FONDATION
MEDIC

Consortium “Tumor-Host Interaction” supported by the MEDIC Foundation

Annual Report 2013

1. Introduction

The theme 'Tumor-host interaction' chosen for the consortium remains timely, notably in view of the renewed interest in immunotherapy, which finally seems to acquire a confirmed position among the available therapeutic modalities. Of the 12 projects presently supported by MEDIC, 1 was renewed in 2013 based upon review of a full new grant application. All developed favorably. For the first time since its creation, a certain level of competition had to be introduced for funding as three new projects were submitted, only one of which could be funded in spite of positive evaluation of all three. The different groups in the consortium continued to interact, in terms of exchange of materials, new questions that popped up during the MEDIC day being entertained in the laboratory or bioinformatics support. Some studies might not have taken off had it not been for partnerships developed within the MEDIC consortium. The external Scientific Advisory Board, already strongly involved in selecting the MEDIC prize awardee, might have to be more actively involved in decisions on funding in view of the competitive element, which will contribute to maintaining high quality research. The annual reports and the annual meeting presentations provide the SAB the tools to critically follow the progress made in the studies. The web-site was completed and now provides detailed information of the groups (see www.fondation-MEDIC.ch). A tool was provided to the consortium members allowing them to continuously update their data on the website.

Also this year the consortium members met in Lausanne to present in symposium format the progress made and the new projects under submission for funding. The one and a half day approach was appreciated as it allowed more time for discussion. The program of the 2013 october meeting is presented in table 1.

Table 1 Programme of the MEDIC day 2013

Marie-Agnès Doucey	Pro-angiogenic monocytes in breast cancer
Agnese Mariotti	Function of the transcription factor Sox10 in the mammary gland and in breast cancer
Didier Picard	Regulators of estrogen receptor alpha activity and their role in breast cancer
Anita Wolfer	The role of MYC in cancer cell motility
Laurence Buisseret	MEDIC prize 2012 lecture: Organized Immune Responses in Breast Cancer
Ivan Stamenkovic	Energy regulation of cancer stem cells
Curzio Ruegg	Unraveling the role of MAGI1 and CYR6 in cancer progression and therapy
Christine Desmedt	Molecular heterogeneity drives metastasis in breast cancer
Philippe Martiat	Role of immune micro-environment in acute leukemia
Pedro Romero	Role of miRNA-155 in CD8+ T-cell function
Laurence de Leval	Molecular characterization of nodal peripheral T-cell lymphomas

Ghanem Ghanem/ Fabrice Journé	Melanogenesis in melanoma progression and therapy
Mauro Delorenzi/ Edoardo Missiaglia	Molecular genetic heterogeneity of colon cancer
Tania Petrova	Role and targeting of Prox1 in colon cancer metastasis

Productive collaborations continue to develop, feed-back of the participants is very positive so the chosen approach was continued. With the creation of the Department of Oncology in Lausanne most of the Lausanne groups are now in the Department of Oncology. The future of the consortium was discussed with the Head of the Department and in the future the Department might play a leading role in the further evolution of MEDIC funded cancer research.

2. Research groups, themes and received support

Table 2 lists the research projects that are supported by the MEDIC Foundation, the project title and the total amount of annual support received.

Table 2 List of projects funded in 2013 by the MEDIC foundation

C.Ruegg	Role of CYR61 in tumor progression and metastasis	CHF	238'000,-
A.Mariotti	Elucidation of the function of Lactadherin and Sox10 in breast cancer	CHF	134'000,-
D.Picard	Molecular and pharmacological investigation of the factors contributing to tamoxifen resistance of ERa-positive breast cancers	CHF	164'000,-
M.Delorenzi	Linking tumor heterogeneity with clinically useful subtypes of colon cancer	CHF	122'000.-
L.de Leval	Characterization of molecular biomarkers relevant to the biology, diagnosis and prognosis of peripheral T-cell lymphomas	CHF	71'000,-
T.Petrova	Role and targeting of PROX1 in colon and small cell lung cancer	CHF	201'000.-
I.Stamenkovic	Mechanisms that govern energy regulation in cancer stem cells *	CHF	0.-
P.Romero	Role of microRNAs in CD8 T cell function	CHF	126'000.-
M-A. Doucey	Tie-2 expressing monocytes and their ligands: appealing targets in breast cancer angiogenesis	CHF	112'000,-
C.Sotiriou	Interrogating breast cancer molecular heterogeneity	€	246'000.-
G.Ghanem	Search for new prognosis markers, new targets for therapy and new drug combinations in high risk melanoma	€	179'000.-
Ph.Martiat	Functional characterization of T cells and their regulatory subset in bone marrow and blood of acute leukemia patients. Correlations with leukemia free survival.	€	162'000.-
*	Funding on hold in view of positive balance		

It is relevant here to note that the total volume of MEDIC supported research conducted in Lausanne has grown and in 2013 7 groups continued to profit from MEDIC support. A new project was submitted from Geneva and will be (partially) funded in 2014. Two new projects were submitted from Lausanne of which one was funded, the other remaining without support for lack of funds in spite of good reviews. One group is in the Department of Medicine of the Faculty of Sciences in Fribourg.

3. Research program

Three clusters of activities can be distinguished: general aspects of tumor biology, the pathobiology of breast cancer, pathobiology of colon cancer and cancer immunotherapy.

3.1 General aspects of tumor biology

This heading puts together research lines which address questions concerning the development and behaviour of cancer cells more in general and not necessarily limited to an organ or organ system. Two research lines fall into this category: the complex interactions between a variety of cells and molecules that make up the host response to growing tumor cells and basic aspects of cell function that are disrupted in cancer cells.

1. **Role of CYR61 in tumor progression and metastasis**
   Principal Investigator: Curzio Ruegg

The generation of cancer cells from normal cells involves the accumulation of transmissible modifications of the cell's genome resulting in uncontrolled proliferation and survival. In addition, cues from the immediate environment and distant tissues such as the bone marrow, are critical mediators of tumor initiation, and progression, including invasion and metastasis formation. Recent evidence suggests that this interaction between the tumor and its host also modulates the tumor response to anti-cancer treatments. The Rüegg laboratory is investigating selected aspects of tumor-host interactions, with emphasis on inflammation-related pathways and mechanisms mediating metastasis and modulating response to therapy with potential for translation to the clinics.

In addition to the planned experiments and reported results we have initiated experiments, which are related to the study of tumor-host interactions and relevant to MEDIC project.

A. Chemotherapy-mediated breast cancer cell dormancy.

Chemotherapy (CTX) is widely used as a systemic treatment modality in cancer patients and provides survival benefits for a significant fraction of treated patients. While it is generally assumed that survival benefits are due to CTX –mediated tumor cell killing, alternative mechanisms have not been investigated in depth. By using the syngeneic metastatic 4T1 murine breast cancer model, we observed that chemotherapy treatment and selection of chemotherapy-resistant cancer cells in vitro can induces two opposite phenotypes: a dormant one and a relapsing-metastatic one. We found that CD11b+ cells play important roles in inducing metastasis or dormancy in vivo. Dormant tumor cells were able to induce an in vivo immune-inflammatory response in the draining lymph node, which is normally absent due to the immunosuppressive effects of tumor-recruited myeloid derived-suppressor cells (MDSCs). Genome-wide gene expression analysis revealed the enrichment of immune response-related genes in the dormant tumor cells. Interestingly, CD11b+ cells derived from the microenvironment of growing-metastatic tumors, but not CD11b+ cells derived from the spleen of tumor-free mice, were able to instigate outgrowth of dormant tumor cells in vivo. Also, dormant cells formed growing and metastatic tumors when injected into immune-compromised NGS mice. These results point to a role of chemotherapy in enabling treated tumor cells to acquire immune response-inducing capabilities, while impairing the recruitment of CD11b+ cells and their differentiation into an immune-suppressive cell. The molecular mechanisms underneath these effects are being further investigated.

