FONDATION
MEDIC

Consortium "Tumor-Host Interaction" supported by the MEDIC Foundation

Annual Report 2009

1. **Introduction**

The consortium, created in 2006 around the project groups funded by grants from the MEDIC Foundation, has functioned well. Presently all applications (for 3 year grants) are peer reviewed and only positively reviewed grants are funded. The stability of the present situation calls for new more rigorous review of the scientific activities in the consortium. The decision was therefore taken to create an external Scientific Advisory Board. Annual scientific and financial reports are received (a condition for continued funding). The consortium members 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 programme of the 2009 meeting is presented in table 1. The annual reports and the annual meeting presentations will provide the SAB the tools to critically follow the progress made in the studies.

Table 1 Programme of the MEDIC day 2009

I. Stamenkovic	Tumor-host interactions in cancer progression and metastasis
A. Mariotti	Study of the role of lactadherin in tumor progression
C. Ruegg	Unraveling heterotopic interactions in the tumor micro-environment
V. Piguet	Gene expression profiling of pigmented skin lesions and primary melanoma
G. Ghanem	New treatments, new markers in melanoma
GC. Alghisi	Bone marrow and peripheral blood cells in human cancer: use for diagnostic, prognostic and therapeutic purposes
M. Delorenzi	In silico modeling of tumor stroma
C. Sotiriou	Integrating tumor microenvironment, micrometastasis and the host properties in the study of breast cancer progression.
T. Petrova	Analysis of PROX1 role in colon and small cell lung cancer
P. Martiat	Leukemia-host interactions: interplay between leukemic cells, marrow immune and mesenchymal stem cells
P. Romero	Role of microRNA's in CD8 T-cell function

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. The new groups have integrated well into the consortium and restructuring of long standing research projects, focusing more on possible synergies between groups, has continued and this interaction has grown into one of the assets of the consortium.

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	Unraveling heterotypic interactions in the tumor micro-environment	CHF	311'440.-
A.Mariotti	Study of the role of lactadherin in melanoma progression	CHF	153'095.-
D.Picard	Mechanisms and functions of unusual estrogenic signaling by the host and the environment in breast cancer	CHF	173'720.-
M.Delorenzi	Tumor expression profiling In silico modeling of tumor stroma	CHF	120'240.-
V.Piguet	Molecular pathways in melanoma progression	CHF	84'000.-
GC. Alghisi	Study of bone marrow-derived peripheral blood cells in human cancer progression: evaluation of their potential use for prognostic, diagnostic and therapeutic means.	CHF	103'000.-
T.Petrova	Analysis of PROX1 role in colon and small cell lung cancers	CHF	168'000.-
I.Stamenkovic	Tumor-host interactions in cancer progression and metastasis	CHF	83.000.-
P.Romero	Role of microRNAs in CD8 T cell function	CHF	112’012.-
C.Sotiriou	Tumor-host interaction in breast cancer	€	360'000.-
G.Ghanem	Regulation of Ras/Raf/MEK/ERK Map kinase pathway and melanoma progression	€	153'000.-
Ph.Martiat	Leukemia-host interactions	€	226'864.-

It is relevant here to note that the total volume of MEDIC supported research conducted in Lausanne has grown and presently 8 groups profit 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 participates with 2 groups.

3. Scientific reports

Signatures predictive for response to chemotherapy in breast cancer
(Delorenzi)

Main results obtained

1. Universal Gene Coexpression Modules (collab. with Pascale Anderle, IOSI)
We have analyzed gene expression profiles of different types of cancer, using mainly the public ExpO collection, that includes over 2'000 samples. Fairly well represented are tumors from the following organs: breast, colon, lung, kidney, prostate ovaries and endometrium. We have constructed methods to identify "hub" genes, which in all these types of cancer identify a consistent net of coexpressed genes linked to their biological role, thus extending the gene modules used for breast cancer in previous publications. These new "ubiquitous" gene modules will be used in the next phase to characterize heterogeneity within tumors from each organ and then to test their association with patient survival and response to treatment in a set of suited public datasets.

2. Testing Modules Predictive of Treatment Response to Anthracyclines (collab. with Fabrice André, IGR)
We have applied the same gene coexpression modules we tested earlier to gene expression profiles of 50 patients (24 responders) treated essentially with anthracycline-only neoadjuvant chemotherapy in ER negative breast cancer. In this dataset two of our metagenes were significant predictors of response: the interferon module ("hub" gene MX1) and the cell proliferation module ("hub" gene TPX2). Earlier, we had significance for the tumor stroma module ("hub" gene DCN) in two datasets and for the MX1 module in one dataset.
In recent work by the Soritiou group, predictive power was found for modules linked to the stroma ("hub" gene PLAU, which is part of our DCN network), to the immune system ("hub" gene STAT1, which is part of our MX1 network) and to proliferation, as well as by amplification of the TOP2A gene (which is part of our proliferation module).
In summary, we and others might have identified three informative motifs linked to response to anthracycline-based chemotherapy related to proliferation, activated stroma and immune activity. All datasets analyzed so far are small and the treatments not exactly identical, so comparisons and summary conclusions are still difficult. Larger studies and / or a meta-analysis of the existing studies would be useful, but researchers are being reluctant to make data available quickly for further analyses. The best predictor might be a combination of two or three weaker predictors. We have some evidence for that in the André study, but sample size is too small for confident conclusions. We and others have shown that similar modules are also prognostic in subtypes of breast cancer and larger studies are also needed to separate drug-specific predictive and broadly prognostic (drug unspecific) effects, as only the first ones are useful for deciding the best individualized treatment.

