FONDATION
MEDIC

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

Annual Report 2012

1. Introduction

The theme 'Tumor-host interaction' chosen for the consortium, created in 2006 around the project groups funded by grants from the MEDIC Foundation, remains of primary interest, even though the creativity of the investigators sometimes required a fairly wide interpretation of this theme. Of the 11 projects presently supported by MEDIC, 4 were renewed in 2012 based upon review of a full new grant application (some conditional, following critical reviews) and all developed favorably. 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, which has been instrumental in the decision making process that culminated in awarding the first MEDIC prize, played a role at a distance but an essential one in terms of maintaining high quality research. Annual scientific and financial reports were reviewed. 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 developed and went live (even though the detailed information of the groups was not yet fully developed; see www.fondation-MEDIC.ch).

The consortium members continue to meet annually in Lausanne and present in symposium format the progress made and the new projects that will be or have been submitted for funding. The MEDIC day was expanded to a day and a half, to allow more intense interaction. The program of the 2012 october meeting is presented in table 1.

Table 1 Programme of the MEDIC day 2012

Anita Wolfer	MEDIC prize 2011 lecture: The role of Myc in cellular invasion and metastasis
Ivan Stamenkovic	Energy regulation of glioblastoma stem cells
Mauro Delorenzi	Linking molecular genetic heterogeneity of colon cancer with clinically useful subtypes
Tania Petrova	Role and targeting of Prox1 in colon cancer metastasis
Curzio Ruegg	Unraveling the role of MAGI1 and CYR6 in tumor progression and therapy
Christos Sotiriou	Molecular heterogeneity drives metastasis in breast cancer
Agnese Mariotti	Lactadherin and Sox10 in breast cancer
Didier Picard	Pharmacological and genetic investigations of estrogen-regulated and other cancers
Marie-Agnès Doucey	Pro-angiogenic monocytes in breast cancer
Ghanem Ghanem	Melanogenesis in melanoma progression and therapy

Edoardo Missiaglia/ Laurence de Leval	Molecular biomarkers in peripheral T-cell lymphoma
Philippe Martiat	Role of immune micro-environment in acute leukemia
Jan Dudda (P.Romero)	MicroRNA-155 is promoting tumor-specific CD8+ T cells

Productive collaborations continue to develop, justifying the decision to choose for this approach. The chosen theme "tumor-host interaction" continues to be timely and allows both a common focus in the research projects as well as significant latitude in the development of the individual research lines. One new group was accommodated. With the creation of the Department of Oncology in Lausanne some groups changed their affiliation (for the better).

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 by the MEDIC foundation

C. Ruegg	Role of CYR61 in tumor progression and metastasis	CHF	351'000.-
A. Mariotti	Elucidation of the function of Lactadherin and Sox10 in breast cancer	CHF	118'830,-
D. Picard	Molecular and pharmacological investigation of the factors contributing to tamoxifen resistance of ERa-positive breast cancers	CHF	161'340.-
M. Delorenzi	Linking tumor heterogeneity with clinically useful subtypes of colon cancer	CHF	209'240.-
L. de Leval	Characterization of molecular biomarkers relevant to the biology, diagnosis and prognosis of peripheral T-cell lymphomas	CHF	64'000,-
T. Petrova	Role and targeting of PROX1 role in colon and small cell lung cancer	CHF	191'000.-
I. Stamenkovic	Mechanisms that govern energy regulation in cancer stem cells	CHF	117'000.-
P. Romero	Role of microRNAs in CD8 T cell function	CHF	121'012.-
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.	€	142'000.-

It is relevant here to note that the total volume of MEDIC supported research conducted in Lausanne has grown and in 2012 7 groups profited from MEDIC support. With the expansive growth of cancer research in the Lausanne academic community this does not come as a surprise. The second biggest site is Institut Jules Bordet with 3 project groups. Geneva participated with 1 group and attempts are underway to expand in Geneva. One group is in the Department of Medicine of the Faculty of Sciences in Fribourg.

3. Research programme

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. **Heterotypic interactions is the host response to tumor cell growth**
   Principal Investigator: Curzio Ruegg

The main focus of the laboratory is the study of the tumor microenvironment (tumor-host interaction). In recent years we have learned that the tumor microenvironment plays an important role promoting tumor progression. Tumor angiogenesis and bone marrow-derived and inflammatory cells recruited at tumor sites have emerged as critical determinant of tumor progression. Our understanding of the functional relationship between the tumor microenvironment and tumor cells is still limited. This is becoming particularly relevant in view of the potential targeting of stromal event to inhibit tumor progression. The main questions addressed in the laboratory include:

- How do cells of the microenvironment, in particular inflammatory cells, promote tumor growth and metastasis, and how do therapeutic interventions modify the tumor microenvironment and how do these modifications impact tumor behavior?

- How does the cross-talk between tumor cells and the microenvironment evolve during progression to metastasis?

- How do the microenvironment react to anticancer therapies and what are the consequences to tumor progression?

During this year we have progressed in the characterization of MAGI1 and CYR61.