B. Characterization of MAGI1, CYR61 and Tumor cell-Fibroblast interaction.

MAGI1. We previously identified MAGI1 as a tumor suppressor gene upregulated by COXIB in colorectal cancer (CRC) cells. In order to study MAGI1 function in CRC formation, we generated double transgenic mice with Tetracycline-regulated MAGI1 expression in the intestine (Vilin::rtTA-M2 x CMV/tet::MAGI1, in collaboration with Pr. R. Fodde, Rotterdam) and in

endothelial cells (EC) (VEC::rtTA-M2 x CMV/tet::MAGI1, in collaboration with Pr. T. Petrova, Lausanne) and initiated their characterization. We confirmed MAGI1 expression in the intestine and in endothelial cells in the respective lines upon Tetracycline induction. MAGI1 expression in the intestine is patchy with a baso-apical gradient, with only about 30% of the enterocytes expressing it. Vilin::rtTA-M2 x CMV/tet::MAGI1 are being treated with Azoxymethane + DSS to induce intestinal carcinogenesis. Using the Matrigel plug assay we observed that forced MAGI1 expression in EC inhibits angiogenesis. To monitor for effects of MAGI1 in developmental angiogenesis we are inducing MAGI1 during pregnancy and we are analyzing embryos at different time points. In vitro experiments revealed that MAGI1 localizes at cell-cell contacts in response to shear stress and that MAGI1 is essential for shear stress-induced EC alignment to flow. Importantly, we found that MAGI1 induces eNOS phosphorylation, expression of KLF4, a transcription factor mediating flow-induced EC responses, and integrin-mediated adhesion. Thus MAGI1 emerges as a novel mediator of EC response to shear stress.

CYR61. We further characterized the role of CYR61 in metastasis. Silencing of CYR61 in the metastatic breast cancer cell line MDA-MB-231 suppressed lung metastasis formation. We observed that CYR61 inhibits MDA-MB-231 anoikis, promotes anchorage independent growth, transendothelial migration, spreading and migration on matrix proteins. In vivo experiments indicate that the critical step in CYR61-mediated metastasis involves enhanced survival of disseminated cancer cells and facilitated entry into the lung. The survival effect of CYR61 involves integrin ligation.

Tumor cell-fibroblast. We are investigating the mechanisms of fibroblasts (Fb) mediated tumor cell (TC) invasion. Using 2D and 3D co-culture systems consisting of Fb derived from skin, normal colon and colon cancer and the CRC cell lines SW680 and HT29. In co-culture with Fb we demonstrated that TC acquire elongated morphology and increased motility in a cell-cell contact dependent manner. In 2D and 3D spheroid assays Fb induced contact-dependent TC migration and invasion. In cell signaling experiments we identified Src as critical mediator of TC elongation and increased motility in response to interaction with Fb. This effect is associated with the rearrangement of the actin cytoskeleton. Other signaling pathways, in particular MAPK, Pi3K-AKP and PKC are not essential. Furthermore, TC-Fb contact promotes the expression of inflammatory genes in Fb and EMT-relevant genes in TC. This effect, however, is not sufficient to induce TC elongation, motility and invasion. Also, conditioned medium of TC-Fb co-cultures does not promote TC elongation and migration. Current experiments focus on the identification of cell surface receptors involved in the cross-talk between TC and Fb. While Fb-mediated TC invasion was generally attributed to Fb-derived motility factors, these results clearly demonstrate that direct Fb-TC interaction is critical in promoting TC invasion.

Publications

2. Mechanisms that govern energy regulation in cancer stem cells

Principal Investigator: Ivan Stamenkovic

Growth of numerous cancer types is believed to be driven by a subpopulation of poorly differentiated cells, often referred to as cancer stem cells that have the capacity for self renewal, tumor initiation and generation of non-tumorigenic progeny. Despite their potentially key role in tumor establishment and maintenance, the energy requirements of these cells and the mechanisms that regulate their energy production are unknown. Our ongoing work is exploring the bioenergetics of cancer stem cells, focusing mainly, but not exclusively, on Ewing's sarcoma family tumors and glioblastoma. We have identified cancer stem cells in glioblastomas and have demonstrated that these dpend on oxydative phosphorylation for their energy production and survival. We explore the mechanisms involved in ensuring oxydative phosphorylation maintenance.

Project temporarily suspended

3.2 The pathobiology of breast cancer

Several groups in the consortium work on breast cancer, which is the most frequently encountered type of cancer in women since 1 out of 9 will develop breast cancer and unfortunately one third of these will subsequently die from this disease. The currently used factors for predicting survival and response to treatment do not sufficiently explain why in some patients the tumors progress and in others do not or why some women respond well to therapy whereas in others the tumors continue to grow. During the last years, several prognostic predictors have been developed in breast cancer using gene expression profiling technologies. Although these predictors outperform the currently used clinico-pathologic factors, they remain suboptimal. This means that in order to get a better picture of breast cancer biology, additional elements need to be considered, such as: 1) the tumor microenvironment and 2) the disseminated and circulating tumor epithelial cells. These key elements constitute the main research axes of this group of projects. Four research projects in the consortium focus on aspects of breast cancer.

1. **Interrogating breast cancer molecular heterogeneity**
   Principal Investigator Christos Sotiriou

Despite the progress made during the last decade from gene expression-profiling studies and preliminary data on mutational events related to breast tumorigenesis, very little is known regarding breast cancer heterogeneity and its implication during
disease progression, dissemination and distant colonization. Moreover, there is very
little progress in identifying potentially "druggable" mutations that drive tumor
progression and development of distant metastases, which are responsible for breast
cancer death. We aim to address this question by studying at the genome (whole exome) level breast tumor heterogeneity at the primary site (tumor initiation and progression), circulating tumor cells (tumor dissemination) and at the metastatic sites (tumor colonization) using next generation sequencing technology.

A. Molecular heterogeneity of multifocal breast cancer

The question whether multifocal breast cancers are due to the spread of a single carcinoma throughout the breast or is due to multiple carcinomas arising simultaneously has been a matter of debate. Some earlier studies suggested, using either comparative genomic hybridization or the PGK gene inactivation assay in 3 and 4 MFBC patients respectively, that MFBC result from intramammary spread of a single primary tumor. Others, by studying immunohistochemical markers or the distribution of allele loss of chromosome 16q in 24 and 26 patients respectively, suggest that in some cases MFBC arise from different clones. We have shown that all ductal MFBC share a common genetic background suggesting spread of tumor cells throughout the breast rather than simultaneous growth of tumor cells with different genetic origin.
We do not know yet what causes some cancers to be multifocal, i.e. what triggers the intramammary spreading of the cancer cells to form geographically distinct lesions. In our study, no common molecular denominator to the MFBC could be observed. Recent observations that MFBC are associated with a significant down-regulated expression of E-cadherin compared to unifocal breast cancer, might partly explain the increased invasive potential of MFBC. No differences between multifocal and unifocal breast cancers were instead observed in the distribution of molecular subtypes defined by ER, PR, HER2, and the basal-like CK5/6, CK14 and EGFR markers, suggesting that multifocality cannot be attributed to the prevalence of a more aggressive subtype.
Although different lesions of MFBC are clonally related, they can display considerable molecular heterogeneity. The inter-lesion genetic heterogeneity, evaluated at the level of mutations in cancer-related genes, was evident in 40% of the patients, suggesting that most of the mutations observed in these patients occurred after the seeding of the different lesions. Of note, intra-lesion genetic heterogeneity, which mainly concerned genes with low percentages of mutated reads, was also observed for those patients without important inter-lesion genetic heterogeneity. Consequently, this suggests that inter-lesion genetic heterogeneity is more than merely a

reflection of the level of intra-lesion genetic heterogeneity. Interestingly, for the majority of the patients with strong inter-lesion genetic heterogeneity, the inter-lesion distance was considerably higher than for the remaining patients. The potential association between the distance separating the lesions and the inter-lesion genetic heterogeneity needs however to be further investigated. The inter-lesion heterogeneity was also present at the gene expression and DNA methylation level in all but one patient. Some transcriptomic differences could be predicted by the nature of the microenvironment, such as increased lymphocytic infiltration in one lesion compared to another, or by certain genomic changes, such as MYC amplification; however the majority of these differences could not.
The implications of the inter-lesion differences in terms of treatment response to the various standard and targeted therapies, as well as for the progression of the disease deserve further investigation as they could be of clinical relevance. We are currently planning to validate our initial findings using a clinically well-annotated cohort of primary multifocal, unifocal and contralateral breast cancer specimens with available matched metastatic lesions (part of our next round grant proposal).

