These differences could be observed instantly after injection and persisted during embryonic development where cells cultured under hypoxia were found to form tumour-like structures in the chick embryo and its extra embryonic blood vessels. In contrast, cells cultured under normoxia could be found only as single cells surrounding the 'tumour-sphere' and showed a weaker survival rate as well as a more differentiated phenotype.

Conclusions: The ability to study the impact of hypoxia on cancer cell dynamics in live intact embryos using high-resolution imaging is an important step toward understanding neuroblastoma behaviour and the molecular mechanisms underlying its aggressiveness and unpredictable clinical behaviour. In conclusion, we are developing a novel intra-vital imaging technique to follow and track in real-time the fate of cancer and cancer stem cells injected in a chick embryo, allowing us to record the migration and invasion capabilities of cancer cells *in vivo*.

EFFICIENT QUANTIFICATION OF CELL MIGRATION AND INVASION IN THREE-DIMENSIONAL TIME-LAPSE VIDEO SEQUENCES

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Introduction: Cell migration and metastasis are complex phenomena regulated by the mechanobiology of the cells and the mechanical and chemical properties of the surrounding microenvironment. Since metastasis is a primary cause of cancer-related cell death, the development of strategies to quantify cell movement is of paramount importance to measure, for instance, the efficacy of cytoskeleton-targeted drugs. Using in-vitro three-dimensional assays, migrating cells can be imaged in realistic tissue environments. Given the amount of time-lapse image data produced in migration experiments, the analysis must rely on automated methods. Therefore we have developed a fast and accurate image-processing algorithm to i) track migrating cells while ii) quantitatively analyzing filopodial extensions in 3D+time fluorescence microscopy sequences. This algorithm provides a mathematically accurate description of cell movement, shape changes, and filopodia dynamics. We show a proof of principle of our algorithm using time-lapse series of migrating lung adenocarcinoma cells that either over-express the tubulin adaptor protein CRMP-2, fundamental for proper microtubule assembly, or express its phospho-defective form.

Methods: Our tracking algorithm was tested on 3D time-lapse videos of either overexpressing or phosphodefective CRMP-2-GFP-transfected lung adenocarcinoma cells embedded in a matrigel/collagen mixture. All videos were acquired using a spinning disk confocal microscope. The algorithm starts with detecting and tracking the cell body using a fast level set-like framework [1] over the whole time-lapse series. Next, each frame of the video is preprocessed using a steerable filter and a sequence of morphological filters is applied to delineate individual filopodia. Finally, we estimate their length using a geodesic distance transform and derive their dynamics by tracking the movement of their tips.

Results: Using our method, we were able to follow migrating cells of both phenotypes, while quantifying the number, average length, and dynamics of the filopodial extensions. The quantification of filopodia showed that altering the dynamics of tubulin polymerization modifies 3D migration of lung adenocarcinoma cells. Concretely increased tubulin dynamics in CRMP-2 lead to aberrant number and size of filopodia, which is especially relevant in the phospho-defective mutants, where it is also accompanied by loss of cell directionality.

Conclusions: Our tracking scheme combines simultaneous quantification of cell movement and the morphological characterization of the migrating cells. This method could be applied to model the phenotype of migratory cells and to test the efficacy of drugs that target the cytoskeleton to impair cell migration.

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LIVE IMAGING OF METASTATIC CELL INTERACTIONS WITH COMPONENTS OF TUMOR MATRIX

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Introduction: Non-malignant tumor stroma can compose up to 85% of volume of tumor mass and its elements can regulate affect tumor growth and metastatic spread. However, details on how matrix is used by tumor cells to reach lymphatics or blood vessels remain largely unknown.

Methods: Here we used time-lapse live immunofluorescence imaging technique and whole-mount confocal imaging to define the effect of tumor remodeled matrix on metastasis.

Results: Seven days after implantation, GFP-expressing B16-F10 melanoma cells formed irregular tumor lesions with metastatic single cells and cell clusters surrounding the tumor mass. Matrix (tenescin C, collagen I and collagen III) and basement membrane (perlecan, collagen IV, collagen III) staining revealed extensively remodeled stroma, damaged nerve fibers, blood and lymphatic vessels at the edges of the tumor mass. Individual tumor cells were also found within lymphatic vessels. Only a small fraction of individual tumor cells appeared motile and these cells exhibited two types of cell motility: fast, single-ameboid and integrin independent cell migration that was paralleled by slow tissue contraction, and slower, matrix-dependent individual and collective movement of mesenchymal-type tumor cells. Mesenchymal cells preferentially moved along tenescin C matrix. The pattern of individually fast migrating cells resembled that of embryonic cell streaming, where cells follow one another in pre-formed tissue channels. By following these matrix channels fast migrating tumor cells could enter lymphatics. Cancer-associated fibroblasts (CAF) were align along the matrix edges and were positive for tenescin C as show by live imaging and confocal analysis. We also show that VEGF-C over-expressing tumor cells are more mobile than control cells, which was related to induction of CCL21 expression and matrix deposition.

Conclusions: We confirm usability of our immunofluorescence intravital imaging system by detecting major types of metastatic cell migration. In addition, by exploring the main advantage of our method, the detection and functional blocking of various matrix components of the tumor stroma, we identified tenescin C as the preferred substrate for migration of melanoma cells. We also propose that metastatic spread can be inhibited by targeting at the interaction between metastatic cells and tumor matrix.

VISUALIZING SRC TARGETING USING INTRA-VITAL FLIM-FRET

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Introduction: Pancreatic Ductal Adenocarcinoma (PDAC) is the 4th most deadly form of cancer, with 5 year survival rates of less than 5%. Pancreatic tumors are poorly vascularized and highly desmoplastic, which limits drug perfusion and contributes to poor therapeutic response. A mouse model of pancreatic cancer based on two common human mutations, KRas and p53 (KPC model (1)), closely mimics the onset and progression of human PDAC. Dasatanib, a clinically approved Src inhibitor, has been shown to severely inhibit metastasis of primary tumours in this model (2) and is currently in clinical trial for treatment following primary tumor resection. Here, we have used intra-vital fluorescence lifetime imaging (FLIM) of a FRET reporter (3) to analyze the spatial and temporal response of PDAC tumor cells to dasatinib treatment.

Methods: Cells derived from primary KPC tumors were stably transfected with a FRET reporter, which changes conformation in response to phosphorylation by Src, and dephosphorylation by antagonistic phosphatases4. Control and Dasatinib treated cells were analyzed in vitro to determine the range of active (2.4-2.5 ns) and inactive (2.2-2.3 ns) reporter lifetimes (respectively), which were subsequently used to classify single cells in vivo as „Src-active" or „Srcinactive". Src-reporter cells were subcutaneously injected into SCID mice and allowed to form tumors, which were treated with Dasatinib using a protocol previously shown to inhibit metastasis by 50%. Collagen SHG was used to identify central and peripheral tumor regions, and QuantumDots were used to identify blood vessels.

Results: In vitro, drug treatment suppressed Src activity, which rebounded to a maximum within one hour of washout and returned to baseline within 6 hours. In vivo, Src activity rebounded to a maximum at 16 hours following drug treatment, and returned to baseline by 24 hours. Src activity was higher at tumor borders than in central regions both before and after drug treatment, however treatment converted the majority of border cells from Src-active to Src-inactive. The fraction of Src-active cells increased with increasing distance from the nearest blood vessel. Dasatinib treatment dramatically reduced the fraction of Src-active cells up to a distance of 100 μm from the nearest blood vessel. The hedgehog inhibitor cyclopamine, which has previously been shown to increase tumor perfusion by reducing stromal content (4), increased the targeting efficiency of cells within 100 μm of a blood vessel, but did not improve targeting of cells beyond 100 μm.

Conclusions: We have used intra-vital FLIM-FRET to analyze pharmacodynamics at the single-cell level in a mouse cancer model. In this approach the same reporter can be used as a read-out for both cell-based assays and animal disease models, which facilitates the drug discovery process. Our results suggest an optimal dosing frequency of 6 hours. We found that Src activity was inversely correlated with proximity to vasculature, quantified the effects of a combinatorial treatment regime, and determined the effective perfusion limit of drug treatment.

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NON-INVASIVE BIOLUMINESCENCE IMAGING ANIMAL MODELS TO STUDY TUMOR GROWTH AND METASTASES PROGRESSION OF HUMAN COLORECTAL CANCER

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Introduction: Development of non-invasive clinically-relevant mouse models which mimic natural tumor progression and metastatic dissemination of human colorectal cancer (CRC) is an essential requirement to better understand the mechanisms of cancer metastasis and to improve clinical therapeutics. A new era of modeling cancer metastasis involves the use of optical imaging technologies to monitor tumor growth and colonization after introduction of cancer cells into the animals. The purpose of this study was to characterize the behavior of the human colorectal cell lines HT-29 and HCT-116 in a subcutaneous, cecum orthotopic and intrasplenic and intracardiac experimental metastases mouse models using non-invasive bioluminescence imaging (BLI) techniques.

Methods: Luciferase expressing HT-29 and HCT-116 cells were injected subcutaneously, orthotopically into the cecal wall, and into the spleen or left ventricle of the heart of immunodeficient nude mice. The tumor growth and metastatic dissemination patterns were quantitatively and continuously followed via BLI. Non-invasive monitoring of tumorigenicity and metastatic dissemination was also compared to the traditional assays of tumor volume or weight measurements and histology. *Ex vivo* BLI and histological analyses were performed to further identify the exact nature and location of cancerous lesions.

Results: Our results, using subcutaneous models, provide a direct validation of BLI as a powerful approach for non-invasive longitudinal monitoring and quantification of colorectal tumor growth and treatment efficacy, and in identifying new metastatic *foci*. The orthotopic colon model resembled the natural primary tumor microenvironment and the clinical pattern of CRC metastases that includes lymphatic, hematogenous and coelomic dissemination. Furthermore, the intrasplenic and intracardiac models resulted in hepatic and bone marrow metastases, respectively, sites with high clinical relevance in CRC. Accordingly, the use of BLI in small animals allows the implementation of more advanced orthotopic and experimental mouse models with almost the same simplicity as traditional ectopic subcutaneous models.