MAGI1. We previously identified the scaffolding protein MAGI1, as a tumor suppressor gene upregulated by COXIB in colorectal cancer (CRC) cells. Here we studied the regulation of MAGI at transcriptional and posttranscriptional level to identify possible regulatory pathways. First, we performed a promoter analysis of the 1500 bp of the gene upstream from the translation start site. This region was predicted to include binding motifs for transcription factors based on in silico studies (e.g. HIF1, LEF1/TCF-4E, NF-kB, cMyc). Overlapping fragments were generated and tested in non-tumorigenic cells, 293T and in SW480 CRC. In both cell types, we found activity between -300 bp and +200 bp of the MAGI1 gene, suggesting the presence of a promoter/enhancer. Between - 500 bp and +1 bp we found repressive activity in both cell types. In the region between -700 bp to -200 bp we found activity in 293T, and to a lesser extent in SW480. Uusing dominant negative TCF4 constructs we demonstrated that Wnt induces MAGI1 expression. As MAGI1 represses Wnt signaling, it appears that MAGI1 provides a negative feed-back to the Wnt pathway. Next, we screened for miR binding sites in the 3'UTR of human MAGI1 gene. We found consensus sequences for at least 7 miRs. Functional assays confirmed suppressive activity for miRs 182 and 205. The significance of MAGI1 regulation by miR is under investigation.

To study the role of MAGI1 as tumor suppressor, we are generating a transgenic mouse with Tet-regulated MAGI1 expression (CMV/tet::MAGI1). We have constructed the plasmid and transgenic mice are generated at EPFL transgenic core facility. The Vilin::rtTA-M2 line allowing regulated expression in the intestine will be obtained from Dr. R. Fodde. Resulting mice will be used in chemical carcinogenesis assays (Azoxymethane + DSS) and crossed to APCmin mice to assess for turmo suppressive activity of MAGI1. In collaboration with T. Petrova, we are generating VEC::rtTA-M2 x CMV/tet::MAGI1 double transgenic mice to study effects ono MAGI1 in the vasculature.

CYR61. We further characterized the role of CYR61 in promoting EMT. CYR61 is transiently induced during EMT in NMuMG and 4T1 cells and is a direct target of Zeb2. Functional experiments demonstrated that silencing of CYR61 in NMuMG cells delays but does not suppress TGFbeta induced EMT. Silencing of CYR61 in the metastatic breast cancer cell line MDA-MB-231 did not affect cell adhesion, but strongly reduced cell spreading and cell migration on matrix proteins, transendothelial cell migration and colony formation on 3D. CYR61 silencing effectively suppressed the formation of experimental lung metastasis. To study the effect of CYR61 on multistep cancer progression we generated a Rip1Tag2CYR transgenic line. Tumors in Rip1Tag2CYR are significantly larger, of higher grading and more invasive compared to Rip1Tag2 mice, but have no metastasis.

Furthermore, we obtained a conditional CYR61knock-out line with a floxed Cyr61 allele, in collaboration with P. Uhrin and J. Breuss, Medical University of Vienna. We are currently back crossing the mice to various backgrounds.

Publications

Kuonen F, Secondini C, Rüegg C. Molecular pathways: emerging pathways mediating growth, invasion, and metastasis of tumors progressing in an irradiated microenvironment. Clin Cancer Res. 2012;18:5196-202

Kuonen F, Laurent J, Secondini C, Lorusso G, Stehle JC, Rausch T, Faes-Van't Hull E, Bieler G, Alghisi GC, Schwendener R, Andrejevic-Blant S, Mirimanoff RO, Rüegg C. Inhibition of the Kit ligand/c-Kit axis attenuates metastasis in a mouse model mimicking local breast cancer relapse after radiotherapy. Clin Cancer Res. 2012;18:4365-74.

Lorusso G, Rüegg C. New insights into the mechanisms of organ-specific breast cancer metastasis. Semin Cancer Biol. 2012;22:226-33 Sleeman JP, Christofori G, Fodde R, Collard JG, Berx G, Decraene C, Rüegg C. Concepts of metastasis in flux: the stromal progression model. Semin Cancer Biol. 2012;22:174-86

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.

Growth of numerous cancer types is believed to be driven by a subpopulation of poorly differentiated cells, often referred to as cancer stem cells (CSC), 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. We have shown that the oncofetal insulin-like growth factor 2 mRNA binding protein 2 (IMP2, IGF2BP2) regulates oxidative phosphorylation (OXPHOS) in primary glioblastoma (GBM) sphere cultures (gliomaspheres), a validated *in vitro* model for CSC expansion. We demonstrated that IMP2 binds several mRNAs that encode mitochondrial respiratory chain complex subunits and that it directly interacts with Complex I (NADH:ubiquinone oxidoreductase) proteins. Depletion of IMP2 in gliomaspheres decreased their oxygen consumption rate and both Complex I and Complex IV activity that resulted in impaired clonogenicity *in vitro* and tumorigenicity *in vivo*. Importantly, inhibition of OXPHOS but not of glycolysis abolished GBM cell clonogenicity. Our observations suggest that gliomaspheres depend on OXPHOS for their energy production and survival and that IMP2 expression provides a key mechanism to ensure OXPHOS maintenance by delivering respiratory chain subunit-encoding mRNAs to mitochondria and contributing to Complex I and Complex IV assembly.

Publications

Janiszewska M, Suvà ML, Riggi N, Houtkooper RH, Auwerx J, Clément-Schatlo V, Radovanovic I, Rheinbay E, Provero P, Stamenkovic I. Imp2 controls oxidative phosphorylation and is crucial for preserving glioblastoma cancer stem cells. Genes & Dev 2012;26:1926-1944

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 fromgene 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 (tumorcolonization) using next generation sequencing technology.