B. Comparison of matched primary and metastatic breast cancer

In this project, we investigated metastatic progression in breast cancer through temporal and geographical whole-exome sequencing, followed by deep re-sequencing, and copy number profiling of several sections of primary tissue and multiple matched metastases. By reconstructing the phylogenetic history of metastatic progression, we were able to infer the natural evolution of the disease, something which has never been done before and had up to now only been deduced from case reports. Our novel findings can be summarized as follows:

1) ‘Single seeding event’: We have demonstrated that although tumors disseminate a large number of cells, in the majority of the patients analyzed in this study, a single distant metastasis arose from a successful seeding event and subsequent secondary metastases descended from this metastatic precursor. To our knowledge, this is the first time that the concept of ‘metastatic precursor’ is introduced in the literature and we believe that this clone acquired all the alterations necessary to overcome the barriers that would otherwise lead to death of cells disseminated by the primary tumor. If proliferation at the distant organ is left unchecked, this first seeding event ultimately leads to further re-seedings in other organs showing that the first metastasis acts as a ‘boot camp’ for further metastases.

2) ‘Horizontal re-seeding’: In the minority of patients, we found evidence for multiple seeding events. Interestingly, we were able to show that one independently established metastasis re-seeded to another metastasis. Whilst the provocative tumor self-seeding hypothesis has been previously described in murine cancer models by the group of Joan Massagué, its existence in human settings has so far been controversial. On the other hand, our results show for the first time that circulating cells can travel between metastases making the tumor self-seeding hypothesis in human settings more than tenable.

3) ‘Axillary lymph node metastases as ‘metastatic dead-ends’ or ‘transit points of the metastatic process’: Axillary lymph node metastases proved to be "metastatic dead-ends" in two of the three investigated cases. In particular, in these patients we observed that tumor cells in lymph nodes did not give rise to any of the distant metastases. This suggests that, rather than acting as a transit point from where distant metastases develop, axillary lymph node involvement and the negative prognostic value associated with it, reflect the propensity of the primary tumor to metastasize. Yet, in one patient, the axillary lymph node metastases displayed a mutation profile similar to the one inferred for the ‘metastatic precursor’, suggesting that in this patient the axillary lymph node could have given rise to the distant metastasis. Overall, this shows that the metastatic process in breast cancer can be very complex and may be conceptually different between patients.

4) ‘Dynamics of genomic alterations’: We established that the extent of genomic alterations private to both primary tumors and distant metastases is positively correlated with the time between diagnosis and death. These results suggest that patients who relapse rapidly have a nearly complete repertoire of genomic alterations necessary for successful metastatic outgrowth, whereas additional alterations might be required as ‘late’ oncogenic events for others. Clinically, these results imply that it is highly relevant to characterize distant metastases in addition to the primary tumor, particularly for patients who relapsed a few to many years after the initial diagnosis;

Overall, by characterizing the genomic alterations that reshape metastatic genomes during progression, we have generated novel insights into the dissemination process of breast cancer metastasis. We plan to validate several of the above hypotheses and in particular the lymphatic dissemination results using an extra cohort of well-annotated tumors samples with matched primary, positive lymph nodes and distant metastases (part of our next round grant proposal).

C. Molecular characterization of circulating tumor cells

We aimed to demonstrate that plasma circulating DNA could be the initial tissue for targeted gene screen using next-generation sequencing. For that purpose we interrogated whether plasma can be used as an alternative tissue to metastatic biopsies. The Ion AmpliSeqTM Cancer Hotspot Panel v2, covering approximately 2,800 COSMIC mutations from 50 cancer genes was used to analyze 101 samples including primary and / or metastatic lesions and serial plasma samples from 17 metastatic breast cancer patients.

We identified at least one mutation (median 1 mutation per patient, range 0-3 mutations) in at least one of the p53, PIK3CA, PTEN, AKT1 and IDH2 genes in 13 of 17 patients. We demonstrated spatial (between different tissues/lesions) and/or temporal (between different time-points) heterogeneity. Plasma circulating tumor DNA analysis identified 9 of 13 patients with detectable mutations. This is one of the first studies demonstrating the feasibility of applying next generation sequencing technologies to identify genomic alterations in the plasma.

We currently aim to validate our initial results in a large series of neoadjuvant and metastatic breast cancer patients (NeoAltto dataset, IJB series). The ultimate goal of this project is to demonstrate that liquid biopsy captures tumor heterogeneity in the metastatic setting and therefore can be used as a tool for disease monitoring and for molecular screening.

For whole genome sequencing of single CTCs we initially performed spiking experiments using the HCC38 tumor cell line and single / pool of 10 HCC38 cells. These were recovered using the CellSearch and the DEPArray system as described in the methods. DNA extraction and amplification were performed using the Ampli1 Kit. Average coverage depth was 0.68x. Paired-end sequences were obtained for 100 bases per read. Reads were trimmed at a 79 bp length in order to remove Ampli1 adaptor sequences in the first 21 base positions from the analysis. At a binning window of 50 kb, single-cell CNV profiles were highly concordant with CNV profiles of a non-amplified multi-cell sample. On average, 72% copy number concordance was observed per bin genome-wide.

Publications

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2. Mechanisms of functioning of the estrogen receptor

Principal Investigator Didier Picard

Estrogen receptor (ER) plays an important role in breast cancer, both in terms of cancer biology and as predictor of response to therapy: ER positive tumors are likely to respond to anti-estrogen drugs (notably tamoxifen). Estrogen receptor positive breast cancers tend to develop resistance for tamoxifen, however. The Picard laboratory studies the molecular biology of the estrogen receptor against this background. Mechanisms of resistance are explored as well as possibilities to overcome this resistance.

The project comprised of several sub-projects:

A. Alternate ERα-mediated responses

We have pursued our efforts to understand the molecular biology and the pathological relevance of the activation of ERα by alternate pathways, notably cAMP. In collaboration with Wilbert Zwart (NKI, Amsterdam), we had found that the cAMP-induced ERa binding sites (ERBS) of breast cancer cells are largely a subset of those induced by estradiol (E2). We then correlated ERBS with the genes whose expression is regulated by cAMP in an ERα-dependent fashion, and discovered that the upregulated genes are predictors of a poor outcome. After validating several genes, we decided to focus on two from a functional angle. One is NFkB2, an anti-apoptotic transcription factor, which has already been linked to cancer and the response to tamoxifen. The other is RalBP1, which has been linked to drug resistance and metastasis. In parallel, since ERα, CARM1 and Hsp90 all come together in shared complexes and are functionally required for this unusual response, we are preparing to compare their cistromes (i.e. their genome-wide distribution) by ChIP-seq

B. The arginine methylase CARM1 as cAMP-regulated ERα activator

We have followed up on our discovery that CARM1 mediates the activation of ERa by cAMP and thereby contributes to tamoxifen resistance in breast cancer. We have known all along that other factors must be involved as well, notably at least one other factor that responds to cAMP-signaling. One candidate that we are currently investigating is the demethylase LSD1. It appears to be involved in the response of ERα to both E2 and cAMP. For both CARM1 and LSD1, we are in the process of clarifying and mapping the PKA phosphorylation sites using mutagenesis as well as mass spectrometry.

C. cAMP signaling crosstalk, a human trait?

We have discovered that mouse andhuman ERα differ in their responses to cAMP signaling. While the hormone binding domain (HBD) of the human ERα is sufficient to mediate a cAMP response (through the recruitement of CARM1), there is no response with the mouse ERα HBD. Since the two species differ by only 8 amino acids within the core of the HBD, this will allow us to map the determinants of this response more precisely. Moreover, this analysis might at least indirectly provide insights into the physiological significance of this crosstalk as it would have to be something that is specific to humans.