Conclusions: By combining the more advanced tumor models with non-invasive imaging technologies like BLI, we have broadened the range of clinically relevant experimental mouse models available to investigate CRC disease, providing powerful tools to further study the molecular bases underlying tumor and metastatic disease progression as well as aid in the development of new therapies or improvement of current ones. As a result, these BLI models will greatly help to improve and accelerate the evaluation of cancer treatment response and new biomarkers.

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HALLMARKS OF CANCER: REPROGRAMMING OF ENERGY METABOLISM

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One of the first biochemical abnormalities of cancers to be noticed was reprogramming of energy metabolism. In the early 1920s Otto Warburg found that cancer cells perform aerobic glycolysis, i.e. the conversion of glucose to lactate in the presence of an adequate oxygen supply, whereas normal tissues would metabolise the glucose oxidatively[1]. Warburg received the Nobel Prize in 1931 for his work on oxidative metabolism, and until his death in 1970 he argued that the lactic acid produced by cancer cells would make them acidic, and that this low intracellular pH (pHi) and low pO2 were the cause of cancer. Nobody believes that theory nowadays, of course, but the Warburg Effect continues to fascinate researchers and has been the subject of much recent work.

When non-invasive metabolic measurements of living tumours by Magnetic Resonance Spectroscopy (MRS) became possible in the 1980s they didn't show the massively disordered metabolism and low pHi that Warburg's work had led us to expect: 31P MRS showed that tumours in vivo had fairly normal amounts of ATP and inorganic phosphate, and - a big surprise - that their pHi was neutral or even a little alkaline[2, 3]. In 1994 Gillies et al.[4] showed that it is the extracellular pH (pHe) of cancers that is acidic, suggesting that despite having the elevated rate of glycolysis found by Warburg, tumours can export lactate as fast as they make it. Lactate itself was measurable in rat tumours by 1H MRS[5] (note that the signals derive from lactate in both the extracellular and intracellular compartments) but in subsequent clinical 1H MRS studies on brain tumours (lactate is most easily measured in brain) lactate concentration has not correlated closely with tumour grade or with glucose uptake determined by FDG-PET[6].

Several other MRS-based methods can be used to study cancer energy metabolism. 13C MRS can be used to detect the metabolism of 13C-labelled substrates such as [U-13 C]Glucose in animals or even patients[7]. Tumour tissues or cultured cancer cells can be studied by in vitro 1H NMR by making extracts of the metabolites (note that although HR-MAS can be used to study metabolites in solid tissue specimens, it should be used with caution in work on energy metabolism, as pathways such as glycolysis could remain active in the specimen while the spectra are being accumulated[8]). This use of NMR to measure a number of metabolites simultaneously is effectively "metabolomics", a field in which it is more usual to use more sensitive mass spectrometry methods to measure larger numbers of metabolites. However, because it requires no preliminary chemical derivatisation or chromatographic separation, NMR has compensating advantages: it is more quantitative, sample preparation is easier, and there is less scope for sample bias due to factors such as ion suppression. Thus from NMR we know with good precision and reproducibility the relative concentrations of a relatively small number of metabolites in a sample. As well as the concentrations of metabolites in cultured cells (a "metabolic fingerprint") one can also use NMR to measure the changing concentrations of substrates and excretory products in the culture medium (a "metabolic footprint"). This approach is ideal for monitoring metabolomic experiments in which energy metabolism is perturbed, e.g. by genetic modification or the administration of drugs[9].

The detection of 18F-fluorodeoxyglucose (FDG) by Positron Emission Tomography (PET) is a quite different approach to studying tumour metabolism that was also developing in the

1980s10; unlike MRS it soon became a standard clinical method. Because of their reliance on glucose as their main substrate, most tumours take FDG up avidly, and once it is phosphorylated by hexokinase they cannot metabolise it further or (in most cases) dephosphorylate it, so the radioisotope becomes trapped, highlighting the tumour tissue. Mathematical models are used to infer the amount of intracellular FDG signal since the signal detected also includes both intravascular and extracellular components. Nowadays PET is invariably combined with CT, and 18FDG scanning has become routine in most hospitals for highlighting tumour deposits and distinguishing the ones that are metabolically active11. A number of other PET methods, notably 11C acetate and 11C choline, are used for some clinical purposes, but they do not appear to target energy metabolism.

In the 1990s the discovery of the HIF-1 pathway12 provided a mechanism to explain the Warburg effect13. Cancers tend to be hypoxic, which activates the HIF-1 pathway14 and HIF-1 expression correlates with tumour aggressiveness and poor outcome15; HIF-1 activity can also be upregulated by oncogenic signalling in some cancers16. In response to hypoxia, HIF-1 upregulates the transcription of hundreds of genes, including almost all the glycolytic enzymes and glucose transporters (GLUTs), suggesting a mechanism for the upregulation of tumour glycolysis. However, we have shown that tumours lacking HIF, and with downregulated expression of glycolytic enzymes and GLUTs, still have normal glycolysis17, suggesting that classical metabolic regulation is also involved. Carcinogenesis involving energy metabolism and the HIF-1 pathway was initially shown in rare, cancer-inducing renal and uterine genetic abnormalities18,19. These tissues lack the tricarboxylic acid cycle enzymes succinate dehydrogenase or fumarate hydratase; the resulting accumulation of succinate or fumarate activates HIF-1 (pseudo-hypoxia). More recently a much more common HIF-1 dependent carcinogenesis mechanism has been shown in brain tumours20. Mutations of the metabolic enzymes isocitrate dehydrogenase 1 and 2 are common in low-grade gliomas, astrocytomas and some leukaemias; they cause a gain-of-function resulting in formation of the oncometabolite 2-hydroxyglutaric acid. It is still not certain, however, that the carcinogenic effects of these mutations are actually mediated by HIF-1, or that if they are, that they involve energy metabolism.

After a long period in which genetic and cell biology studies of cancer predominated, tumour metabolism is once again of great interest. Several recent studies on the Warburg Effect have highlighted the role of pyruvate kinase M2, which redirects glycolytic intermediates into anabolism rather than ATP synthesis21,22. Switching expression from PKM2 to PKM1has been shown to abolish the Warburg effect in some tumour cells22. Another recent theme has been the role of glutamine as a fuel or as a source of anabolites for cancer cells23. Most of the studies mentioned so far have been performed on cultured cells. However, when solid tumours were studied in vivo a quite different pattern emerged. In the 1980s and 90s, Vaupel's group performed many classical metabolic balance studies on "tissue-isolated" subcutaneous tumours in vivo, which were grown in rats in such a way that they were supplied and drained by a single artery and vein. One of their papers gives substrate (almost entirely glucose) uptake and waste product (lactate and CO2) output for numerous tumour types.24 A little arithmetic on these results shows that, although the tumours did indeed secrete more lactate than normal tissues, at least half the glucose they consumed was oxidised to CO2, and because oxidative metabolism is much more efficient than glycolysis they therefore made 88% of their ATP aerobically25. In another study, the same group found that tissue-isolated tumours in vivo showed no net uptake of glutamine26, again contradicting the results from more recent cultured cell studies20.

The tissue-isolated tumour model is quite artificial and is technically challenging: could the anomalous (but in Vaupel's hands very reproducible) results it gives be artefactual? No, some metabolic balance studies on colon tumours in patients have also been reported27. These tumours, which had grown entirely normally, give results that were similar to those of the Vaupel group and in some ways even more anomalous. The tumours again subsisted almost entirely on glucose and took up negligible glutamine; only about a third of the glucose consumed was excreted as lactate which implies that up to 97% of their ATP was generated oxidatively25.

A plausible explanation for these anomalies is that the cancer cells exchange metabolites with the host cells within the tumour matrix. It is normally assumed that the lactate produced by tumour cells is distributed via the blood stream to host organs that then use it as an oxidative substrate or, in the case of the liver, recycle some of it to glucose. But perhaps a major proportion of the lactate produced by tumour cells is taken up and oxidised by the host matrix cells, so that it never leaves the tumour mass. Another possibility is that some well-oxygenated tumour cells take up and oxidise the lactate produced by other, anaerobically metabolising tumour cells. Either mechanism would result in solid tumours in vivo having an overall oxidative metabolism even if some or all of the cancer cells within them The metabolism of skeletal muscle is nowadays thought to involve the release of lactate by some (anaerobically metabolising) muscle cells and its uptake and oxidation by other muscle cells; furthermore, these muscle cells frequently interchange roles28. Something similar may take place in tumours, since transient periods of hypoxia frequently occur due to blood flow instability. Indeed Lisanti has proposed a "Reverse Warburg Effect" in which tumour cells instruct host fibroblasts to perform aerobic glycolysis, secreting lactate that the tumour cells use for oxidative metabolism29. Similarly, tumour cells may be able to take up glutamine from host cells. Such phenomena could account for the very different metabolic balances displayed by cultured tumour cells and solid tumours in vivo.

Non-invasive imaging methods combined with metabolic balance studies would seem to be ideal for clarifying these frustrating ambiguities, but none have been reported. My own group found the tissue-isolated tumour model too unstable to be usable in an MRS magnet. In a recent (highly invasive) study, 13C labelled glucose was infused i.v. into patients who were about to undergo surgery for removal of brain tumours7; tumour samples were then taken for analysis by mass spectrometry. The tumours produced lactate, as expected, but there was also significant oxidative metabolism in the TCA cycle; surprisingly however, much of the acetyl-CoA entering the TCA came from substrates other than glucose. Another unexpected finding was that glutamine was not taken up.