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

Tumor Rearrangements:

We identified 334 somatically acquired rearrangements across the 5 patients, with considerable interpatient heterogeneity in terms of numbers of rearrangements, with 3 patients having few (<10) whilst 2 patients showed more than 100 rearrangements with a

particular tandem duplication phenotype. All patients had rearrangements common to both foci, suggesting a shared genetic background. In 4 patients, we observed a branched evolutionary pattern since private rearrangements were present in each focus. In contrast, we observed a linear evolutionary pattern in 1 patient, since private rearrangements were only found in 1 of the 2 foci.
Interestingly, for 4 pts some of the identified rearrangements were found in parts of tumoradjacent histologically normal breast tissue.

Targeted screen sequencing:
The targeted gene screen identified 121 and 7 unique somatic substitutions and short indels, respectively, across all samples. There were no shared variants between the 2 lesions from 2 patients. The lesions from the 3 other patients shared variants of 3 (PIK3CA, FOXP4, MAP3K1), 5 (PIK3CA, MLL3, PRKCE, PIK3R1, NTRK3) and 9 genes (PTEN, TP53, XPC, GAB2, UBR5, YAP1, CCND2, PIK3C2G, TUBB4), respectively. Interestingly, some variants in driver cancer genes were not shared between the 2 lesions of the same patient. This suggests that although multifocal tumors seem to have a shared genetic background, different lesions could rely on different genetic abnormalities for further cancer development

Gene expression & DNA methylation:
To obtain a comprehensive comparison between foci in each patient, an overview of methylation and gene expression patterns was additionally sought and presented as differentially methylated CpGs (absolute delta beta>0.2) and differentially expressed ProbeSets (absolute fold change>1.5).
In one patient there were only minor differences in transcriptomic and methylation patterns between the 2 foci. Contrastingly, in the second patient, DNA methylation patterns were dramatically discordant between the foci, suggesting that genomic, transcriptomic and epigenomic patterns are not necessarily correlated. Only in 1 patient of the 5 investigated, the 2 foci were extremely different, with only 14% of common rearrangements. These genetic differences were associated with dramatic differences in methylation and transcriptomic profiles. Interestingly, for 4 patients some of the identified rearrangements were found in parts of tumor adjacent histologically normal breast tissue.
To understand the potential clinical relevance of these transcriptomic differences, we computed different gene expression signatures representing the main molecular breast cancer subtypes, the contribution from the tumor microenvironment and molecular pathways which have been associated to breast cancer prognosis and response to some anticancer therapies. The various signature scores were very similar between the lesions of 2 patients but not for the 2 other ones for which expression profiles were available. These differences might imply a differential response of multifocal lesions to cancer therapies targeting or making use of these pathways or biological processes.

b. Comparison of matched primary and metastatic breast cancer

We do have some preliminary results for a single patient with at autopsy metastases in the brain, lung, kidney, ovarium, and in the spleen.We started by sequencing the normal tissue, the primary, as well as the ovarium and spleen metastases.One hundred and eighty nine unique somatic substitutions were identified for this patient. Twentyeight percent could be validated by Sequenom, 32% were false positive, 40% could not be validated by the Sequenom because of their presence in repetitive regions (11%) or because the Sequenom assay could not be designed (7%) or because the Sequenom assay failed (22%). For the last two categories, we will try to validate the variants using an alternative technology such as capillary sequencing.

c. Molecular characterization of circulating tumor cells

We have performed spiking experiments using the MDA231 tumor cell line and single/pool of 10 MDA MB231 cells were recovered using the CellSearch and the DEPArray system. The mutation profiles of three single tumor cells and one group of 10 tumor cells isolated with the DEPArray were compared to that of genomic DNA extracted from 35 million cells harvested from the same culture flask. We have studied the presence of 10 different mutations in 6 genes with a customized iPlexassay. Call rates of the amplified genomic sequences were 80% for the single cell samples and 100% for the 10 cell sample. Of the 10 mutations covered by the iPlex assay, 4 (BRAF_G1391T, KRAS_G38A, TP53_G839A and NF2_G691T) have previously been documented in the MDAMB231 cell line. All 4 expected mutations were reliably detected in 2 out of 3single cell samples and the 10 cell sample.

The prospective study will continue and we aim to collect frozen metastatic biopsies and CTCs from 5 additional women with metachronous metastatic breast cancer that have frozen primary tumor available. Moreover, we are currently comparing different protocols for whole genome amplification of single/pool of tumor cells in order to perform whole genome sequencing. The best protocol will be selected for whole genome sequencing of CTCs from our prospective cohort of 8 metastatic breast cancer patients. Whole genome sequencing (x40) results of biopsies of liver metastases and matched CTCs of the 8 patients will be compared. Validation of identified variants will be performed on the same samples using an alternative technology such as Sanger sequencing. Selected identified variants in CTCs or metastasis will be also evaluated in the primary tumor using Sanger sequencing. In the 5 additional women with matched samples from frozen primary tumor, frozen metastasis and CTCs, whole genome sequencing (x40) will be performed to compare primary tumor vs metastasis vs CTCs. Validation of identified variants will be performed on the same samples using Sanger sequencing.

Publications

Criscitiello C, Azim HA Jr, Schouten PC, Linn SC, Sotiriou C.Understanding the biology of triple-negative breast cancer. Ann Oncol. 2012;Suppl 6:vi13-vi18.

Desmedt C, Voet T, Sotiriou C, Campbell PJ.Next-generation sequencing in breast cancer: first take home messages. Curr Opin Oncol. 2012;24:597-604.

Ignatiadis M, Sotiriou C, Pantel K.Minimal residual disease and circulating tumor cells in breast cancer: open questions for research. Recent Results Cancer Res. 2012;195:3-9.