D. Interactome analyses

Even though a large numberof ERa interactors have been reported (see http://www.picard.ch/downloads/ERinteractors.pdf), the list may still be incomplete and their functional relevance is poorly understood. We have developed novel bioinformatic tools to interrogate large protein-protein interaction networks such as that of ERα. (the figure shows the virtual ERa interactome with 268 proteins being engaged in 1443 connections between them)

E. Screens for novel ERα regulators both in yeast and by RNAi in mammalian cells

The validation of hits from the yeast screen is continuing in mammalian cells. We are currently focusing on links with membrane traffic and a histone H2B ubiquitination activity. We have found that components of the CORVET/HOPS complex, which is known for its role in membrane traffic, modulate ERα function. In their absence, ERα activity is augmented. In contrast, interfering with endocytosis using a dominant-negative mutant lowers ERα. We speculate that ERα activity may be increased by the sustained activity of endocytosed growth factor receptors. The monoubiquitination of histone H2B by Rnf20/40 has previously been linked to cancer. We have found that this activity interferes with ERα, at least at some target genes. The next steps include connecting these new regulatory mechanisms with physiological and/or pathological states.

F. New transactivation assays

To facilitate future transcriptional assays, we have developed a novel dual luciferase system that would be a cheap, simple and sensitive alternative to the commercial Promega kit. It is based on two secreted luciferases that can easily be measured without even lysing the cells. We expect to submit this new system for patenting and publication in the next few months.

Publications

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3. New prognostic factors in breast cancer: lactadherin

Principal Investigator Agnese Mariotti

We have found that the secreted protein MFG E8 is highly expressed in breast cancer in association with the transcription factor Sox10 , suggesting that Sox10 may play a role in breast carcinogenesis possibly by functionally interacting with MFG E8. In this project we have started to address this hypothesis by analyzing the role of Sox10 during normal mammary gland development, and how its function is affected by transformation events that will lead to tumorigenesis. Our final goal is to understand if and how active Sox10 can cooperate with oncogenes to promote breast cancer.

By analyzing the gene expression profiles of over 500 breast carcinomas, we have found that the transcription factor Sox10 is highly expressed in triple-negative breast cancer and present at lower levels in other breast carcinoma subtypes. We have confirmed this by immunohistochemistry analysis in breast carcinoma specimens. We have also found that Sox10 is expressed during normal mammary gland development in a subset of basal and luminal cells that are proliferating and have no or low expression of Estrogen receptor. During the past year we have extended our analysis of the expression and function of Sox10 in both the normal and transformed mammary gland by employing several in vivo and in vitro models.
Results:

- By using a reporter mouse expressing a fluorescent-tagged version of Sox10 we have isolated by FACS primary mammary cells expressing or not Sox10 and have analyzed them in vitro and in vivo. We have confirmed that Sox10 is expressed in a subpopulation of basal and luminal cells. Only Sox10-expressing cells are able to survive and form spheres in vitro and form 3D branched structures in Matrigel and Collagen. Even more interesting, when Sox10-expressing cells are injected in the cleared mouse fat pad, they regenerate mammary gland while Sox10 negative cells do not. These results suggest that Sox10-expressing mammary epithelial cells have features of stem or progenitor cells. We are currently characterizing Sox10 expressing cells by analyzing the expression of stem/progenitor cell markers by RT-PCR.
- We have found that Sox10 is expressed during mammary tumor development in the PyMT mouse model of mammary cancer. In particular, high expression is present during the hyperplastic stage and in lung metastases. We have crossed PyMT mice with the Sox10-fluorescent reporter mouse and observed that in fact tumors express fluorescent Sox10 during different stages of development. We plan to FACS sort Sox10 expressing tumor cells and analyze their gene expression profiles to identify the deregulated genes associated with Sox10 during cancer development.
- We are also evaluating the function of Sox10 during tumor development using a Sox10'-conditional knockout mouse. We will isolate primary mammary cells and transduce them with PyMT with or without Cre recombinase, and then inject the resulting cells orthotopically in

NODSCID mice. This experiment will reveal if the transforming activity of PyMT is influenced by the expression of Sox10.

- We have also overexpressed Sox10 in human mammary epithelial cells (non-transformed HMT-3522-S1 and Ras-transformed MCF10AT1). While parental cells form spheres in 3D Matrigel with defined borders, composed of multiple, regular layers of cells, Sox10-overexpressing cells form irregular spheres characterized by Matrigel-invading protrusions and composed of non-polarized layers. In addition, when grown in Collagen, Sox10 overexpressing cells proliferate more compared to control cells, as assessed by their higher expression of phospho-histone H3, and form longer duct-like structures.
- Interestingly, MCF10AT1 cells overexpressing Sox10 form more and larger lung metastases than control cells after t.v. injection in immunodeficient mice, indicating that Sox10 promotes one or more steps of lung colonization in this system.

In conclusion, our results so far indicate that Sox10 is expressed in mammary epithelial cells with stem/progenitor cell features. We hypothesize that these cells may be preferential targets of oncogenes during transformation of the mammary epithelium. If this is the case, Sox10-regualted signaling pathways in mammary cells might cooperate with oncogenic pathways to promote tumor development. The experiments that we plan to perform in 2014 will clarify this issue and help identify Sox10 targets that may contribute to mammary tumor development.

Final report, project terminated.

4. **Tie-2 expressing monocytes and their ligands: appealing targets in breast cancer angiogenesis**
   Principal investigator Marie-Agnès Doucey

Tumor vascularization is essential for tumor growth and cancer progression. In breast cancer, monocytes are angiogenic i.e. able to induce tumor vascularization. In patients, blood circulating monocytes drastically increase their angiogenic activity when reaching the tumor suggesting that the tumor microenvironment shapes their angiogenic activity. The identification of the tumor signals inducing the angiogenic activity of monocyte is of paramount significance because it represents the rationale for anti-angiogenic therapies in breast cancer. We address these issues in a combination of in silico modeling approaches and experimental studies in mouse models of breast cancer.

We have identified the critical pathways and tumor factors controlling TEM (Tie2-expressing monocytes) hemangiogenic activity in breast cancer. In addition to their hemangiogenic activity, TEM display lymphangiogenic and immune-suppressive activities. Interestingly, we show this year that these three functions share the following features:

- They are coordinated by synergistic interplay between angiogenic and inflammatory pathways.
- They are specific for the tumor microenvironment which shapes both TEM functions and phenotype.
- They are controlled by Tie2 and VERGFR kinase activities.
- They can be reversed by specific treatments into efficient antigen presenting cells sharing features of myeloid DC and promoting anti-tumor immune responses.

We have identified critical pathways controlling TEM suppressive, haem- and lymphangiogenic activities. Our observations on TEM lymphangiogenic phenotype and lymphangiogenic activity in BC tissue are currently under submission. Our phenotypical observations were based mainly on confocal and FACS analyses. We have now sorted by flow cytometry TEM from dissociated breast tumors and confirmed their expression of the lymphatic marker PROX-1 by RT-PCR at the single cell level. This antibody-independent phenotypical characterization strengthened our previous observations that TIE-2-expressing monocytes are lymphangiogenic and incorporate specifically into lymphatics of human breast cancer.

An in vitro cell culture model of TEM angiogenic activities and incorporation of TEM into lymphatics was set up. This model reflects the phenotype and the haem- and lymphangiogenic activities of TEM that we have observed in BC and was used this year to delineate the pathways controlling TEM lympangiogenic activity. We found that in addition to Tie-2 and VEGFR, two other pathways control TEM lymphangiogenic activity. Thanks to this model, the significance of this activity and of TEM insertion into lymphatics in BC will be investigated next year. A paper on this in vitro model system is currently under submission.
We have made progress in understanding the principle of detection of our memristive nanowires. Indeed, we observed that temperature and humidity are critically controlling the memristive effect and we have compared the performances of our memristor-based nanowires with FET (Field Effect Transitor)-based nanowires.

Publications

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3.3 The pathobiology of colon cancer

Colon cancer is the second most frequent cancer type in the western world in females and males. In spite of the fact that it is one of the most widely studied types of cancer and that the concept of stepwise progression of molecular events has been developed based on colorectal carcinogenesis, many questions around this tumor type remain unsolved and overall about 50% of the patients with this disease cannot be cured. It is therefore not surprising that in the MEDIC consortium a significant effort is directed towards contributing answers to these questions. Two research lines address colorectal cancer.