Another exciting possibility for non-invasive imaging of tumour metabolism is hyperpolarised 13C MRS. Most studies on cancer are on potential clinical applications, but Hu et al. have used 13C pyruvate infusion to demonstrate changes in energy metabolism during carcinogenesis in a Myc-driven liver cancer model. Pyruvate was converted to alanine in pre-tumorous lesions particularly in areas where tumour nodules would develop. When tumours did develop, the injected pyruvate was converted to lactate (along with increased lactate dehydrogenase and glycolytic enzyme expression); pyruvate to lactate conversion was rapidly inhibited during regression30. It should be noted, however, that although pyruvate, alanine and lactate are all glycolytic products, studies on the fate of injected hyperpolarised pyruvate will primarily monitor the lactate dehydrogenase reaction rather than glycolytic function. I will conclude by showing a study that supports the notion that both tumours and cultured tumour cells hydrate enough CO2 for bicarbonate to be a major acidifier of the tumour extracellular space. Using the pHe probe ISUCA and 1H MRSI, we have mapped the spatial distribution of pHe in a human tumour xenograft model that had been genetically-engineered to express high levels of carbonic anhydrase IX. Tumours over-expressing carbonic anhydrase IX had a more acidic pHe than control tumours.

HALLMARKS OF CANCER: REPROGRAMMING OF ENERGY METABOLISM

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TARGETING AMINO ACID TRANSPORTERS LAT1, ATB[0,+] AND DOPA DECARBOXYLASE DDC FOR TUMOR CHARACTERIZATION BY PET

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Introduction: Amino acid uptake and metabolism are increased in cancer cells. We aimed at developing ^{18}F-labeled probes for Positron Emission Tomography (PET) targeting the amino acid exchange carrier for large neutral amino acids (LAT1, SLC7A5), the concentrative transporter for neutral and cationic amino acids ATB[0,+] (SLC6A14) and the dopa decarboxylase (DDC), respectively. The expression level of LAT1 correlates with tumor malignancy and is, therefore, of prognostic value[1]. LAT1 may in addition serve as a target for anticancer therapy[2]. ATB[0,+] is upregulated in estrogenreceptor positive breast cancers, colorectal and prostate cancers. ATB[0,+] inhibition may evolve as a new therapeutic strategy[3]. Regarding DDC, high activity is a characteristic of endocrine tumors. Non-invasive imaging of the above targets not only allows to diagnose cancer but may also serve to characterize the malignant lesions individually and help deciding on the therapeutic strategy. PET probes for the above targets may furthermore be helpful for the in vivo evaluation of inhibitory drug candidates.

Methods: We synthesized the net neutral tryptophan analogs 6- and 7-(2-[18F]fluoroethoxy)-DL-tryptophan (6FEHTP, 7FEHTP) and 5-(2-[18F]fluoropropyl)-DL-tryptophan (5FPTRP) as well as the cationic tyrosine analog 2(2([18F]fluoroethyl)(methyl)amino) ethyltyrosine (FEMAET) and compared their uptake and efflux by prostate adenocarcinoma PC-3 cells and small cell lung cancer NCI-H69 cells (tryptophans). Xenograft uptake was studied by small animal PET and blood plasma and xenograft tissue were analyzed for metabolites.

Results: The ^{18}F-labeled amino acids were produced in ≥ 9 % radiochemical yield with specific radioactivities ≥ 20 GBq/μmol at end of synthesis. The tryptophan analogs were taken up into PC-3 and NCI-H69 cells in vitro. Uptake was inhibited to > 95 % by BCH, an inhibitor of LAT1/2 and ATB[0,+]. Among the tryptophan analogs, 6FEHTP showed the highest uptake into PC-3 and NCI-H69 xenografts, similar to or higher than O-(2-[18F]fluoroethyl)-L-tyrosine (FET) and L-3,4-dihydroxy-6-[18F]fluorophenylalanine (FDOPA), respectively. 6FEHTP was the only tryptophan analog with significant levels of metabolites in blood and xenografts. In vitro cell uptake of the cationic amino acid FEMAET was inhibited to > 95 % by BCH and to some extent by cationic amino acids. PC-3 cell uptake and efflux studies suggested the concentrative ATB[0,+] as the major uptake transporter. PET revealed accumulation of FEMAET in PC-3 xenografts. The tracer was metabolically stable in vivo and in human liver microsomes.

Conclusions: Compared to other tryptophan analogs including a previously published,[4] 6FEHTP shows surprisingly high uptake into tumor xenografts in mice. 6FEHTP was the only analog with significant metabolism. We are currently investigating whether the tracer is decarboxylated by DDC. All investigated tryptophan analogs are substrates of LAT1. The cationic amino acid PET probe FEMAET is most probably a substrate of ATB[0,+] and may serve as a tool to identify ATB[0,+]-positive tumors and to evaluate potential ATB[0,+] inhibitors in vivo.

HALLMARKS OF CANCER: FROM CONCEPTS TO APPLICATIONS

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The hallmarks of cancer constitute an organizing concept that may provide a rational basis for distilling the diversity and complexity of human cancers so as to better understand mechanisms of the disease and its manifestations (Hanahan and Weinberg, 2011). The conceptualization involves eight acquired capabilities – the hallmarks of cancer - and two generic characteristics of neoplastic disease that facilitate their acquisition during the multistage process of neoplastic development and malignant progression. The distinctive and quasi-independent hallmark capabilities consist of sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, deregulating cellular energetics and metabolism, and avoiding immune destruction. The principal facilitators of their acquisition are genome instability with consequent gene mutation, and tumor-promoting immune inflammation. The integration of these hallmark capabilities in symptomatic disease involves multiple cell types populating the tumor microenvironment, including heterogeneous populations of cancer cells, in particular cancer stem cells, and three prominent classes of stromal support cells – angiogenic vascular cells, cancer associated fibroblasts, and infiltrating immune cells. Notably, these stromal cells populating the tumor microenvironment have the demonstrated capacity to contribute to seven of the eight hallmark capabilities (Hanahan and Coussens, 2012). Thus, while the functional contributions of stromal cells and their pathologic importance will likely vary between different cancers and indeed at different stages of tumorigenesis and tumor progression, the evidence is clear that a sole focus on the transformed cancer cell (and its genome) cannot fully inform us about mechanism of the disease. One premise, to be discussed in this keynote lecture, is that the hallmarks of cancer may prove to be a useful heuristic tool for designing innovative new mechanism-guided (hallmark-targeting) therapeutic approaches for cancer treatment. A second premise to be considered in depth throughout this conference, is that the ability to image the hallmarks of cancer will likely prove to be instrumental in assessing the prominence of particular hallmarks in different types and subtypes of human cancer, and to monitor hallmark changes in response to therapy, to reveal both efficacy and direct/indirect forms of adaptive resistance.

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INTRAVITAL LINEAGE TRACING TO STUDY REPLICATIVE IMMORTALITY OF TUMOR CELLS

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It has been widely hypothesized that, similar to epithelial tissues, tumors are hierarchically organized; only a small number of stem-like cells referred to as cancer stem cells (CSCs) have replicative immortality and is therefore able to maintain and provide tumor growth. However, during tumor progression additional mutations may enable all tumor cells to acquire replicative immortality, leading to loss of hierarchical growth. Until recently, evidence for the presence of growth hierarchy in primary tumors came from transplantation assays, in which the capacity of specific tumor cell subpopulations to reform a tumor identical to the parental tumor is studied upon transplantation into immunodeficient mice. Although these studies clearly demonstrate the clonogenic capacity of specific cancer cells under transplantation conditions, it has been widely disputed whether these assays accurately reflect cell behavior required for growth of an unperturbed tumor. In my seminar, I will discuss how we have developed intravital imaging tools to visualize and lineage trace individual tumor cells in unperturbed tumors in living mice. We have intravitally lineage traced the growth of adenomas and carcinomas, and the presence of growth hierarchy both at early and late stages of tumor progression. Interestingly, we also observed that cells can gain or lose stemness capacity. This process which we referred to as stem cell plasticity could include non-CSCs that temporarily adapt stemness properties, or quiescent CSCs that "wake up" or "active" CSCs that become quiescent. From this we conclude that, in the models that we have investigated, only a small fraction of all tumor cells have the capacity to provide tumor growth, and that these cells show high plasticity.

day three: wednesday january 23, 2013

IMAGE-GUIDED DEVELOPMENT OF METABOLIC INTERVENTIONS TARGETING DNA REPLICATION IN CANCER

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In cancer cells, rapid proliferation, aneuploidy and genomic instability increase the demand for deoxyribonucleotide triphosphate (dNTP) precursors of DNA. To accommodate this demand, tumor cells can rely on two dNTP biosynthetic pathways. The de novo pathway (DNP), via the rate-limiting enzyme ribonucleotide reductase (RNR), utilizes glucose and amino acids to synthesize new dNTPs. An alternative route, the nucleoside salvage pathway (NSP), converts deoxyribonucleosides present in the extracellular environment to cytosolic dNTPs through phosphorylation by nucleoside kinases. The precise contribution of these two pathways to the production of dNTP pools in rapidly dividing cancer cells is unknown. Using liquid chromatography–mass spectrometry to perform metabolic flux analyses of deoxycytidine triphosphate (dCTP) biosynthesis in human acute leukemia cells we show that, under basal conditions, NSP's contribution to dCTP pools is dispensable. However, pharmacological abrogation of de novo dCTP synthesis by RNR triggers a rapid metabolic switch from the DNP to the NSP. Consequently, RNR inhibited cancer cells become dependent on the NSP enzyme deoxycytidine kinase (dCK) to maintain dCTP pools required for DNA replication. Using high-throughput screening and a new PET imaging-guided approach for lead optimization we developed a library of novel small molecule dCK inhibitors, which were screened in vitro across a panel of human and murine cell lines alone or in combination with RNR inhibition. In vivo measurements of pharmacological targeting of tumor dCK activity were performed non-invasively using specialized PET imaging probes developed by our group and were correlated with DNA replication stress, DNA damage induction and tumor regression. As many aggressive cancers demonstrate defects in DNA replication stress and damage response pathways, further studies will determine whether particular genetic and imaging signatures can enable tumor stratification for metabolic interventions targeting dNTP biosynthesis.