Ignatiadis M, Singhal SK, Desmedt C, Haibe-Kains B, Criscitiello C, Andre F, Loi S, Piccart M, Michiels S, Sotiriou C. Gene modules and response to neoadjuvant chemotherapy in breast cancer subtypes: a pooled analysis. J Clin Oncol. 2012;30:1996-2004.

Desmedt C, Majjaj S, Kheddoumi N, Singhal SK, Haibe-Kains B, El Ouriaghli F, Chaboteaux C, Michiels S, Lallemand F, Journe F, Duvillier H, Loi S, Quackenbush J, Dekoninck S, Blanpain C, Lagneaux L, Houhou N, Delorenzi M, Larsimont D, Piccart M, Sotiriou C. Characterization and clinical evaluation of CD10+ stroma cells in the breast cancer microenvironment. Clin Cancer Res. 2012;18:1004-14.

Fumagalli D, Andre F, Piccart-Gebhart MJ, Sotiriou C, Desmedt C.Molecular biology in breast cancer: Should molecular classifiers be assessed by conventional tools or by gene expression arrays? Crit Rev Oncol Hematol. 2012;84 Suppl 1:e58-69

Haibe-Kains B, Desmedt C, Loi S, Culhane AC, Bontempi G, Quackenbush J, Sotiriou C. A three-gene model to robustly identify breast cancer molecular subtypes. J Natl Cancer Inst. 2012;104:311-25

Metzger-Filho O, Michiels S, Bertucci F, Catteau A, Salgado R, Galant C, Fumagalli D, Singhal SK, Desmedt C, Ignatiadis M, Haussy S, Finetti P, Birnbaum D, Saini KS, Berlière M, Veys I, de Azambuja E, Bozovic I, Peyro-Saint-Paul H, Larsimont D, Piccart M, Sotiriou C. Genomic grade adds prognostic value in invasive lobular carcinoma. Ann Oncol. 2013;24:377-84

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 ERα 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. We have now validated several genes by targeted chromatin immunoprecipitation (ChIP) and gene expression analyses (Q-PCR). We also picked several genes for further mechanistic and functional studies. These include NFkB2 and RalBP1. The former is a paralog of the far more extensively studied NFkB. However, NFkB2 is well worth investigating since it has been linked to a variety of cancers. RalBP1 has been linked to drug resistance, which makes it highly interesting in the context of tamoxifen resistance of breast cancer and the role that we believe cAMP signaling plays in this.

b. The arginine methylase CARM1 as cAMP-regulated ERα activator:

We have followed up on our discovery that CARM1 mediates the activation of ERα by cAMP and thereby contributes to tamoxifen resistance in breast cancer. We invested a lot of time and money into raising an antiserum against the PKA-phosphorylated form of CARM1 to be able to probe for this form in tumor biopsies. Unfortunately, all of these attempts failed. In parallel, we have decided to provide formal experimental proof that it is S447 (in human CARM1) that is phosphorylated by using mass spectrometry. Once we have this confirmation, we would be encouraged to explore the possibility to use the advanced mass spectrometric method called "Selected reaction monitoring" (SRM). SRM allows one to home into a specific peptide, e.g. the CARM1 peptide around phosphorylated S447, in a total lysate. In principle, although highly challenging, one may be able to use SRM to monitor the phosphorylation status of S447 in total protein extracts of biopsies.

c. Role of Hsp90 for activation of ERα by cAMP and tamoxifen resistance:

We had previously discovered that Hsp90 is required for activation of ERα by cAMP and that this also requires a histone deacetylase activity as a positive regulator of Hsp90. Using specific Hsp90 or HDAC inhibitors or combinations thereof, we were hoping to restore tamoxifen sensitivity to tamoxifen-resistant breast cancer cells. By now, we have accumulated a whole panel of HDAC6-selective inhibitors, but more tests will be necessary to clarify the pharmacology. Meanwhile, we have also optimized Hsp90 ChIPs to be able to determine the genomic binding landscapes of ERα, Hsp90 and CARM1 in parallel. Comparative ChIP-seq experiments are imminent and will complement the analysis of the cAMP-induced ERα "cistrome" that has been discussed above.

d. miRNAs regulating ERα expression:

We generated a miR-22 KO mouse hoping that we would be able to get genetic proof for its importance in regulating in ERα levels. Unfortunately, it looks like genetics has proven us wrong in this case. We could not detect any change in ERα levels in a panel of tissues, notably tissues with moderate levels of ERα where one might have expected an impact of removing miR-22. Why miR-22 regulates ERα levels in human breast cancer cells but not in the mouse remains a mystery.

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. Although these experiments have been severely disrupted by repeated personnel changes, we are progressing. We are still studying VPS11 and VPS16, two proteins that might link ERα function to membrane traffic. Several candidates from the genome-wide RNAi screen have also been selected for further analysis. These are currently ongoing.

Publications

Picard, D. Preface to Hsp90. Biochim. Biophys. Acta 2012;1823:605-606.

Yoshida, S., Tsutsumi, S., Mühlebach, G., Sourbier, C., Lee, M.-J., Lee, S., Vartholomaiou, E., Tatakoro, M., Beebe, K., Miyajima, N., Mohney, R., Chen, Y., Hasumi, H., Fukushima, H., Nakamura, K., Koga, K., Kihara, K., Trepel, J., Picard, D., and Neckers, L. The molecular chaperone TRAP1 regulates a metabolic switch between OXPHOS and aerobic glycolysis. Proc. Natl. Acad. Sci. USA2013;110:E1604-12

Fierro-Monti, I., Echeverria, P., Racle, J., Hernandez, C., Picard, D., and Quadroni, MDynamic and diverse impacts of the inhibition of the molecular chaperone Hsp90 on the T-cell proteome. Mol. Cell. Proteomics (2013). *submitted*.