1. Linking tumor heterogeneity with clinically useful subtypes of colon cancer
Principal Investigator Mauro Delorenzi

In this project the molecular heterogeneity of colorectal cancer is studied in a large series of cases, with detailed clinical follow-up. The basic aim of this project is to discover new ways for recognizing types of colon cancer that differ in the way they should be treated, thus helping clinicians and patients to take better decisions. Decisions to be taken are if after surgery additional chemotherapy treatment should be given, and if so, which drug should be used. Information that helps taking the decisions is twofold. One concerns the probability that a patient is going to suffer from a metastasis; when this is low the benefit of chemotherapy might not outweigh its side effects. The second valuable information consists in predicting which drug is more likely to be effective. For both questions, the grouping we are uncovering could be useful. On one hand we use gene expression to find new subgroups, which really look different from each other; on the other we search for markers of tumor characteristics that are associated with how well the patient survived with the treatment received. With the first approach we should find subgroups that are real and robust, but their utility might become clear only later on and be of more importance for the choice of drug than for risk estimation. With the second approach we hope to improve the current methods used for risk estimation, but they might have limited utility to decide what the best treatment is for a given patient.

Main results obtained

A. Consensus molecular subtyping in CRC

Upon the publication of our study on the development of a colorectal cancer subtyping our group, in collaboration with others, has promoted the development of an international colorectal subtyping consortium in order to build consensus on the molecular subtypes of colorectal cancer (CRC) among the major researchers and clinicians in this field. The consortium members have agreed to contribute and share their respective data sets to achieve the following goals: (i) to compare and validate the major published colorectal subtypes; (ii) to conduct an integrative analysis across the pooled data sets to establish a robust consensus of molecular subtypes; (iii) to define the clinical and molecular hallmarks of these subtypes; (iv) to investigate the clinical value of these subtypes in patient studies; and (v) to establish a new paradigm within the research community based on collaboration and sharing when defining subtypes in cancer.
The project has been split in two separated phases: the first is mainly confirmatory and aims to reconcile divergent published subtype systems as well as identify their molecular and clinical hallmarks. The second focuses on a new discovery phase using the pooled dataset, which might lead to a more refined subtyping of CRC patients. We are now in the process of completing the first phase having applied our subtyping classifier to 30 public and proprietary gene expression (GE) data sets, outnumbering 3,800 patients. Our results have been compared to those obtained by the other 5 groups which are also part of the consortium, in order to assess concordance of subtype calls across the participating groups and performed clinical, molecular, pathway correlations.
Although the 6 subtyping algorithms evaluated were all trained independently, spanning multiple GE platforms and patient cohorts (mainly stage 2 and 3), a cross-subtype comparison yielded clear CRC consensus molecular subtypes (CMSs). In particular, CMS1 (approx. 15%) is enriched

for MSI, right-sided tumors, older age, females, hyper-mutation rates (including *BRAF*), and immune activation. CMS2 (40%) includes the most frequent, epithelial tumors, that are predominantly high CIN, MSS, left-sided, *TP53* mutated with upregulation of the EGFR, WNT and proliferation pathways, and have better survival outcomes. The rarer CMS3 (10%) is characterized by epithelial tumors that are heterogeneous in terms of MSS/MSI status, rather low CIN but have higher proportions of *KRAS*/*PIK3CA* mutations. CMS4 (20%) is characterized by mesenchymal/TGF-beta signaling, younger age at diagnosis and worse survival. These consensus molecular subtypes are very similar to the groups we described in our publication, with a fairly clear mapping of CMS1 to our "C" (CIMP-H like or inflammatory), CMS2 to our "B" (Lower crypt like), CMS3 to the "A" (Surface crypt like) and CMS4 to "D" (Mesenchymal).
Some 10-20% of the tumors cannot be classified easily or are called differently by the systems under comparison. The existence of additional subclasses needs further investigation, like the group that was least supported in our data (E or "mixed") and that is intermediate between the CMS2 and CMS4 could be kept as a separate group or merged with others. The pharmaceutical or clinical relevance of this gene expression-based taxonomy of colon cancer remains an open question to be explored. Their use in retrospective studies to assess potential association with differential benefit from specific pathway-based therapies would be very informative. These results are reported in an abstract that will be submitted at ASCO 2014 meeting.

B. Left-Right CRC

In this study, we focused on the clinic-patological and molecular differences between right- and leftsided stage II and III Colon Cancer (CC). In fact, it has been shown that tumors arising in the proximal and distal colon have distinctive clinical and molecular features, but very little is known concerning the differences in the mechanism of tumorigenesis and the effect that this could have on therapy. The analysis was performed on a population of patients enrolled in the PETACC-3 adjuvant chemotherapy trial for which detailed clinico-pathological data for right- (N=1116) and left-sided colon carcinomas (N=1733) were available. A subset of N=1404 samples had molecular data, including gene expression and DNA copy number profiles for 589 and 199 samples, respectively. Clinical-pathological and molecular information was also investigated in 413 patients from the TCGA project. The role of tumor site in anti-EGFR therapy was assessed in 650 chemo-refractory metastatic CRC patients. Overall we observed that mucinous histology, MSI-High, and mutations of BRAF and PIK3Ca were more frequent in right- than in left-sided colon carcinomas. Left-sided carcinomas were more frequently chromosomal instable, had higher frequency of EGFR or HER2 amplification, and overexpression of Epiregulin. Right-sided tumors showed enrichment for serrated pathway and the BRAF-like colon carcinomas subtypes as well as higher mutation rate in key tumorigenic pathways. Right-sided stage III carcinomas treated with adjuvant therapy had poorer SAR (N=285; HR=1·95 95% CI(1·6-2·4) P<0·001) in a multivariable model. Response to anti-EGFR therapy was restricted to metastatic left-sided colorectal carcinomas. In conclusion, our study has showed that proximal and distal colon tumors have distinctive patterns of clinical-pathological and molecular features. These biological differences likely have significant prognostic and therapeutic implications.

Publications

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2. The role of PROX1 in colon cancer progression and metastasis

Principal Investigator Tatiana Petrova

In this project one particular molecular mechanism in colorectal cancer is studied. This consists of PROX1, a molecule that has been shown to be involved in intestinal adenoma development in mouse models. The experiments conducted have shown that PROX1 is involved in the interaction between the cancer cells and the host stromal cells. Furthermore, PROX1 is closely associated with the signaling pathway involved in regulation of cell differentiation in intestinal mucosa (the Wnt pathway) and also in transformation of intestinal epithelial cells into cancer cells. Present data suggest that PROX1 is involved in progression to later stages (e.g.metastasis) rather than in the initial stages of colorectal cancer development. Analysis of the mechanism demonstrated that PROX1 acts by enhancing survival of cancer cells in hypoxia or nutrient‑low conditions through the productive use of autophagy.

The main goal of this project is to investigate the role of PROX1 in colon cancer metastasis. Previously, using a panel of 160 primary CRC tumors, we found that PROX1 expression is restricted to microsatellite stable cancer subtype, which has worse prognosis in comparison to microsatellite instable CRC (in collaboration with Dr. G. Marra, University of Zurich, and Dr. F. Bosman, IUP, CHUV). Furthermore, we found that PROX1 expression is increased in approx. 40% of metastatic CRC lesions in lymph nodes and liver (in collaboration with Dr.I. Letovanec, M. Gonzales and H. Bouzourene, IUP, CHUV). Using two different cell lines, we showed that PROX1 promotes the development of metastases. Moreover PROX1suppression, after the establishment of primary tumor and metastases, further prevented the development of metastases, demonstrating that targeting PROX1 could be an important clinical goal. Analysis of the mechanism demonstrated that PROX1 acts by promoting metabolic adaptation of tumor cells, in particular by enhancing survival of cancer cells in hypoxia or nutrient‑low conditions through the productive use of autophagy. During this year we addressed the questions of the mechanisms, underlying the ability ofPROX1 to promote outgrowth of metastases, and potential therapeutic strategies to targetPROX1+ cells in metastases.