VISUALIZING THE DNA DAMAGE RESPONSE IN CELLS AND TUMORS

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The integrity of our DNA is constantly challenged by DNA damaging agents, such as radiation and exposure to chemicals. The cells in our body have various repair systems to counteract this damage and to regulate cell cycle progression and apoptotic responses, collectively called the DNA damage response (DDR). Most, if not all, tumors have lost one or more elements of the DDR, which allowed them to accumulate a multitude of mutations, which was necessary to become a tumor.

These defects in the DDR not only cause an increased frequency of mutagenesis, but also sensitize these cells to drugs that target another pathway of the DDR. This observation can be used to target tumor cells while leaving normal cells in the body relatively untouched. A compelling example of this approach is the application of PARP inhibitors to kill BRCA1 or BRCA2 deficient tumor cells. The current challenge is to find similar strategies to target other DDR defects and to develop analysis tools to identify tumors that would benefit from such a treatment.

Development of novel treatment strategies requires better understanding of the various DDR pathways and their interplay. We have developed several strategies to visualize DNA double strand break repair protein assembly at sites of DNA damage and repair kinetics in living cells using fluorescently tagged proteins. These approaches can subsequently be used to predict more precisely what the effect of therapeutic interventions will be.

Furthermore, we are also developing techniques to identify human (breast) tumors with defects in the homologous recombination (HR) pathway of DNA double strand break repair, which can subsequently be used to select these patients for PARP inhibitor treatment. For this purpose, we developed 'ex vivo' irradiation protocols and studied accumulation of the HR protein RAD51 at sites of damage (so-called IR induced foci). We have been able to show that this can indeed identify BRCA1 and BRCA2 deficient tumors (because they fail to support RAD51 foci formation). We are currently analyzing a larger panel of tumors from the clinic, where we identified a few tumors that did not support RAD51 foci formation, suggesting that this method might be useful to select patients for PARP inhibitor treatment. Similar visualization assays for other DNA damaging tumor treatments are currently under development.

IMAGING THE HALLMARKS OF CANCER: TUMOR ANGIOGENESIS

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Angiogenesis is one of the hallmarks of cancer that is considerably easy to characterize with many different imaging modalities and methods. Generally one can distinguish between the assessment of vessel micromorphology, physiological function and molecular characteristics. When characterizing these vascular characteristics at one single time point, however, one should consider that this is static information that does not really tell us much about the process of vessel formation and maturation but only about its consequences. Thus, in the strict sense longitudinal assessments are required to conclude about angiogenesis, which can favorably be done with non-invasive imaging methods. In order to characterize micro-vessel morphology, microscopic methods like two photon microscopy and optical coherence imaging techniques but also μCT and optoacoustic imaging have been established. All these methods are capable of resolving 3D structure of vessels down to capillary level.

Vascular physiology can easily be investigated with dynamic contrast enhanced ultrasound, MRI, and CT, which provide information about perfusion, relative blood volume and in case for the latter two also about vessel permeability. Many studies have proven that these parameters are highly suited to assess anti-angiogenic therapy effects sensitively at very early therapy stages. In addition, vessel maturation can be investigated with BOLD MRI, vessel size imaging (MRI) and ultrasound. The blood oxygenation can be characterisaed with photoacoustic imaging. Tumor hypoxia can further be characterized with PET using 18F-MISO or 18F-FAZA or 64Cu-ATMS.

Recent studies have shown that for characterizing tumors and in particular for distinguishing more malignant highly angiogenic ones from more benign ones, molecular imaging can be superior to functional imaging. For example, molecular ultrasound imaging of VEGFR2 proved to better discriminate differently aggressive breast carcinomas than contrast-enhanced determining the rBV. Other imaging modalities being regularly used to characterise angiogenesis with molecular imaging agents are PET, SPECT, MRI, optical and photoacoustic imaging. Of those, PET and ultrasound are also already used in clinical trials.

This talk will give an overview of the possibilities of microstructural, functional and molecular imaging of tumor angiogenesis. It will show how excellently imaging biomarkers of this tumor hallmark are suited to characterize tumors and to assess anti-angiogenic therapy response. In addition, the high clinical potential of some imaging methods will be highlighted.

VEGFR-2 EXPRESSION CHANGES DURING ANTI-ANGIOGENIC THERAPY:ASSESSMENT BY IMMUNOHISTOCHEMISTRY AND ULTRASOUND MOLECULAR IMAGING

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Introduction: Signaling through vascular endothelial growth factor receptor 2 (VEGFR-2) plays a key role in tumor angiogenesis. Numerous agents which block interaction with its ligand and/or its signaling pathways are either approved or in clinical trials. However, many patients' tumors respond poorly to these agents or develop resistance after variable lengths of treatment. To assess whether molecular imaging of VEGFR-2 expression could be used to measure the response to anti-angiogenic treatment at an early stage, we performed immunohistochemistry on rat tumors harvested after different lengths of sunitinib treatment and determined whether the protein-level VEGFR-2 expression reflected the anti-angiogenic effect of the treatment. BR55, an ultrasound contrast agent targeting VEGFR-2 (1,2) was used to image the expression of this receptor in the tumors prior to removing them.

Methods: The spontaneous rat mammary tumor model (N-nitroso-N-methylurea treatment) was used. When tumor cross-sectional areas reached 1.5 cm^2, animals received an anti-angiogenic treatment (sunitinib, LC-Laboratories, Woburn MA). Immunohistochemistry was performed on rat tumors harvested after different lengths of treatment (up to 3 days) using blinded readers to semi-quantitatively assess the protein-level expression of VEGFR-2. Vascular density was assessed by immunohistochemical detection of CD31 and also graded by blinded readers. Tumor response was monitored by ultrasound molecular imaging (USMI) periodically until sacrifice. Targeted contrast enhancement due to bound BR55 microbubbles was assessed 10 minutes after BR55 injection when most non-bound bubbles were removed from circulation.

Results: Before treatment, tumors were highly vascularized and demonstrated high VEGFR-2 expression as assessed by immunohistochemistry. Following treatment with sunitinib, a rapid and dramatic decrease in overall VEGFR-2 staining was observed which preceded changes in vascularity, starting within 12h of treatment. Within tumor cell islands this effect was much more rapid and pronounced than in the bands of stroma that segmented the tumor into multiple large foci (islands) of malignant cells. Perfusion and BR55 binding in the tumor as measured by USMI, also showed a rapid parallel decline, which was greater and more consistent for BR55 binding than perfusion. Tumor size also decreased with treatment, but more slowly and to an even lesser extent.

Conclusions: These data indicate that treatment with a VEGFR-2 signaling inhibitor resulted in a rapid and dramatic decrease in VEGFR-2 expression within NMU-induced mammary tumors which was successfully measured in vivo using USMI with BR55. This suggests that imaging with BR55 may be useful in detecting early responses to some angiogenesis inhibitors before morphological changes can be measured.

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keynote lecture

EVALUATION OF PRECLINICAL, MULTIMODAL THERAPY MONITORING BASED ON MULTIPARAMETRIC MR USING A HNIS- EXPRESSING TUMOUR XENOGRAFT MOUSE MODEL

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Introduction: On the way to individual therapy concepts for patients early diagnosis and therapy response assessment is mandatory. Improvements of preclinical methods to validate therapeutic effects of a novel drug candidate are therefore urgently needed. Novel in vivo imaging methods such as MRI and SPECT offer exact estimation of the total tumour mass noninvasively as well as functional and biological characterisation of the tumour tissue including the grade of vascularisation. As a result quantitative assessment of therapeutic effects in the tissue should further improve local and systemic tumour control. Dynamic contrast-enhanced MRI is an emerging imaging method to assess not only morphologic changes but also tumour angiogenesis and validate the changes of physiological alterations. A multiparametric MR analysis was established using various mouse tumour xenograft models in order to determine parameters such as apparent diffusion coefficient (ADC) and pharmacokinetic parameters for exact tumour tissue characterisation. In order to perform tumourspecific SPECT, tumour cells were transfected with sodium iodine symporter (NIS) and NIS-specific tracers such as TC^{99m} pertechnetate was used.

Methods: Tumour cells of human origin (colon, breast) were stably transfected with the hNIS gene. Mouse tumour xenograft models were established by inoculating ~10^6 cells over the right scapula subcutaneously. MRI Imaging was performed on a dedicated preclinical small animal 7T MR (ClinScan, Bruker) and SPECT imaging using a preclinical small animal SPECT/CT (NanoSPECT, Mediso). To monitor tumour growth by MRI, tumour size was measured using a T2-weighted sequence. Additionally, diffusion weighted imaging (DWI) and dynamic contrast enhanced (DCE) MRI (0.15 mmol/kg Gd-DTPA) were performed. To visualize tracer accumulation and measure tracer retention time, SPECT imaging was performed using Tc^{99m} (100 MBq).

Results: hNIS- expression in both tumour entities was visualized by a strong Tc99m-uptake via SPECT-imaging when compared to tumour xenografts bearing untransfected tumour cells. MR imaging allowed a precise delineation of tumour mass and estimation of tumour volume. Signal enhancement measured by DCE was predominantly found in the tumour periphery, whilst homogenous tumour tissue showed a more uniform contrast agent distribution. Increased apparent diffusion coefficient (ADC) in the tumour centre gave the indication of necrotic tissue.

Conclusions: The NIS-transfected tumour xenograft model seems to be a promising tool for tumour imaging and monitoring. Due to a more exact estimation of tumour mass even little effects in tumour volume shrinkage can be monitored compared to the current method using a calliper. Further validation will be performed by pharmacokinetic modelling of the contrast agent uptake and parameters such as perfusion and permeability used as correlated measures of the tumour tissue in order to predict therapy response. Tumour vascularisation and heterogeneity can be additionally visualised.