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.

In a paper we published recently we have demonstrated that the secreted protein MFGE8 is highly expressed in Estrogen Receptor (ER) and Progesterone Receptor (PR) negative breast cancer and enhances the tumorigenic potential of mammary cancer cells in vivo. In order to define signaling networks involving FG E8 and identify its functional partners we have looked for genes highly coexpressed with MFG E8 in breast cancer by analyzing the gene expression profiles of 808 breast carcinomas reported in three publicly available datasets (GSE20194, GSE3494, and GSE6532). We have found that the expression of the transcription factor Sox10 presents the highest positive correlation with MFGE8 expression, suggesting a possible functional interaction between the two proteins. We have started to analyze Sox10 expression and function in breast carcinoma. All together our results indicate that Sox10 is expressed in normal mammary epithelial cells having proliferative potential and progenitor features, and suggest that it may regulate mammary epithelial cell growth and possibly the maintenance of luminal progenitor cells. Our observations are also supported by recent publications which identified Sox10 as a transcriptional target of Sox9 in mammary epithelial stem and progenitor cells, important for Sox9/Slug dependent maintenance of the stem cell state. On the basis of our preliminary results we hypothesize that oncogene activation in mammary epithelial cells that express Sox10 can result in cell transformation, causing the expansion of the Sox10/oncogene expressing cell population, and ultimately cancer development. In other words, Sox10 may cooperate with oncogenes to promote breast carcinogenesis. Interestingly, a synergy between Sox10 and an oncogene has recently been demonstrated in giant nevi derived melanomas in which Sox10 is required for mutant NRas to induce progression from a benign lesion to melanoma. Our results lead us tohypothesize that oncogenic activation in the subpopulation of mammary epithelial cells that express Sox10 may result in cell transformation and eventually cancer development, and that active Sox10 may thus cooperate with oncogenes in mammary tumorigenesis.Our ongoing experiments will allow us to explore this hypothesis and clarify the role of Sox10 role in breast cancer.

Publications

Carrascosa C, Obula RG, Missiaglia E, Lehr HA, Delorenzi M, Frattini M, Rüegg C, Mariotti A. MFG-E8/lactadherin regulates cyclins D1/D3 expression and enhances the tumorigenic potential of mammary epithelial cells.Oncogene. 2012;31:1521-32.

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.

By combining Boolean modeling and experimental approaches, we predicted *in silico* all minimal perturbations transitioning the highly pro-angiogenic phenotype of tumor TEM to the weak pro-angiogenic phenotype of blood TEM and vice versa. *In silico* predicted perturbations were validated experimentally using patient TEM. Inferred TEM regulatory network accurately captured experimental TEM behavior and highlighted crosstalk between Tie2, VEGFR1, TGFR1 and TNFR pathways to control their pro-angiogenic and pro-tumoral activities. Furthermore we show that Ang-2, PIGF and TNF are specifically enriched in tumor zones containing TEM and promote tumor growth and TEM pro-angiogenic activity.

In human breast tumors, TEM express the canonical lymphatic markers Lyve-1, podoplanin, VEGFR-3 and PROX-1 and display lymphangiogenic activity in vivo in addition to their known hemangiogenic function. Furthermore, the acquisition of TEM lymphatic phenotype coincides with the stable insertion of TEM into lymphatic vessels specifically in the tumor but not in adjacent non-neoplastic breast tissue. These observations suggest that the breast tumor microenvironment shapes both TEM phenotype and spatial distribution. Moreover, we show that combined blockade of Tie2 and VEGFR kinase activities effectively inhibits tumor angiogenesis.

Publications

Ibberson M, Bron S, Guex N, Faes-van't Hull E, Ifticene-Treboux A, Henry L, Lehr HA, Delaloye JF, Coukos G, Xenarios I, Doucey MA.TIE-2 and VEGFR kinase activities drive immunosuppressive function of TIE-2-expressing monocytes in human breast tumors.Clin Cancer Res. 2013;19:3439-49

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 to take better decisions about how to treat a given patient. 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 for applications.

Main results obtained

A. BRAF-like CRC tumors

We developed a classifier for detecting tumors harbouring the BRAF activating mutation V600E in gene expression profiles of colorectal cancer. The classifier had very high sensitivity, finding virtually all tumors with this mutation, but not very high specificity. There are tumors without the mutation that have high classification scores, close to those of the tumors with the mutation. A cleassifier training method yielded a 64 gene (32 pairs) classifier with 96% sensitivity and 86% specificity. Therefore, 14% of the tumors without mutation were wrongly called positive. We call this subpopulation of BRAF wild type tumors, the BRAFm-like tumors. They include 30% of the tumors with an activating mutation in the KRAS gene and 13% of those that are wild type for BRAF and KRAS. The BRAFm-like group shares a number of features with the BRAF mutated tumors, both are enriched in mucinous tumors, tumors from the proximal colon and tumors with microsatellite instability). In particular the group forms a prognostic subgroup characterized by poor survival after relapse like the group with the BRAF mutation.