1. By combining the data of PROX1 ChIP‑seq and gene expression profiling of colon cancer cells after PROX1 overexpression or suppression, we established a short‑‑‑list of direct candidate target genes. For two of them, BCL2L15 and CAV1, we could show that their overexpression overrides the ability of PROX1 to promote the survival of colon cancer cells in hypoxia in vitro, and the development of metastases in vivo. These results suggest that BCL2L15 and CAV1 are functionally important effectors ofPROX1 transcriptional network.
2. During the year 1, we found that autophagy inhibition and hypoxia state induce a synthetically lethal phenotype in PROX1‑expressing cells in vitro. During this year we demonstrated that the administration of small molecule autophagy inhibitor (chloroquine) induces a profound central necrosis in PROX1+ liver metastases *in vivo*, strongly suggesting that autophagy underlies metabolic adaptation of PROX1+cells in CRC metastases, and furthermore, that metastatic PROX1+ cells can be targeted with autophagy inhibitors.

3. In order to identify a more relevant in vitro model, we established intestinal organoids derived from genetically engineered mouse model, bearing most relevant colon cancer mutations (loss of Apc, overexpression of KrasG12V, loss of p53), and we showed that suppression of Prox1 prevents a clonogenic expansion of organoids, demonstrating an important role of Prox1 in the maintenance of colon cancer stem cells.
4. In order to identify small molecule inhibitors of PROX1 pathway, we screened a panel of FDA---‑approved cancer drugs for their ability to kill PROX1+ cells under hypoxic conditions or nutrient---‑low conditions. We identified a restricted number of such substances, which we further plan to test in vitro and in vivo. Our goal here is to provide additional means for targeting PROX1+ cells in metastases. Our results show that PROX1 transcriptional network, contributes to the outgrowth of micrometastases. We identified two potential effectors, BCL2L15 and CAV1, and demonstrated that targeting autophagy is a potential theurapeutic approach for eliminating PROX1+ cells, residing in hypoxia areas of metastases.

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3.4 The pathobiology of melanoma

Melanogenesis in melanoma progression and therapy

Principal Investigator Ghanem Ghanem

The Ghanem laboratory studies the biology of melanoma, with the intention to identitfy markers which are prognostic (distinguish melanoma with low risk of progression from high risk melanomas) and new therapies for melanoma. A promising new marker is TYRP1, of which the biology is studied in melanoma cell lines. A promising new therapy for melanoma could be Dasatinib. The mechanisms of action of this drug are explored, in order to allow identification of patients which might benefit from Dasatinib treatment.

In our preliminary study from microarray analysis, we measured the tyrosinase-related protein 1 (TYRP1) mRNA expression in cutaneous metastases from melanoma patients by real-time PCR. We found significantly correlation with distant metastasis-free survival, overall survival and Breslow thickness, suggesting that TYRP1 is a prognostic marker in skin metastases particularly useful when prognostic pathology parameters at the primary lesion are lacking.

Recently, we evaluated the prognostic value of TYRP1 in 104 lymph node metastases of stages III and IV melanoma patients. We quantified TYRP1 by real-time PCR and normalized to S100 calcium binding protein B (S100B) mRNA expression to correct for tumor load. We found that a high TYRP1/S100B mRNA ratio significantly correlated with reduced disease-free and overall survival (Cox-regression analysis, $p=0.005$ and 0.01, respectively), increased Breslow thickness (Spearman's rho test, $p<0.001$) and presence of ulceration (Mann-Whitney test, $p=0.02$) of the primaries. Moreover, high TYRP1/S100B was of better prognostic value (lower p-value) for overall survival than Breslow thickness and ulceration. Finally, it was well conserved during disease progression with respect to high/low TYRP1 groups. Thus, high TYRP1/S100B mRNA expression in lymph node metastases from melanoma patients is associated with unfavorable clinical outcome. Its evaluation in lymph node metastases may refine initial prognosis for metastatic patients, may define prognosis for those with unknown primaries, and may propose more aggressive therapy for high TYRP1 patients. Altogether, these data support the interest in prognostic value of melanogenic markers.

We recently evaluated responses to vemurafenib of a panel of wild-type and mutated melanoma cell lines and observed that even if six of eight V600EBRAF lines were sensitive to vemurafenib with IC50 < 2 μM, five of nine lines with WTBRAF were also sensitive to vemurafenib with IC50 between 1 to 3 μM. The three cell lines with NRAS mutation were resistant (IC50>10μM). We found that vemurafenib had a concomitant inhibitory effect on MAPK and PI3K/AKT signalling pathways in sensitive cells. Combination of vemurafenib and a PI3K inhibitor demonstrated synergic effects in vemurafenib-resistant V600EBRAF cells, while combination of vemurafenib with a MEK1/2 inhibitor showed a synergic effect in vemurafenib-resistant WTBRAF cells. Interestingly, as a previous study reported that inhibition of mutant BRAF (interfering RNA) increased pigmentation in melanoma cells (Rotolo et al, 2005), we evaluated the impact of vemurafenib on melanogenesis in V600EBRAF and WTBRAF melanoma cell lines. We found that three of six V600EBRAF cells had a substantial increase of visible pigmentation after 2 weeks of exposure to vemurafenib (no effect of tyrosine alone), while pigmentation was increased by tyrosine in five of six WTBRAF cell lines (no effect of vemurafenib). In V600EBRAF cells, pigmentation was accompanied by strong dose-dependent inductions of MITF, TYR, TYRP1 and gp100 protein expression after 24 hours (weak effects on DCT and melan-A, already expressed at high levels). MITF activation could explain the melanogenic effect of vemurafenib as it explains the pigmentation induced by tyrosine in WTBRAF cell lines. Thus, oncogenic BRAF may block pigmentation in some melanoma cell lines and its specific inhibition may restore it by upregulating MITF and inducing differentiation. By contrast, pigmentation

could not be induced in WTBRAF cells by vemurafenib confirming that melanogenesis is linked to some potent regulations of MAPK/ERK pathway. These data indicated that as pigment polymers are known to function as powerful free radical scavengers, ion exchangers and drug traps, the evaluation of pigmentation during vemurafenib therapy may be of clinical relevance as it may influence the sensitivity/resistance to the drug.

Publications

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3.5 Immunotherapy of cancer

Immunotherapy of cancer has been an important research focus for several decades and remains to be so. Key questions are: why the immune system responds initially to the presence of abnormal (cancer) cells in the body but fails to eliminate these cells. Conceptually, this might be due to failure of the immune system to recognize the cancer cells as harmful, hence no longer attacking them. Alternatively, this may be due to failure of immune-competent cells to kill the cancer cells. Effective ways to reconstitute and reinforce the immune system in its efforts to eliminate cancer cells would constitute a major breakthrough in cancer research and treatment. Two groups are working in this area, one on melanoma as a model system with emphasis on the role of miRNAs in the regulation of the function of CD8+ T-cells and the other on leukemia, with similar approaches and productive interaction between the groups.

1. **Role of miRNA species in regulating the immune response to melanoma**
   Principal Investigator Pedro Romero

The Romero laboratory studies mechanisms deployed by the immune system to attack cancer cells. The ultimate goal is to develop effective immunotherapies. In this project regulation of the function of a specific set of immune-competent cells (CD8+ T-cells) is examined. It was found that their function is at least in part regulated through micro-RNA, a new species of RNA with important general gene regulatory functions and potentially important as diagnostic tool as well as target for new therapies. The group focuses on melanoma. miRNA 155 has been identified as particularly promising.