THE BEHAVIOR OF CELLS IN BREAST TUMORS DURING INVASION AND METASTASIS

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Multi-photon microscopy (MPM) allows the direct observation of the behavior of individual cells in vivo. In mammary tumors MPM demonstrates that invasive/migratory carcinoma cells form migratory streams and intravasate when associated with macrophages. Taking advantage of this macrophage tropism allows collection of the migratory competent macrophages and tumor cells as live cells directly from the primary tumor. Expression profiling of these co-migratory tumor cells and macrophages has led to the surprising conclusion that both cell types exhibit embryonic expression patterns. Further analysis indicates that the tumor cells express genes associated with breast cancer stem cells, apoptosis and cell cycle arrest, and DNA repair. Consistent with this unusual expression pattern are the phenotypes of the isolated tumor cells: radiation and chemotherapy resistance, arrest in G0-1 and a greatly amplified ability of tumor cells to find and co-migrate with macrophages and intravasate. Consistent with the embryonic expression pattern is the observation that tumor cell migration with macrophages in vivo in mammary tumors is reminiscent of cell migration during morphogenesis in the embryonic breast.

The expression pattern unique to the migratory tumor cells is called the Invasion Signature. Invasion, adhesion and motility pathways identified in the Invasion Signature converge on the RhoC/Cofilin/Mena pathway identifying it as a master regulator of chemotaxis, invasion and dissemination of breast tumor cells in vivo. Using markers derived from the RhoC/Cofilin/Mena pathway, anatomical landmarks have been developed for use with breast cancer patients. One of these, composed of an intravasating carcinoma cell marked by Mena over-expression, and a peri-vascular macrophage, is called TMEM (Tumor Micro-Environment for Metastasis) in human breast tumors. Related markers of metastatic risk are MenaCalc (relative expression of Mena isoform 11a), Mena ratio (MenaINV/Mena 11a in fine needle aspirates of breast tumors), and cofilin x P-cofilin, a marker of activation of the Cofilin/Mena pathway in tumor cells. These related markers can be organized to represent progression from EMT to migration to intravasation. They predict metastatic risk in human invasive ductal carcinomas of the breast as shown in 4 retrospective clinical studies. The molecular mechanisms behind these markers are a major focus going forward.

IMAGING THE HALLMARKS OF CANCER - INDUCING ANGIOGENESIS

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Being a prominent hallmark of cancer, the process of tumor angiogenesis is an important target of diagnostic imaging and image-guided therapy. This presentation will highlight some of the many tools that are offered by Magnetic Resonance Imaging (MRI) and nuclear imaging to address key aspects of tumor angiogenesis as well as anti-angiogenic therapies.

MRI provides a window on tumor anatomy, structure, function and metabolism. This remarkable versatility, combined with its broad availability (both in the clinic and in biomedical research centers) explains the widespread use of MRI techniques in cancer diagnostics and therapy monitoring. The nuclear imaging techniques of Positron Emission Tomography (PET) and Single-Photon Emission Computed Tomography (SPECT) require injection of radioactive compounds, the radioactive decay of which leads to the generation of g-photons that are detected by photo-detectors. Since PET and SPECT provide no anatomical background image, these techniques are typically combined with Computed Tomography (CT) to place the sources of gamma emission in the anatomical context. PET and SPECT are very sensitive and therefore low levels of radiotracers suffice to produce usable signals. The spectrum of tracers for PET and SPECT is phenomenal, explaining the great utility of these techniques in the diagnostics and therapy monitoring of cancer, as well as basic oncological research.

The presentation will address MRI as well as PET and SPECT tools that report on the function as well as molecular markers of tumor angiogenic blood vessels. The maintenance of effective perfusion in tissues, which undergo remodeling and vascular expansion, requires tight control of endothelial sprouting and subsequent recruitment of perivascular contractile cells and vascular maturation. However, this regulation is often impaired in tumors and consequently causes tumor vasculature to respond differently to vasoactive stimuli than normally matured blood vessels. This phenomenon is the basis of the use of blood oxygenation level dependent (BOLD) contrast MRI in oncological studies. In this setting, BOLD MRI maps spatial differences in the response to vasoreactive interventions, such as hypercapnia. Matured blood vessels do, while tumor-associated immature blood vessels do not show a strong response Thus, the BOLD assay can be used as a functional imaging biomarker for vascular maturation. Closely linked to this is the use of PET to assess tumor oxygenation, or rather, tumor hypoxia with the use of ^{18}F-MISO, the prototype agent for hypoxia imaging.

Next, the utility of MRI and nuclear imaging methods for visualizing molecular markers that are associated with tumor angiogenesis, will be presented. The use of MRI for molecular imaging of angiogenesis requires powerful contrast agents, in order to overcome the low intrinsic sensitivity of the technique in face of the often low-levels of molecular angiogenesis markers. MRI produces relatively high spatial resolution *in vivo* images, enabling the analysis of angiogenesis markers to be carried out with great detail. Nanoparticles equipped with a high payload of MRI contrast agent are often used in molecular MRI and since these are also well suited as drug carriers, MRI is widely used for theranostic applications, also in the setting of angiogenesis-specific imaging and anti-angiogenic therapies. The high sensitivity of PET and SPECT and the plethora of nuclear imaging contrast agents that have been developed for oncological applications explain the widespread use of these imaging methods in cancer diagnostics as well as for therapy monitoring.

MULTI-MODAL PET/MR IMAGING OF ANGIOGENESIS INDUCTION AND INHIBITION IN AN ORTHOTOPIC RAT MODEL OF GLIOBLASTOMA

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Introduction: Glioblastomas (GBM) are highly vascularized tumors, and angiogenesis is playing a major role in tumor progression. Our goal was to evaluate steady state contrast enhanced magnetic resonance imaging (SSCE-MRI) for the non-invasive determination of vessel size, density and blood volume in a rat model of primary human GBM. Imaging of vessel characteristic changes was combined with C11-methyl-L-methionine (MET), and F18-fluoro-L-thymidine (FLT) positron emission tomography (PET) for the evaluation of tumor angiogenesis and proliferation.

Methods: MET- and FLT-PET, together with diffusion-weighted imaging (DWI) and SSCE-MRI, were performed 3 weeks after intracranial implantation of human GBM spheroids in nude rats (n = 8). Total cerebral blood volume (tCBV), blood volume present in microvessels (µCBV), vessel density and vessel size were calculated. Rats were then treated with weekly injections of bevacizumab (n = 4) or vehicle (n = 4) for 3 weeks. Imaging was performed again at week 6, before immuno-histochemistry validation.

Results: Combination of MET-, FLT-PET and SSCE-MRI allowed us to determine the relationship between radiotracer uptake and characteristics of tumor vasculature in good agreement with histological changes. 3 weeks after implantation, MRI analysis showed an increase of vessel density and tCBV. 3 weeks later, non-treated rats showed a pronounced increase of MET and FLT uptake. tCBV and vessel size increased between week 3 and 6, whereas vessel density and the µCBV decreased. The changes in tumor vessel characteristic and tumor cell proliferation induced by bevacizumab could be non-invasively characterized and confirmed using immuno-histochemistry. µCBV decreased in treated compared to non-treated rats, whereas the mean vessel size increased. Accumulation of both radiotracers was less intense for the treated versus the non-treated group.

Conclusions: These results demonstrate that SSCE-MRI allows a non-invasive determination of the characteristics of glioblastoma vasculature. Combination of SSCE-MRI and MET/FLT-PET could be of great interest in clinical application to non-invasively depict GBM response to therapy and in preclinical studies to understand mechanisms of resistance to new treatment paradigms.

MULTIPARAMETRIC IMAGING OF PATIENT DERIVED HUMAN CERVIX CANCER XENOGRAFTS

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Introduction: Multiparametric imaging provides a valuable tool to probe the phenotypic features of the tumour microenvironment. Used individually, modalities such as: positron emission tomography (PET) can be used to probe metabolic (e.g. [18]FDG) and hypoxic (e.g. [18]FAZA, ^{18}F-MISO) features of tumours; while computed tomography (CT) and magnetic resonance imaging (MRI) can be used to probe angiogenic and metastatic features. Used in combination, imaging can probe into the potential interplay between the aforementioned phenotypes. The primary objective of this study was to establish a multiparametric imaging approach using dynamic contrast enhanced (DCE)-MRI, DCE-CT and [18]FDG to characterize the tumour microenvironment, specifically the relationship between glucose metabolism and perfusion. Additionally, we set out to determine to what extent the primary xenografts recapitulate their human tumour counterpart.

Methods: Cervix cancer biopsies from 4 patients were used to generate 9 orthotopic cervix xenografts in mice. DCE-CT, DCE-MRI and [18]FDG-PET were performed on xenografts and DCE-CT and DCE-MRI were performed on two patients. Co-registration of xenograft data sets was facilitated using the Minerve System (Bioscan, USA) and no co-registration was performed for patient tumours. A software package was developed to facilitate simultaneous multiparametric analysis of each data set. The mouse and human data was analysed on 3 spatial scales: whole tumour, voxel and clustered. Clustering was used to define 3 regions (hyper, normally, and hypo- perfused) based on the extent of enhancement of each voxel compared to muscle. The SUV_{mean} was used for the PET and the parameters K_{trans}, V_e and V_p were derived from the DCE-CT and DCE-MRI data using the modified Tofts model. Immunohistochemistry (IHC) of human and xenograft tumours was used to validate the imaging results.

Results: Xenograft tumors had a significantly elevated [18]FDG-PET uptake compared muscle. Clustered voxel analysis showed that on average $37\pm23\%$ of xenograft tumours are hyper-perfused compared to muscle and $26\pm21\%$ were hypo-perfused. There was no significant difference in the SUV_{mean} between the hyper-, normally, and hypo-perfused regions. Overall, there was no correlation between DCE-MRI\DCE-CT and [18]FDG-PET activity on all three scales in xenografts. The lack of correlation was reflected in the IHC staining for GLUT-1 (glucose) and CD-31 (blood vessels) in the xenografts. Comparing the mouse and human results, we found that $96\pm1\%$ of the volume in human tumours was hyper-perfused compared to muscle. This is significantly higher compared to the xenografts and was likely caused by significantly lower perfusion observed in human muscle compared to mouse muscle (K_{trans} = 0.23 ± 0.18 min^{-1} vs 0.053 ± 0.014 min^{-1}). A comparison of K_{trans}, V_e and V_p in the whole tumour and on a voxel by voxel level indicated that xenografts have significantly different and inconsistent perfusion properties compared to the corresponding patient tumors from which they were derived. The differing perfusion properties may be related to xenograft passage number, however this has yet to be confirmed.