B. CRC Gene expression subtypes

We have analysed gene expression profiles of 1113 stage II and III CRC microarray samples from PETACC3 and 4 additional public datasets to define a set of subtypes and we confirmed their occurrence in an independent set of 720 samples from several public datasets. Subtypes were defined based on hierarchical clustering, with similarity between tumors based on a set of 52 metagenes obtained by grouping genes with similar expression profiles.

We identified 5 major gene-expression subtypes A-E in stage II-III CRC, with different levels of expression. The subtypes do not significantly differ in stage, age or gender. A subtype (called D) that shows higher stroma and EMT and low differentiation has poorer relapse-free survival, survival after relapse and overall survival. A second subtype (called C), which is enriched in tumors with a particular set of pathological characteristics (mucinous, proximal, MSI tumors) high in the expression of immune genes and some differentiation markers, shows higher risk in overall survival and survival after relapse. Two subtypes (called A and B) are lowest in the stroma / EMT component and high for many markers of differentiated colon cells. They are closer to the normal tissue samples, in particular the subtype A. The last subtype (called E) is a smaller group with high stroma / EMT, high in some differentiation markers, but with no apparent increased risk of relapse.

By an unsupervised approach, we identified and validated distinct molecular subtypes in stage II and III CRC. This molecular characterization can complement the clinico-pathological characterization of tumors and has implications for prognostic models, stratification in clinical trials and the search for the most effective drug in each patient group.

Publications

Rossi S, Di Narzo AF, Mestdagh P, Jacobs B, Bosman FT, Gustavsson B, Majoie B, Roth A, Vandesompele J, Rigoutsos I, Delorenzi M, Tejpar S.microRNAs in colon cancer: a roadmap for discovery. FEBS Lett. 2012;586:3000-7.

Roth AD, Delorenzi M, Tejpar S, Yan P, Klingbiel D, Fiocca R, d'Ario G, Cisar L, Labianca R, Cunningham D, Nordlinger B, Bosman F, Van Cutsem E. Integrated Analysis of Molecular and Clinical Prognostic Factors in Stage II/III Colon Cancer.
J Natl Cancer Inst. 2012 7;104:1635-46.

Tian S, Roepman P, Popovici V, Michaut M, Majewski I, Salazar R, Santos C, Rosenberg R, Nitsche U, Mesker WE, Bruin S, Tejpar S, Delorenzi M, Bernards R, Simon I. A robust genomic signature for the detection of colorectal cancer patients with microsatellite instability phenotype and high mutation frequency. J Pathol. 2012 ;288:586–595

Popovici V, Budinská E, Tejpar S, Weinrich S, Estrella H, Hodgson G, T. Xie T,
Van Cutsem E, Bosman F, Roth A, Delorenzi M.
Identification of a poor prognosis BRAF-mutant-like population of colon cancer patients.
J Clin Oncol. 2012;30:1288-95.

Budinska E, Popovici V, Tejpar S, D'Ario G, Lapique N, Sikora KO, Di Narzo AF, Yan P, Hodgson JG, Weinrich S, Bosman F, Roth A, Delorenzi M. Gene expression patterns unveil a new level of molecular heterogeneity in colorectal cancer. J Pathol. 2013;231:63-76.

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.

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, CHUV). Furthermore, PROX1 expression was observed in metastatic CRC lesions in lymph nodes and liver (in collaboration with Dr. H. Bouzourene, IUP, CHUV). Suppression of PROX1 in PROX1+ SW620 cells, derived from the metastatic lymph node lesion, strongly reduced development of metastases in an orthotopic model of CRC. In line with these results, PROX1 overexpression in PROX1-negative DLD1 CRC cells significantly enhanced the development of metastases. Surprisingly, growth of primary tumor was not affected in either cell line, suggesting that PROX1 has distinct roles in adenomas vs. carcinomas. To establish whether targeting PROX1 pathway is a potentially viable clinical approach, we have studied the effects of PROX1 suppression after the establishment of primary tumor and metastases. While control mice developed rampant metastatic lesions, suppression of PROX1 arrested growth of metastases. During this year we addressed the questions of the mechanisms, underlying the ability of PROX1 to promote outgrowth of metastases.

1. We have analysed the ability of PROX1 to regulate colon cancer cell survival in response to hypoxia and nutrient deprivation. We found that PROX1+ cells proliferate and survive significantly better then PROX1- cells when cultured in hypoxic conditions. We also analysed the metabolism of PROX1+ vs PROX1- cells, and we found that PROX1+ cells demonstrate more efficient switch to oxidative phosphorylation under the conditions of glucose deprivation in normoxia and to enhanced glycolysis in hypoxia. Therefore these data suggest that PROX1 acts by promoting metabolic adaptation of tumor cells to changes in fuel, which would explain better survival of metastatic cells in the microenvironment of new host organ.

2. We further addressed the mechanism of enhanced adaptation of PROX1+ cells. One of the key pathways that mediates stress-induced metabolic adaptation is autophagy, which is important for elimination damaged cellular components, but also for recycling to maintain nutrient flux and energy homeostasis. We found that PROX1+ cells have increased basal level of autophagy. Moreover, inhibition of autophagy with chloroquine and 3-methyl adenine, which act at different steps, completely prevented the induction of cell survival and proliferation by PROX1+ in hypoxic conditions. These results demonstrate that autophagy inhibition and hypoxia state induce a synthetically lethal

phenotype in PROX1-expressing cells, and strongly suggest that autophagy underlies metabolic adaptation of PROX1+ cells in CRC metastases.
3. We have carried out gene expression profiling of SW620 PROX1+, SW620PROX1-, DLD1PROX1+ and DLD1PROX1- cells (in collaboration with Mauro Delorenzi). We have identified a subset of 36 genes, which are regulated by PROX1 in both DLD1 and SW620 cells. Using previously obtained PROX1 ChIP-seq data, we further restricted this list to several direct target genes. We have cloned cDNAs and obtained targeting siRNAs to study the role of these genes in PROX1 induced autophagy and enhanced cell proliferation/survival in hypoxia. These results suggest that PROX1-regulated transcriptional network contributes to the outgrowth of micrometastases through the regulation of autophagy. Therefore, we propose to study the mechanisms of autophagy induction by PROX1 and whether inhibition of autophagy represents a clinically relevant treatment approach for colon cancer metastasis.