In order to check the potential effect of miR-155 on memory cell generation, we transduced bone marrow cells from OVA specific OT-I CD8+ T cells with a miR-155 or control expressing retrovirus before transfer into irradiated recipients. Consecutively, we adoptively transferred the emerging naive cells into Listeria-OVA infected mice. Whereas miR-155 overexpression did not alter the phenotype of naive cells, upon transfer into infected mice miR-155 overexpression strongly promoted the accumulation of highly activated effector CD8+ T cells. In contrast, the percentage but not number of memory cells was strongly reduced. This suggests that miR-155 is strongly expanding the effector response without affecting the number of memory cell differentiation. Interestingly, upon antigen clearance, also the miR-155 overexpressing response contracted (importantly without showing signs of lymphoma formation over 2 month), suggesting that effects of miR-155 are dependent on extrinsic or temporal modulators of the T cell response. In this context and in collaboration with the De Palma lab/EPFL, we were testing a GFP reporter vector for measuring miR-155 activity in vivo.
We were also interested if miR-155 would alter WNT signaling as predicted by several algorithms. Comparing naive, effector and memory T cells from LCMV infected mice, we did not find differences in Conductin mRNA expression by quantitative PCR, which is a gene induced by WNT signaling. These results are suggesting that there is no major effect of miR-155 on WNT signaling and memory cell generation.
We further investigated the dynamics of miR-155 in chronic infection and for tumor control in a melanoma model and found that initial high levels are decreasing rapidly although antigen is persisting, suggesting room for therapeutic induction of miR-155. We now generated and are testing an inducible retrovirus in order to test the therapeutic effect of increasing miR-155 levels in `exhausted` T cells. This is promising as STAT5 cytokine signaling is beneficial for these cells and miR-155 is promoting these pathways.

For establishing new strategies of clinical use of miR-155, we have generated PSMA specific chimeric antigen vectors co-expressing miR-155. In order to avoid uncontrolled overexpression potentially promoting lymphomas, miR-155 is under control of an 3xNFAT/minimal IL-2 promotor for T cell receptor signal dependent expression. Experiments in vitro using a GFP reporter and also by measuring miR-155 levels by PCR, revealed indeed an antigen dependent

activity of the promotor, although with unsatisfying activity. GFP expression was low and miR-155 induction in the range of 1.5 fold. However, preliminary results show increased proliferation of miR-155 but not control vector containing cells in vitro upon stimulation with antigen expressing tumor cell lines. We are now testing a reportedly more efficient 6xNFAT element as well as vectors containing constitutive promotors for the proof of principle. For the readout of these new CAR vectors, we have established a humanized mouse model using PSMA and Luciferase co-expressing MS-1 tumor cells in immunodeficient mice, allowing for in vivo imaging of the efficacy of CAR/miR-155 expressing cells. In parallel, we have generated similar mouse vectors now ready to be tested in vivo.

Publications

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2. The role of the immune response in leukemia development

Principal Investigator Philippe Martiat

Like the Romero laboratory, the Martiat laboratory studies mechanisms deployed by the immune system to attack cancer cells. The high incidence of relapse in human cancers demonstrates the failure of the immune system to control cancer cells naturally. Fundamental investigations in the last decades have clearly established the concept that crosstalk between the immune system and tumor cells is important in cancer patient outcome. Tumors develop many ways to escape immune surveillance and favor their own growth. Even when a potent immune response is demonstrated ex vivo in laboratory, tumors induce regulatory, suppressive and inhibitory mechanisms to protect themselves from this immune response in patients. The interplay between the tumor and her immune microenvironment has been recently defined as a key element in the goal to achieve a definitive cure in cancer patients. The primary objective of our project is to understand in details the mechanisms responsible for the lack of effective anti-tumor immune response in leukemia.

Our approach is to characterize the functionality of the immune system of leukemic patients in order to define reliable, relevant and cost effective immune signatures. These immune profiles will help us to determine firstly which group of leukemic patient could benefit from immunotherapeutic approaches to eradicate leukemic cells and secondly to follow the patient treatment response and risk of relapse.

Since a few years, there is increased evidence that the immune microenvironment plays a fundamental role in the outcome of leukemia, either after high-dose chemotherapy or allogeneic stem cell transplantation. In particular, several groups have recently highlighted the role of regulatory T cells. Known prognostic factors include leukemic burden, age, cytogenetic or specific molecular abnormalities and residual disease at the end of the induction treatment. Nonetheless, few studies had concentrated yet on a thorough investigation of the immune cellular environment as a potential independent prognostic factor. We started to investigate the entire marrow and blood T cell compartment, which can be regarded, in the case of leukemia, as tumor-infiltrating lymphocytes. We compared those T cells to the ones purified from healthy donors' marrow and blood.

In AML patients, we have assessed quantitatively and qualitatively the purified T cell population, using flow cytometry and Affymetrix microarray studies, at diagnosis and in complete remission,

in bone marrow and peripheral blood samples, in an effort to better correlate the role of the absolute number and percentage of the various T cell subpopulations to the outcome of the disease in terms of relapse-free survival and overall survival, in otherwise undistinguishable leukemia as far as known prognostic factors are concerned. Unsupervised analyses revealed important significant differences between leukemic patients and healthy individuals in the gene expression profile of their T lymphocytes. To better understand the dissimilarities between the different samples, we have also performed an analysis of the cytokine mRNAs produced by infiltrating T cells, using quantitative RT-PCR arrays (Human Cytokines, Chemokines and Receptors StellARray™ qPCR array, T Regulatory Phenotyping 96 StellARray™ qPCR Array) from LonzaTM. T cell polarization bias in AML patients vs healthy individuals consist in the fact that type 1 T cell response associated molecules are downregulated, type 2 T cell response associated molecules are upregulated, regulatory T cell associated molecules are upregulated, innate immunity is inhibited, immunosuppressive molecules are expressed, T cell activation, inflammation and immune cell recruitment genes are expressed.

Another objective of this work was to determine whether inter-individual differences in the immune microenvironment had an impact on the outcome of the disease, in a given patient subgroup, having an identical prognosis when using the classical approaches currently in use. We could observe patient subgroups with distinct immune signature. This could, with a sufficient number of patients and a 5 years follow-up, reveal potential new prognostic markers, not directly linked to the leukemic cells themselves, but rather to the way how the host immunity reacts to the presence of these cells and, perhaps more importantly how it controls (or fails to) potential residual cells in patients after the end of treatments.

An already important conclusion is that, despite inter-patient differences, all exhibit a skewed CD3 profile, when compared with healthy individuals, but also a highly inflammatory environment. Age is a known prognostic factor in AML. When we performed an age-supervised analysis of patients' T cell gene expression profile, we could observe highly significant differences. Similarly, before waiting for a longer patient follow-up in order to look after prognostic value of our data, we stratified our patients in risks factor groups using cytogenetics and molecular biology classically used. Then, we performed analyses and observed significant differences in T cells from the different groups, showing that high risk AML patients have T cells that display a distinct genetic program than those from intermediate or "favorable" risk group.

Most of AML studies were focused on the leukemic blast biology, but circulating immune cells in patients seem to reflect an important message. Further study of leukemia microenvironment is needed to understand its role in this pathology and maybe reveal new possible therapeutic approaches or more patient specific therapy. In its current status, this study suggests the immediate possibility of initiating clinical studies aiming for example at inhibiting Tregs after chemotherapy for patients not assigned to allogeneic HSCT, but also to associate immunomodulatory drugs to classical therapy.

This last year we have doubled the number of samples in acute myeloblastic and lymphoblastic leukemia (AML and ALL) and gather more complete remission samples from patients we had in diagnosis state. We could perform new analyses and finished those for T lymphocytes from AML patients at diagnosis. We are also collecting and processing samples for the study of the methylome profiling of patients' T lymphocytes. We have optimized the extraction of all nucleic acids from the type of samples we receive, and we are checking, on control samples, the use of the Human Methylation 450 BeadChip researchers (Illumina HighScan platform), which can quantitatively interrogate 485,000 methylation sites per sample at single nucleotide resolution.

For Treg phenotyping gene expression array, we could see differences between healthy donors and leukemia patients' T lymphocytes but also inter-patients. Interestingly, those differences were concerning functional immune suppressive genes, Toll-like receptors signaling genes, STAT genes and genes implicated in microRNAs generation. We will continue to analyze all the samples we already have at our disposal. This should give us more information about the immune

leukemic environment of leukemia, and confirm a difference not only between patients and healthy volunteers, but also between subsets of patients (children versus adults for example) or inter-patients that could be correlated with relapse-free survival, and serve as individual biomarkers.