Conclusions: In conclusion, this work demonstrates the ability to perform pre-clinical multiparametric imaging in a patient derived cervix xenograft model. Additionally, demonstrated perfusion derived parameters where not conserved between xenograft and human tumors.

Notes

day four: thursday january 24, 2013

IMAGING TUMOUR METABOLISM WITH HYPERPOLARIZED MRI

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Patients with similar tumour types can have markedly different responses to the same therapy. The development of new treatments would benefit, therefore, from the introduction of imaging methods that allow an early assessment of treatment response in individual patients, allowing rapid selection of the most effective treatment [1].

We have been developing methods for detecting the early responses of tumours to therapy, including magnetic resonance (MR) imaging of tumour cell metabolism using hyperpolarized ^{13}C-labelled cellular metabolites. Nuclear spin hyperpolarization can increase sensitivity in the MR experiment by >10,000x. This has allowed us to image the location of labelled cell substrates and, more importantly, their metabolic conversion into other metabolites. These substrates include pyruvate [2], lactate [3], glutamine [4], glutamate [5], fumarate [6], bicarbonate [7] and ascorbate [8]. We have shown that exchange of hyperpolarized ^{13}C label between lactate and pyruvate can be imaged in animal models of lymphoma and glioma and that this flux is decreased post-treatment [2,9]. We showed that hyperpolarized [1,4 ^{13}C] fumarate can be used to detect tumour cell necrosis post treatment in lymphoma [6] and that both the polarized pyruvate and fumarate experiments can detect early evidence of treatment response in a breast tumour model [10] and also early responses to anti-vascular [11] and anti-angiogenic drugs [12]. Fumarate can also be used to detect necrosis in other tissues, such as the kidney [13]. We have shown that tissue pH can be imaged from the ratio of the signal intensities of hyperpolarized $H^{13}CO_3^-$ and $^{13}CO_2$ following intravenous injection of hyperpolarized $H^{13}CO_3^-$ [7] and that tumour redox state can be determined by monitoring the oxidation and reduction of [1-^{13}C]ascorbate and [1[13]C]dehydroascorbate respectively [8]. More recently we have shown that we can monitor tumour glycolysis by measuring the conversion of hyperpolarized [U-^{2}H, U-^{13}C]glucose to lactate. Labelled lactate production was higher in the tumour than in surrounding normal tissue and was markedly decreased at 24 h after treatment with a chemotherapeutic drug.

We have recently obtained funding for clinical trials with polarised pyruvate and fumarate to detect treatment response in lymphoma, glioma and breast cancer patients.

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USE OF HYPERPOLARIZED ^{13}C-MRS TO MONITOR TUMOR RESPONSE TO SORAFENIB TREATMENT, IN COMPARISON WITH DW-MRI

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Introduction: Modulating pathways that are involved in oncogenesis by using targeted agents represents a challenge for current research. Conventional anatomically based endpoints may be inadequate to monitor the tumor response to targeted agents that usually do not result in tumor shrinkage in monotherapy. Therefore, non-invasive biomarkers are needed to optimize novel therapeutic approaches, as well as in the transition towards personalized molecular medicine. [13]C magnetic resonance spectroscopy (MRS)-detectable hyperpolarized (HP) fumarate-to-malate conversion has been validated as a marker of cell necrosis and treatment response in tumors[1]. In addition, ^{13}C MRS-detectable HP pyruvateto-lactate conversion has been suggested as a maker of response to a MAPK inhibitor[2]. The aim of the current study was to assess the response to Sorafenib using a combined HP ^{13}C-fumarate and ^{13}C-pyruvate study in mammary xenografts, in comparison with DW-MRI and histological markers.

Methods: Mice bearing MDA-MB-231 xenografts, were imaged at day 0, day 2, and day 5 of daily treatment with 40 mg/kg of Sorafenib. [1-^{13}C] pyruvic acid or [1,4-[13]C$_2$] fumaric acid, were mixed with 15mM trityl radical and hyperpolarized by an Oxford DNP Polarizer, HyperSense®. The polarized substrate was quickly dissolved in Tris/EDTA, NaCl and NaOH at 37°C, yielding 80 mM pyruvate or 20 mM fumarate at neutral pH, before injection to the mouse via jugular vein catheter. Mice were imaged using a double tuned [1]H-^{13}C volume coil in a Agilent ASR 310 7T small animal imaging system. After administration of 0.45 ml of hyperpolarized fumarate, ^{13}C spectra were acquired (TR: 2000 ms, flip angle 15°, 5 min acquisition) from a 3 – 4 mm thick slice across the tumor. After one hour of fumare injection, 0.35 ml of hyperpolarized pyruvate were administered and ^{13}C spectra were acquired (TR:1000 ms, flip angle: 9°,acq.: 5 min) from the same tumor slice. DW-MRI was performed before the injection.

Results: Daily sorafenib injections for 9 days were able to significantly reduce MDA-MB-231 tumor growth. Malatetofumarate (MA/FA) ratio was progressively increased from day 2 (n=5) until day 5 (n=3; P<0.05). This is in accordance with the changes observed in ADCw with Sorafenib at day 2 (n=9) and day 5 (n=5). A positive correlation was established between the relative change in MA/FA and the relative change in ADCw over time (P<0.05). No significant change was observed in the lactate-to-pyruvate ratio over time during treatment with Sorafenib. H&E histological analysis did show a significant increase in tumor necrosis (40.7% increase) between untreated and Sorafenib treated tumors (for 5 days).

Conclusions: Hyperpolarized MRS using ^{13}C-fumarate is showed to be an early in vivo marker of response to Sorafenib and is positively correlated with DW-MRI, with a higher sensitivity for malate-to- fumarate ratio with respect to DW-MRI (2.8 vs 1.3 respectively at day 5). Results are in accordance with ex vivo H&E. The lactate-to-pyruvate ratio does not seem to be an in vivo marker of tumor response to the MAPK inhibitor in the MDA-MB-231 tumor model.

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THE ROLE OF P53 IN THE LIFE OF STEM CELLS: P53 COUNTERACTS REPROGRAMMING BY INHIBITING MESENCHYMAL-TO-EPITHELIAL TRANSITION

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The process of somatic cell reprogramming is gaining increasing interest as reprogrammed cells are considered to hold a great therapeutic potential. However, with current technologies this process is extremely inefficient. Recent studies reported that inhibition of the p53 tumor suppressor gene profoundly facilitates reprogramming and attributed this effect to the ability of p53 to restrict proliferation, apoptosis, senescence and immortalization. Interestingly, Mesenchymal-to-Epithelial Transition (MET) was shown to be necessary and a rate-limiting step in the reprogramming of fibroblasts. Since p53 was shown to regulate diverse differentiation pathways, we aimed to investigate whether p53 counteracts reprogramming by regulating MET. We found that MET is restricted by p53 during the early phases of reprogramming and that this effect is primarily mediated by the ability of p53 to inhibit Klf4-dependent activation of epithelial genes. We also found that the expression of E-Cadherin, an epithelial marker, negatively correlates with p53 activity in a variety of mesenchymal cells. We conclude that loss of p53 in mesenchymal cells predisposes them to the acquisition of epithelial characteristics and renders them more prone to reprogramming. Our study sheds light on a novel function by which p53 restrains reprogramming and highlights the role of p53 in the regulation of cell plasticity.

IMAGING BRAIN METASTASIS: SPECT IMAGING OF TUMOUR INDUCED GLIOSIS

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Introduction: 20-40% of cancer patients develop brain metastasis, yet the mechanisms under-lying disease pathogenesis are yet to be fully elucidated. As with primary tumours, and inflammatory tumour microenvironment appears to be established in the brain. Microglial cells, the brain's resident immune cells, infiltrate metastases, and recent studies implicate astrocytes early in disease progression [1-3]. Astrocytes have a wide range of roles in the CNS, but notably their inflammatory response to brain insult appears to be of relevance in brain metastases. Here, we aim to quantify astrocyte activation during metastasis induction and pathogenesis in a mouse model of metastatic breast cancer. Secondly, we use the microglia and astrocyte response to brain metastasis as a surrogate for imaging tumour growth with SPECT imaging, using ^{123}I-DPA713, a compound which binds to the translocator protein (TSPO) on activated glia [4].

Methods: Two model of brain metastases were induced via either intra-cardial or intracerebral injection of 4T1-GFP cells into female BALB/c mice (6-7 weeks old). For analysis of the temporal and spatial profile of astrocyte activation with time, brains were perfusion-fixed at days 10, 14, 21 and 28 post-injection (n=5 per group). Astrocyte and microglial activation was detected immunohistochemically. For the SPECT study, DPA713 (synthesised as previously described [5]) was labelled with non-carrier added ^{123}I, in an overall 30% isolated radiochemical yield (non-decay corrected). For the intra-cerebral model BALB/c mice were imaged at 13 days post-injection of either 4T1 cells (n=6) or saline (n=3). For the intra-cardiac model, BALB/c mice were imaged at 21 days post-injection of either 4T1 cells (n=5) or saline (n=5). SPECT/CT was performed 1h after i.v. injection of *ca*. 20MBq [123]I. After imaging, the mice were transcardially perfused with saline, the brains removed and sectioned for autoradiography and immunohistochemistry.

Results: Immunohistochemical data showed that brain metastases are surrounded by a ring of activated astrocytes and microglia, and that the area of astrocyte activation is positively correlated with tumour size throughout a 28 day time course. Using both SPECT and autoradiography, ^{123}I-DPA713 was shown to accumulate at sites of metastases in the brain, in both the intra-cerebal and intra-cardiac model of disease, with the extent of binding correlating spatially with the extent of gliosis. Minimal ^{123}I-DPA713 uptake was seen in the brains of control mice, with the extent of increased binding correlating spatially with the extent of gliosis.