3.4 The pathobiology of melanoma

1. Molecular characterisation of melanoma progression: the role of BCSC1

Principal Investigator Vincent Piguet

Using state of the art molecular biology techniques the Piguet laboratory has identified a new gene in melanoma, which functions as a tumor suppressor gene: its expression is decreased in human melanoma and melanoma cell lines. When the gene is introduced in melanoma cells, the cells switch from a proliferating to a migratory behavior, which would fit with a role of BCSC in melanoma progression. The characteristics of this gene and its function are further explored in this project. This project has not been continued.

Understanding the molecular alterations involved in the development and progression of metastatic melanoma is essential for a better diagnosis and targeted therapy In this project we identified BSCS-1 as a novel tumor suppressor in melanoma using a global analysis of alternative splice variants. By using *in silico* analysis of human publicly available microarray data, qRT-PCR and Western blot techniques on human biopsies we could confirm that BCSC-1 expression is decreased in human melanoma and in melanoma cell lines. Moreover, its ectopic expression blocked tumor formation in vivo and melanoma cell proliferation in vitro. We could show that BCSC-1 downregulates MITF at the transcriptional level via its interaction with Sox 10, resulting in a switch of melanoma cells from a proliferative to a migratory phenotype. We thus identified BCSC-1 as a novel regulator of MITF. BCSC-1 could be used as a marker for melanoma progression and prognosis.

Publications

Anghel SI, Correa-Rochal R, Budinska E, Boliganl KF, Abraham S, Colombetti S, Fontao L, Mariotti A, Rimoldi D, Ghanem GE, Fisher DE, Lévy F, Delorenzi M, Piguet V. Breast cancer suppressor candidate-1 (BCSC-1) is a melanoma tumor suppressor that down regulates MITF. Pigment Cell Melanoma Res. 2012;25:482-7.

2. **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. Melanogenesis and response to targeted therapy.**

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

Aftimos PG, Wiedig M, Langouo Fontsa M, Awada A, Ghanem G, Journe F. Sequential use of protein kinase inhibitors potentiates their toxicity to melanoma cells: A rationale to combine targeted drugs based on protein expression inhibition profiles. Int J Oncol. 2013 ;43:919-26

El Hajj P, Journe F, Wiedig M, Laios I, Salès F, Galibert MD, Van Kempen LC, Spatz A, Badran B, Larsimont D, Awada A, Ghanem G.Tyrosinase-related protein 1 mRNA expression in lymph node metastases predicts overall survival in high-risk melanoma patients. Br J Cancer. 2013;108:1641-7

Anghel SI, Correa-Rocha R, Budinska E, Boligan KF, Abraham S, Colombetti S, Fontao L, Mariotti A, Rimoldi D, Ghanem GE, Fisher DE, Lévy F, Delorenzi M, Piguet V.Breast cancer suppressor candidate-1 (BCSC-1) is a melanoma tumor suppressor that down regulates MITF.Pigment Cell Melanoma Res. 2012;25:482-7

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.

We have been focussing on the mechanisms underlying the role of miR-155 in CD8 T cells in several mouse models. We were able to more deeply confirm the targeting of SOCS-1 by miR-155 as an important mechanism for supporting CD8 T cell accumulation in response to virus infection and cancer. Upon trying three different antibodies for SOCS-1, we were finally able to establish a SOCS-1 specific immunoblot which confirmed higher SOCS-1 levels in miR-155 knockout cells, already suggested from mRNA levels. In the acute LCMV infection, we found that SOCS-1 overexpressing transgenic cells show a similar impairment of survival and expansion as miR-155 knockout cells, but no impairment of effector functions, thereby mirroring our results from studying miR-155 deficient CD8 T cells. Moreover, in collaboration with the group of Nicolas Restifo at NIH we found that inhibition of SOCS-1 is profoundly increasing the tumor control in a therapeutic setting of CD8 T cell transfer, similar to overexpression of miR-155. Furthermore, we clarified that the lack of efficient pSTAT5 activation in miR-155 knockout cells found in 2011 was indeed due to increased SOCS-1 levels, as knockdown of SOCS-1 in miR-155 deficient cells was able to rescue pSTAT5 levels in response to IL-2 stimulation. Interestingly, monitoring of miR-155 expression kinetics in vitro and in vivo suggested that miR-155 levels are highest towards the peak of CD8 T cell accumulation and furthermore depend on the TCR affinity for a given antigen. This was also shown with human CD8 T cells transduced with NY-ESO specific TCRs of increasing affinity (collaboration Michael Hebeisen/Natalie Rufer). This result suggests that higher miR-155 levels provide an advantage for high-affinity T cells in terminal accumulation, which is an established phenomenon. On the other hand, SOCS-1 was surprisingly strongly downregulated on transcriptional level immediately upon antigen contact and came back towards to peak of the response, then modulated by miR-155. This again suggests that interplay of SOCS-1 and miR-155 are shaping the late accumulation of CD8 effector cells.