We are also exploring further the role of microRNAs in Treg biology, and analyzing Treg samples from acute leukemia patients to compare their molecular profile to normal Tregs. A first preliminary analysis suggests that adaptive Tregs in PB of AL patients qualitatively differs from normal ones as revealed by our identification of a distinct miRNA signature. We are studying whether these differences could be linked to different immunosuppressive mechanism or function.

Circulating miRNAs are emerging biomarkers in many diseases and cancers such as type 2 diabetes, pulmonary disease, colorectal cancer, and gastric cancer among others; however, defining a plasma miRNA signature in acute myeloblastic leukemia that could serve as a biomarker for diagnosis or in the follow-up was not done yet.

We identified several microRNAs in the plasma of AML patients at diagnosis that were differentially expressed compared to healthy donors. Among these miRs, two were upregulated (Let-7b, miR-523) and four were downregulated (let-7d, miR-150, miR-339, and miR-342). To note that, the expression level of these microRNAs didn't show any significant difference between the male and female donors implicated in this study as revealed by Student's *t*- test statistical analysis which was performed on the basis of results obtained by normality analysis tests, Kolmogorov-Smirnov and Shapiro-Wilk, that revealed that the results follow a normal distribution. Among these microRNAs, miR-150, and miR-342 were very significantly downregulated in the plasma of AML patients as confirmed using ROC curve analysis (AUC of 0.835 and 0.8125) that revealed that miR-150, and miR-342 were promising candidate biomarkers for AML at diagnosis. These data suggest that microRNA expression signature in plasma can serve as a valuable diagnostic and potential prognostic marker adding new information. This possibility of serving as a potential biomarker was further confirmed by qRT-PCR results that showed that the expression level of these two microRNAs, once patients were in complete remission, was similar to that of healthy donors.

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3. Molecular classification of peripheral T-cell lymphomas

Principal investigator L.de Leval

The Leval group studies peripheral T/NK cell lymphomas (PTCLs): PTCL, not otherwise specified (PTCL, NOS), angioImmunoblastic T-cell Lymphomas (AITL) and systemic ALK-positive and ALK-negative anaplastic large cell lymphomas (ALCL). The pathogenesis underlying most of these entities is poorly understood, and their boundaries are unclear. The aim of the project is to use state of the art molecular profiling methods to gain more insight in molecular characteristics and use these for more detailed classification.

Our hypothesis is that the combination of overall gene expression patterns and (epi)genetics of a large series of PTCLs will improve their biomolecular classification. and should identify clinical traits that will allow therapeutic stratification.

The aim of this project is to gain new insight into the molecular classification of the most common subtypes of peripheral T/NK cell lymphomas (PTCLs): PTCL, not otherwise specified (PTCL, NOS), angioImmunoblastic T-cell Lymphomas (AITL) and systemic ALK-positive and ALK-negative anaplastic large cell lymphomas (ALCL). The pathogenesis underlying most of these entities are poorly understood, and their boundaries are unclear. Our hypothesis is that the combination of overall gene expression patterns and (epi)genetics of a large series of PTCLs will improve their biomolecular classification and should identify clinical traits that will allow therapeutic stratification. Specifically, we aim to address the following questions:

- Can molecular profiles improve the classification of PTCLs?
- Are molecular signatures associated with distinct clinical traits, prognoses and/or response to treatment?
- Can these molecular studies help to characterize new potential therapeutic targets and be used as a rationale for specific therapies?
- Are distinct oncogenic mechanisms associated with different PTCL entities?

The following results were obtained:

1. Unsupervised class discovery

We have identified potential subtypes within PTCLs, including two subclasses within AITL characterized by different levels of mRNA expression of microenvironment-related genes (i.e. microenvironment-rich and microenvironment-poor groups), and four within PTCL, NOS. Among the subtypes within PTCL, NOS, we have found a subclass enriched for CD30, and another subclass enriched for the cytotoxic signature. We are currently characterizing the subgroups in terms of their biology and clinical features. For this part of the project, we have developed a robust pipeline for identifying each of the subtypes and generating classifiers from GEP and miRNA data; currently, we are extending the pipeline to integrate aCGH data.

2. Characterization of TFH-like PTCL,NOS

Analysis of TFH-like PTCL,NOS cases (defined by a combination of morphologic and immunohistochemical criteria) has shown that relevant clinical and mutational characteristics are significantly similar to AITL compared to its non-TFH-like counterparts; we have also observed a significant overexpression of previously-published molecular signatures (TFH, AITL, and AITL microenvironment) within the TFH-like subgroup, suggesting that AITL and TFH-like PTCL,NOS may share a common cell of origin and/or similar tumorigenic mechanisms. Supervised analysis of aCGH were consistent with these results.

3. CD30 expression in PTCL, NOS cases

Brentuximab vedotin has been recently approved by the FDA for the treatment of relapsed or refractory Hodgkin's lymphoma and systematic ALCL. Its efficacy is currently being evaluated for PTCLs. A critical question remains, however, as to what should be considered CD30+ in PTCLs. Previous work have given various thresholds, in some cases arbitrary, for CD30+ status based exclusively on IHC scores. By correlating IHC and mRNA expression levels of CD30, we were able to define an IHC score (staining of at least 50% of the tumor cells) that corresponds with the CD30 mRNA expression level that clearly splits negative from positive cases.

4. Comparison of mRNA and miRNA expression profiles in sorted TFH AITL cells and normal TFH cells

Since AITL tumors are characterized by abundant reactive infiltrate, tumor cells were isolated in order to characterize their specific molecular profile. We performed extended analysis of the genes and miRNA expression profiles between TFH AITL and normal TFH cells. Experimental validation of downregulated miRNAs of interest (e.g. hsa-miR-203) and its targets/target pathways (e.g. ETV1) are currently being performed to improve our understanding of AITL oncogenesis.

5. Characterization of EATL patients

Gene expression profiles of EATL tumors were compared to HSTL tumors (Hepatosplenic T-cell lymphoma) and NK/TCL tumors (natural killer/T-cell lymphoma) in order to identify a gene expression signature specific this entity in comparison with other extranodal NK/T cell tumors with a cytotoxic phenotype. This work is still in progress.

Publications

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Bisig B, de Reyniès A, Bonnet C, Sujobert P, Rickman DS, Marafioti T, Delsol G, Lamant L, Gaulard P, de Leval L.CD30-positive peripheral T-cell lymphomas share molecular and phenotypic features. Haematologica. 2013 ;98:1250-8

4. Outlook

The way the MEDIC foundation supported consortium 'Tumor-host interaction' continues to evolve confirms that high quality research can be effectively supported in an approach that is competitive in a (topographically and in terms of field of interest) limited setting. A high standard continues to be reached through auto-evaluation, internal review within the consortium and external peer review of new applications. The research program with its consortium approach continues to foster new interactions and new projects that individual groups might not have taken on. External peer review of the consortium constitutes a significant effort but remains an essential step towards a scientifically valid modus operandi. The independent external Scientific Advisory Board (prof. F.Lejeune, prof.G.Christofori, prof. H.Moch, prof. M.Mareel), evaluates the overall performance of the consortium, in participating (in part) in the annual research meeting and evaluating the annual reports submitted by the groups. The board members function as jury in the MEDIC prize applications. The trustees are satisfied with the structures developed and have confirmed their intention to continue to support the consortium - to the extent of what is financially possible - at the present level.
As the Foundation did not seek a high profile but better visibility, the website serves an important purpose (www.fondation-MEDIC.ch). The site allows MEDIC member groups to remain informed as to the activities of the consortium. Important element is also the obligation of investigators supported by MEDIC to specifically mention MEDIC support in their publications. In The 'MEDIC prize' for a particularly promising young clinician scientist, which was awarded for the first time in 2010, has been awarded in 2013 to dr. Lukas Flatz from Lausanne. The call for applications for the 2014 prize has resulted in several high quality applications. Awardees of the MEDIC prize are beginning to seek grant support from MEDIC, which is a perfect approach towards maintaining a dynamic evolution of the consortium.

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