Conclusions: Gliosis is an integral part of the tumour microenvironment, contributing to an inflammatory melieu. Here we have shown that it enables early detection of brain metastases through the use of a radiolabelled compound that binds to activated microglia and astrocytes. The ability to image tumour-induced gliosis will not only aid understanding of disease mechanisms, but also has potential for diagnostic imaging.

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MRI OF REPORTER GENE EXPRESSING CANCER ASSOCIATED FIBROBLASTS ENABLES *IN VIVO* MEASUREMENT OF TRANSGENIC CELL FRACTION AND PERI-VASCULAR LOCALIZATION

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Introduction: The involvement of cancer associated fibroblasts (CAF) in all hallmarks of cancer has turned the recruitment of CAFs into an attractive therapeutic target. Several studies have sought to examine the recruitment of fibroblasts to tumors, however existing *in vivo* imaging techniques are unable to provide highly quantitative data on the dynamics of fibroblast recruitment. We used over-expression of ferritin heavy chain (FHC) as an MRI reporter gene in combination with several additional MRI methods in order to quantify the spatio-temporal dynamics of fibroblast recruitment to solid ovarian cancer tumors.

Methods: FHC over-expressing fibroblasts were generated using standard transfection techniques. Tumors were generated via subcutaneous injection of 4×10^6 human ovarian cancer cells into the hind limbs of CD-1 nude female mice. Longitudinal *in vivo* studies of 2 models of fibroblast recruitment were performed after (1) 1×10^6 FHC overexpressing fibroblasts were co-injected with cancer cells, or (2) 2×10^6 FHC over-expressing fibroblasts were injected remotely via an intraperitoneal injection 4 days following tumor initiation. Multi-slice spin echo imaging was performed on a 9.4T Bruker Biospec (Ettlingen, Germany) MRI scanner, and whole body fluorescence imaging was performed on an IVIS Spectrum (Caliper, USA) spectrometer. MR images were analyzed using multi-exponential relaxometric modeling of tissue MR properties in order to measure the FHC over-expressing cell fraction. Vascular mapping was performed by measuring fractional blood volume (fBV) following intravenous administration of an intravascular gadolinium based contrast agent as described in [1].

Results: In both models, MRI revealed preferential recruitment of FHC over-expressing fibroblasts to the rims of solid tumors. Relaxometric modeling enabled measurement of dynamic changes in the FHC over-expressing cell fraction at spatial resolutions of $100um^2$. By the conclusion of experiments in both models of recruitment, the FHC over-expressing cell fraction increased to approximately 40% at the tumor rims. When combined with vascular mapping, the preferential recruitment of FHC over-expressing fibroblasts, specifically to the vascular niche within the tumor rim, was revealed. MRI measurements of FHC over-expressing cell fraction were confirmed by quantitative immuno-fluorescent analysis.

Conclusions: FHC over-expression, relaxometric modeling, and MRI mapping of fractional blood volume enabled *in vivo* quantification of the dynamic process of fibroblast recruitment to the vascular niche of ovarian cancer tumors. The ability to non-invasively quantify the cell fraction of reporter gene expressing cells within a mixed cell population at the superior spatial resolutions afforded by MRI represents a significant advance over conventional reporter gene imaging techniques used for cell tracking in cancer research. When combined with additional MRI protocols, this technique can be used to more accurately assess, at the sub-tissue level, novel therapies that target specific actions of CAFs in promoting tumor growth and metastasis. Importantly, these techniques can further be applied to elucidate the actions of other cellular players in the hallmarks of cancer.

TRACKING IMMUNE CELLS IN ACTION

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Immunotherapy aims to re-engage and revitalize the immune system in the fight against cancer. Research in the past decades has shown that the relationship between the immune system and human cancer is complex, highly dynamic and variable between individuals. Considering the complexity, enormous effort and costs involved optimizing immunotherapeutic approaches clinically applicable tools to track immune cells *in vivo* are most warranted. However, development of such tool is complicated by the fact that a developing immune response involves multiple locations, such as lymph nodes (LN) and the tumour site. Moreover, immune cells are highly mobile and differentiate in function over time.

At our institute we study immunotherapy in melanoma patients in a highly controlled setting; autologous dendritic cells (DC) are activated and loaded with tumour antigens before re-injection into the patient. This controlled setting not only allowed optimization of key parameters, such as the route of administration, the subset of dendritic cells and the class of tumour antigens; it also facilitated the development of immune monitoring tools.

To trigger an effective immune response, *ex vivo* generated tumor antigen-loaded DC need to relocate to lymph nodes. Using scintigraphy and MR, we examined various routes of administration to optimize the delivery of the *ex vivo* labeled dendritic cell based vaccine. We directly compared intradermal and intranodal delivery of [111]Indium labeled DC. Surprisingly, our results show that upon intradermal vaccination the induced T cells were more often able to recognize endogenously processed tumor antigens as compared to intranodal vaccination. Thus, the more laborious and variable intranodal route of administration does not offer an advantage over intradermal vaccination. Although intradermal (i.d.) delivery is more feasible; migration rates never exceeded 4%. We investigated whether ^{111}In-labeled DC migration *in vivo* in humans would improve after pretreatment of the injection site, using unloaded but activated DC, TNFa or a synthetic TLR7/8 ligand; or co-injection with GM-CSF. We show that reduction of cell density, not pretreatment of the injection site, is crucial for improved migration of DC to lymph nodes *in vivo*. However, current imaging modalities for *in vivo* tracking of DC are insufficient to study migration of small numbers of ^{111}In-labeled DC in the clinic. Next to correct delivery of the vaccine, timely assessment of antigen-specific immune responses is critical to aid physicians in clinical decision making. Therefore, we have developed a method for the direct assessment of immune responses *in vivo* in a clinical setting. Melanoma patients with lymph node (LN) metastases received a ^{18}F-labeled 3'fluoro-3'-deoxy-thymidine (^{18}F-FLT) PET/CT scan at varying time points after vaccination. Control LNs received saline or DCs without antigen. *De novo* immune responses were readily visualized in treated LNs early after the prime vaccination in a sensitive manner. Importantly, the level of LN tracer uptake significantly correlates to the level of antigen-specific responses in peripheral blood. So, ^{18}F-FLT, but not the routinely used ^{18}F-FDG PET offers a tool to study the kinetics, localization and involvement of lymphocyte subsets in response to vaccination, allowing early discrimination of responding from non-responding patients.

TRACKING IMMUNE CELLS IN ACTION

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IMMUNE-MEDIATED CYTOTOXIC ACTIVITY IN TUMORS: LESSONS FROM IN VIVO IMAGING

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Cytotoxic T cells and NK cells (CTLs) are key players of anti-tumor immune responses. CD8 T cells can recognize and lyse cells expressing tumor antigens while NK cells can kill tumor cells that have dowregulated MHC class I molecule and/or up-regulated ligands for NK cell activating receptor. To counteract the host immune response, tumors rely on a wide array of mechanisms to escape destruction by infiltrating cytotoxic effectors. Despite our fundamental knowledge on the interplay between the immune system and tumor microenvironments, we have a poor understanding on the efficiency with which intratumoral immune effectors find, interact and kill their targets. Clearly, a better understanding of how these events occur and change during the course of tumor development will help identify why immune responses often failed to clear tumors. We will discuss how intravital two-photon imaging combined with fluorescent to probes to track tumor cell apoptosis in real time can provide quantitative informatation in tumor cell killing in situ. Moreover, we will present recent data showing that NK cells and CTL use strikingly different dynamics during tumor cell regression.

"ALL YOU CAN EAT"; TARGETED INDUCTION OF TUMOR CELL PHAGOCYTOSIS

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Notes

Currently, MAb-based immunotherapy of cancer is still far from perfect and urgently requires study to overcome therapeutic resistance and optimize clinical response rates. This is exemplified by MAbs Rituximab and Trastuzumab, which are not curative in their own right and must be combined with dose-limiting cytotoxic regimes to reach clinical benefit.

Most clinically-approved human(ized) and chimerized anti-tumor MAbs not only have the capacity to directly target and kill tumor cells, but also to activate Complement Dependent Cytotoxicity (CDC), Antibody Dependent Cellular Cytotoxicity (ADCC) and Antibody Dependent Cell-mediated Phagocytosis (ADCP), which may elicit additional beneficial adaptive anti-tumor immune responses. Unfortunately, cancer cells frequently develop resistance mechanisms to current MAb-based approaches. In this respect, over-expression of e.g. CD47 at the tumor cell surface appears to act as a "don't eat me" signal resulting in evasion of antibody-mediated phagocytosis. Moreover, higher CD47 expression independently predicts adverse clinical outcomes in various types of cancer. Tumor cell-expressed CD47 effectively ligates SIRPα, an ITIM motif containing inhibitory transmembrane molecule that is expressed on phagocytic cells, including macrophages and dendritic cells. This CD47-SIRPα interaction initiates a signal transduction cascade that results in potent inhibition of phagocytic activity of myeloid cells. Recent reports indicate that the therapeutic activity of Rituximab and Trastuzumab is enhanced when combined with a CD47-blocking MAb. However, the clinical applicability of CD47-blocking MAbs is limited due to the ubiquitous expression of CD47 on numerous normal cells and tissues. During my presentation I will review some new possibilities to selectively target and inhibit "don't eat me signals" on malignant cells.

day five: friday january 25, 2013

DYNAMIC IMAGING REVEALS SERIAL KILLING OF CANCER CELLS BY CTL: NEW STRATEGIES TO OVERCOME TUMOR RESISTANCE NICHES

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Introduction: Immunological control of tumor progression requires the activation and expansion of tumor-specific cytotoxic T-lymphocytes (CTL) followed by an efficient effector phase in the tumor lesion.

Methods: Using dynamic imaging of organotypic 3D assays of tumor cells confronted with antigen-specific migratory CTL we identify a serial conjugation and killing mechanism dependent on sequential CTL-tumor cell interactions and the killing of multiple tumor cells per CTL (up to 11/24h). The serial conjugation and killing mechanism was confirmed in live B16F10/OVA melanoma grafts, monitored by time-lapse intravital multiphoton microscopy, combined with second and third harmonic generation (SHG/THG).