We also tested the role of miR-155 in a chronic virus infection (LCMV clone 13) which is causing T cell exhaustion similar to chronic antigen exposure in tumor patients. Interestingly, we found an even more striking role for miR-155, as miR-155 deficient CD8 T cells were not able to survive beyond 2 weeks upon infection. This resulted in chronically increased virus levels. The lack of virus specific CD8 T cells in miR-155 knockout cells was further confirmed by the absence of cytokine production in response to a cocktail of LCMV specific peptides 3 month upon infection.

The more detailed knowledge on the role of miR-155 and SOCS-1 in CD8 T cells is now going to be used for engaging miR-155 for therapy of cancer.

Publications

Fayyad-Kazan H, Rouas R, Fayyad-Kazan M, Badran R, El Zein N, Lewalle P, Najar M, Hamade E, Jebbawi F, Merimi M, Romero P, Burny A, Badran B, Martiat P. MicroRNA profile of circulating CD4-positive regulatory T cells in human adults and impact of differentially expressed microRNAs on expression of two genes essential to their function. J. Biol. Chem. 2012;287:9910-22.

Mahnke, Y.D., Devêvre, E., Baumgaertner, P., Matter, M., Rufer, N., Romero, P., Speiser, D. Human melanoma-specific CD8+ T-cells from metastases are capable of antigen-specific degranulation and cytolysis directly ex vivo. OncoImmunology 2012;1:467-530

Dudda, J.C., Salaun, B., Ji, Y., Palmer, D.C., Monnot, G. C., Merck, E., Boudousquie, C., Utzschneider, D.T., Escobar, T.M., Perret, R., Muljo, S.A., Hebeisen, M., Rufer, N., Zehn, D., Donda, A., Restifo, N.P., Held, W., Gattinoni, L., Romero, P. MicroRNA-155 and its target SOCS-1 shape the effector CD8+ T cell response to virus and cancer. Immunity 2013;38:742-53.

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.

Publications

Fayyad-Kazan H, Rouas R, Fayyad-Kazan M, Badran R, El Zein N, Lewalle P, Najar M, Hamade E, Jebbawi F, Merimi M, Romero P, Burny A, Badran B, Martiat P. MicroRNA profile of circulating CD4-positive regulatory T cells in human adults and impact of differentially expressed microRNAs on expression of two genes essential to their function.J Biol Chem. 2012;287:9910-22.

Fayyad-Kazan H, Bitar N, Najar M, Lewalle P, Fayyad-Kazan M, Badran R, Hamade E, Daher A, Hussein N, ElDirani R, Berri F, Vanhamme L, Burny A, Martiat P, Rouas R, Badran B. Circulating miR-150 and miR-342 in plasma are novel potential biomarkers for acute myeloid leukemia. J Transl Med. 2013;11:31

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

Auguste T, Travert M, Tarte K, Amé-Thomas P, Artchounin C, Martin-Garcia N, de Reynies A, de Leval L, Gaulard P, Delfau-Larue MH.ROQUIN/RC3H1 alterations are not found in angioimmunoblastic T-cell lymphoma. PLoS One. 2013 25;8:e64536

Gaulard P, de Leval L.VIII. New markers in peripheral T-cell lymphomas: more entities or more confusion? Hematol Oncol. 2013;31 Suppl 1:51-6

Huang Y, de Leval L, Gaulard P.Molecular underpinning of extranodal NK/T-cell lymphoma. Best Pract Res Clin Haematol. 2013 ;26:57-74

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

Moroch J, Copie-Bergman C, de Leval L, Plonquet A, Martin-Garcia N, Delfau-Larue MH, Molinier-Frenkel V, Belhadj K, Haioun C, Audouin J, Swerdlow SH, Marafioti T, Gaulard P.Follicular peripheral T-cell lymphoma expands the spectrum of classical Hodgkin lymphoma mimics. Am J Surg Pathol. 2012 ;36:1636-46

Valleron W, Ysebaert L, Berquet L, Fataccioli V, Quelen C, Martin A, Parrens M, Lamant L, de Leval L, Gisselbrecht C, Gaulard P, Brousset P.Small nucleolar RNA expression profiling identifies potential prognostic markers in peripheral T-cell lymphoma. Blood. 2012 ;120:3997-4005

Lemonnier F, Couronné L, Parrens M, Jaïs JP, Travert M, Lamant L, Tournillac O, Rousset T, Fabiani B, Cairns RA, Mak T, Bastard C, Bernard OA, de Leval L, Gaulard P.Recurrent TET2 mutations in peripheral T-cell lymphomas correlate with TFH-like features and adverse clinical parameters. Blood. 2012 ;120:1466-9.

Travert M, Huang Y, de Leval L, Martin-Garcia N, Delfau-Larue MH, Berger F, Bosq J, Brière J, Soulier J, Macintyre E, Marafioti T, de Reyniès A, Gaulard P.Molecular features of hepatosplenic T-cell lymphoma unravels potential novel therapeutic targets. Blood. 2012;119:5795-806.

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 mightnot 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 will serve an important purpose and has effectively gone live in nov. 2012 www.fondation-MEDIC.ch.), The site will also allow 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 2012 to dr. Laurence Buisseret from Brussels. The call for applications for the 2013 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.

Fred T Bosman
Lausanne
October 2013