Results: Both, *in vitro* and *in vivo,* individual CTL-tumor cell contacts were predominantly dynamic (2-10µm/min) and variable in duration (min to hours) and lag phase to apoptosis (ranging from 10 min to several hours). Besides individual CTL killing target cells sequentially, apoptosis induction was observed upon accumulation of subsequent short and statistically sub-lethal CTL-tumor cell conjugations, suggesting a cumulative death signal delivered by multiple serially conjugating CTL. Thus, kinetic imaging shows CTL efficiency as function of the cumulative CTL dwell time per target cell, which is dependent on local CTL density (promoting multiple sequential CTL contacts) and CTL migration confinement (promoting prolonged individual CTL contacts). To identify how the tumor microenvironment impacts CTL co-operation and serial killing, we modulated parameters such as temperature and pro-migratory chemokine levels and studied the effect of CTL migration confinement by local microenvironmental architecture. The pro-migratory chemokine CXCL12/SDF-1 enhanced sequential CTL-tumor cell interactions, but concurrently decreased CTL dwell time per target cell and serial killing representing a tumor resistance mechanism. Conversely, fever-range hyperthermia enhanced serial killing by increasing the contact efficiency of individual CTL-tumor cell contacts. Combining intravital multiphoton microscopy with higher harmonic generation (SHG/THG) further allowed reconstructing the 3D structural microenvironment consisting of collagen fibers and bundles, muscle strands (SHG) as well as nerves and fat cells (THG) which together form heterogeneous tissue interfaces and micro tracks and determine local CTL density by either providing guidance or confining CTL migration. Dynamic imaging revealed guidance tracks that support both, CTL migration and density, thereby enhancing serial CTL conjugations and consequently, increasing target cell killing.

Conclusions: Thus, dynamic imaging *in vitro* and intravital multiphoton microscopy in combination with higher harmonic generation (SHG/THG) reveals novel microenvironmental niches and mechanisms that control the function or failure of anti-tumor immune effector mechanisms.

HUMAN GLIOMAS AND IMMUNE SYSTEM: A COMPLEX INTERPLAY

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Astrocytomas, oligodendrogliomas and mixed oligo-astrocytomas are the most common primary, intrinsic brain tumours in adults. They are stratified according to their mitotic activity and microvascular proliferation and graded between II and IV by the WHO. Low-grade tumours progress to a higher grade with an average of 5 years from onset. High-grade tumours and particularly grade IV astrocytomas (called glioblastoma) cause death of patients within an average of 18 months from the diagnosis.

Gliomas are characterised by molecular, metabolic and genetic intra-tumoural and inter-tumoural heterogeneity. The contribution of normal tissue within the tumour bulk and in the immediate surroundings adds an extra layer of complexity. Glioma microenvironment is particularly complex. Microglia and macrophages, astrocytes, oligodendrocytes, neurones, progenitor cells, pericytes, and endothelial cells are all constituents of the tumour mass. They are profoundly affected by the neoplastic cells but also actively influence the biology of the tumour by promoting growth and invasion, and contributing to escaping the immune system.

Glioblastoma is the paradigm of cancers that are associated with immune inhibition. The term immunoediting recently introduced to describe a broader view of the relationship between immunity and cancer cells, well defines the interplay between glioma and immune cells.

Attention has currently shifted from mechanisms regulating uncontrolled proliferation and invasion of cancer cells to investigating the mechanisms that allow them to influence peripheral and local immune cells. For instance, microglial cells and macrophages migrate within tumour tissue following the release of chemo-attractant factors by neoplastic cells but glioma cells also produce cytokines that suppress their activation and proliferation. As a result, microglial cells and macrophages have a tumour promoting role as opposed to a pro-inflammatory phenotype.

Immune response to gliomas was initially thought to be only mediated by soluble factors. More recently the expansion of regulatory T cells was shown to exert major immunosuppressive activity. For instance, the increase in expression of genes immune cell lineage-specific genes, and genes related to infiltrating immune cell is significantly associated with long term survival of patients with high grade astrocytoma suggesting that host immunity can control tumour growth in at least a subset of patients.

Finally, systemic immunity seems to play a role. Several studies support the hypothesis that people with allergies have a low incidence of glioma whereas infections do not seem to alter the course of patients with high grade lesions.

DYNAMIC INTRAVITAL IMAGING HIGHLIGHTS THE MAIN ROLE OF MICROENVIRONMENT FOR GLIOBLASTOMA PROGRESSION

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Introduction: Antiangiogenic treatments are commonly used to treat Glioblastoma brain tumor (GBM) under the assumption that tumor progression critically depends on the oxygen and metabolic supply by blood vessels. A recent intravital imaging study has however proposed that tumor regression under anti-VEGF Bevacizumab was independent from the drug induced vascular regression (von Baumgarten 2011). We thus hypothesized that GBM angiogenesis might in fact be an epiphenomenon of tumor development rather than a requirement for its progression. We have set an experimental protocol to test this view and highlighted the prevalent importance of tumor stroma (Hanahan 2011).

Methods: We have optimized protocols of orthotopic GBM graft in mice to have them suitable for bicolor intravital multiphoton microscopy (Winkler 2009) but also able to recapitulate the biophysical constraints normally governing tumor invasion (Ulrich 2009, Levental 2009). We then repeatedly imaged tumor cells and blood vessels during GBM development and performed quantitative correlative analyses of fluorescence images in control conditions, as well as under Bevacizumab or under AMD3100, a CXCR4 antagonist targeting stromal composition (Duda 2011). In vivo observations were confirmed by post-mortem immunostaining.

Results: Dynamic intravital imaging evidenced the independence of tumor cell densities and tumor cell proliferation from the local levels of angiogenesis in control conditions. Tumor progression was also independent from blood supply under Bev. Whereas Bev did not directly impair tumor cells proliferation, its antitumor effect was recapitulated if pre-treating the mouse prior tumor grafting, which suggested an action on the stroma. Moreover AMD3100 that inhibits the Stromal Derived Factor 1 alpha pathway produced larger and sustained tumor inhibition without significantly affecting blood supply.

Conclusions: Whereas AMD3100 and Bev are both FDA approved anti-tumor drugs that exert their main effect by modifying tumor environment, we concluded that modulation of the stroma is prevalent over oxygen and metabolic supplies to inhibit brain tumor progression. The potent and sustained effect of AMD3100 on these primary tumors supports the relevance of clinical trials using this drug as a first line treatment for GBM patients.

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COMPARISON OF [177]LU-DOTA,TYR[3]-OCTREOTATE ([177]LU-OCTREOTATE) VERSUS RAD001 IN RAT AND MOUSE TUMOUR MODELS; INCREASE OF METASTATIC TUMOUR BEHAVIOR BY MTOR INHIBITION

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Introduction: The mammalian target of rapamycin (mTOR) signal transduction pathway is upregulated in many pancreatic neuroendocrine tumours (NETs) and plays a key role in regulating cell growth, metabolism, proliferation and angiogenesis. Inhibition of the mTOR signal transduction pathway in pancreatic NETs by RAD001 (Everolimus) has resulted in anti-tumour effects in patient studies. Another effective and novel therapy for NET is peptide radionuclide receptor therapy (PRRT) using ^{177}Lu-octreotate targeting the somatostatin receptor 2 (sst_2), often over-expressed in NETs. In this preclinical study we compared PRRT versus RAD001 therapy and tested their combination in somatostatin receptor expressing rat (CA20948) and mouse (H69) tumour models.

Methods: RAD001 (5mg/kg body weight) treatment was given orally twice a week either for 4.5 or 12 weeks. [177]Luoctreotate (125MBq/3.4µg peptide) was given as a single IV injection or combined with RAD001. Twice a week tumour size and body weight was monitored.

Results: The anti-tumour effects of RAD001 in both models, alone or in combination, were less effective than those of ^{177}Lu-octreotate alone, both in rats an mice. The combination of both therapies did not result in increased anti-tumour effects. In rats, beyond day 40 after start of treatment with RAD001 as single treatment or in combination with [177]Luoctreotate, most RAD001-treated animals were rapidly losing weight, indicating declining health, whereas control animals and animals treated with ^{177}Lu-octreotate alone did not loose weight. ^{111}In-DTPA-octreotide, a radiolabelled somatostatin analogue for diagnosis, was administered to perform SPECT/CT in animals with decreasing body weight. Unexpectedly, tumour metastasis was visualized in RAD001-treated animals; metastases in lungs, liver en lymph nodes clearly showed sst_2 expression as confirmed by autoradiography. Metastases were not found in control and ^{177}Lu-octreotate-treated rats, not even after surgical resection of the primary tumour to enable longer follow-up. The percentage of rats with metastases was not significantly different between groups treated with RAD001 for 4.5 versus 12 weeks. After development of distant metastasis was validated by SPECT/CT imaging, a new therapeutic dose of ^{177}Lu-octreotate was administered and 24 hours later a second SPECT/CT scan was acquired. The new PRRT cycle slowed down tumour growth, but no complete regressions were reached.

Conclusions: ^{177}Lu-octreotate PRRT appeared to be more effective than RAD001 or their combination. Unexpected metastasis in animals occurred after treatment with RAD001, this process could be clearly visualized using SPECT/CT. If the results of our studies can be translated to humans, patients should be closely watched during and after RAD001 therapy

8TH EUROPEAN MOLECULAR IMAGING MEETING – EMIM 2013

the EMIM 2013 will take place in Torino, Italy from May 26-28, 2013

abstract submitted?

deadline 10 February 2013

the EMIM 2013 will start with 4 EDUCATIONAL SESSIONS on Sunday May 26 from 08:00h to 11:00h:

�ထ **Advanced Microscopy –**
Studying molecular processes at cellular and subcellular level
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✚ **Photoacoustic Imaging –**
From principles to application
organised by Daniel Razansky and Vasilis Ntziachristos

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Merging the best
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✚ **Imaging Cardiovascular**
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To emphasise the **ESMI Study Groups**
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✚ **Intra-operative Imaging**
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