3. Drug specificity of response prediction by the stroma module.
The treatments in the two studies previously analyzed (see report 2008) contain anthracycline (epirubicin), 5-FU and cyclophosphamide respectively an anthracycline (doxorubicin), 5-FU, cyclophosphamide.and a taxane (paclitaxel). We now tested the identified gene modules on the second arm of the EORTC 10994/BIG 00-01 trial, whose patients were treated with an anthracycline (epirubicine) and a taxane (docetaxel). None of the modules was significantly predictive of response in this arm. Unfortunately again the study is small, confidence intervals of estimates are large and statistical support of conclusions weak. The results could suggest that prediction is specific to a combination therapy, and that the presence of 5-FU plays a role.

4. Expression profiling of stage II and III colon cancer (collab. with Fred Bosman, UNIL and Arnaud Roth, HUG)
RNA extracted from formalin fixed slides on an Affymetrix custom chip by the company Almac provided gene expression data of variable technical quality, according to standard indicators of consistency among probes of the same probeset (gene). Taking the qualitatively 244 best of 323 chips, we estimated that the data are of good quality (in the sense that useful information can be extracted by the following criteria: very efficient classifier can be constructed for discrimination tumors with and without a BRAF mutation or for tumors with and without microsatellite instability (MSI vs MSS)). Gene expression difference between MSI and MSS correspond well to results of the same comparison in three public datasets obtained from fresh frozen material, although the estimated log fold change is generally attenuated by about 30% on average in our data. Discrimination between tumors with and without a KRAS mutation turns out to be much more difficult. Possibly, different aminoacid substitutions in the KRAS genes, or the influence of mutations in other genes, are associated with different effects on signalling pathways. It is striking, that BRAF mutations are associated with a very consistent gene expression

pattern, and one very different from those associated with KRAS mutations. The differential gene expression downstream of BRAF indicates unique activation of key developmental pathways, including Wnt, TGF-b and MAPK. Tumors with BRAF mutations are now recognized as particularly aggressive and refractory to current treatment. The uniformity of their molecular profiles might help identification of new therapeutic targets for these tumors.
Another stunning observation is that there are many genes whose expression varies very widely, even inside each gender and insider the MSS and/or MSI class. The list of these genes is rich in genes known to play roles in tumor biology, especially genes linked to the Wnt pathways (for example Lgr5, Axin2, Nkd1) and to immunological functions and tumor-stroma communication. The latter could be a simple indicator of variable degree of tumor infiltration by immune cells, but we would then expect a higher degree of gene-gene correlation among immune function genes than we observe.

5. Analysis of Colon Tumor Profiles with response data to cetuximab treatment (collab. with Sabine Tejpar, Gasthuisberg, Leuven)
Analyses on a set of about 100 tumors are still too preliminary to be discussed. Aims of the current analyses are to find gene expression signatures and differences in underlying biological pathways for

a. Response to Cetuximab treatment in general
b. Differences between KRAS mutants and KRAS wt tumors (as the first class is associated with lack of response to cetuximab while the second class contains a higher fraction of responders)
c. Differences between tumors with different KRAS mutations (as some appear to be less associated with lack of response to cetuximab treatment than other)

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Integrating tumor microenvironment, micrometastasis and host properties in the study of breast cancer progression (C. Sotiriou)

Main results obtained

This summary is divided into three parts describing the ongoing progresses of the three sub-studies: 1) tumor microenvironment, 2) circulating tumor cells (CTCs) and 3) single nucleotide polymorphisms (SNP) subprojects.

1. Tumor microenvironment

There is growing evidence that interaction of stromal and immune cells, notably CD10+ cells with tumor cells is pivotal in breast cancer progression and response to therapy. We therefore developed a CD10+ stromal signature by comparing gene expression profiles from tumor-associated CD10+ cells with their normal counterparts. Investigating this CD10+ stroma signature in our repository of publicly available gene expression data revealed that this signature was associated with worse prognosis in the HER2+ subpopulation of breast cancer only. In parallel, we developed *in-silico* gene expression modules related to the key biological processes in breast cancer, such as stroma. Our in-silico stroma modules were highly correlated to the CD10+ stromal signature and in addition to being associated with worse prognosis in HER2+ patients, these modules were also associated with resistance to anthracycline-based chemotherapy.

Although the CD10+ stromal fraction was initially reported to be a homogeneous cellular entity, recent reports have shown that not only fibroblasts and myo-epithelial cells but also mesenchymal stem cells (MSCs) express CD10. It has been demonstrated that these MSCs integrate into the tumor-associated stroma and increase the metastatic ability of tumor cells. Given the important prognostic and predictive role of these CD10+ cells, we aimed first at characterizing the proportion of the different cell types within the CD10+ fraction, focusing on the MSCs. We investigated the proportion of CD10+ MSCs in bone marrow, lymph nodes, normal and tumoral breast tissue. Furthermore we aimed at better understanding the exact contribution of each CD10+ cell type to breast cancer progression. To do so, we performed co-culture analyses of representative breast cancer cell lines with different CD10+ stromal components (fibroblasts, myoepithelial cells and MSCs obtained from healthy donors). We then compared gene expression profiles of the different CD10 stromal components, which were and were not co-cultured with the different breast cancer cell lines. Co-colturing breast cancer cell lines with MSCs and fibroblasts induced many changes, especially in the HER2+ SKBR3 cell line. We are currently investigating in detail the potential prognostic and predictive impact of these changes.

2. Micrometastasis

Our working hypothesis is that systemic spread of disseminated tumor cells from the primary site may occur early in breast tumorigenesis by specific subpopulations of cells. We studied the presence of CTCs in 81 women with preinvasive breast lesions (Atypical Ductal Hyperplasia, Lobular Carcinoma In Situ and Ductal Carcinoma In Situ), 101 women with early invasive BC and 35 women with metastatic BC. CTCs were defined as Cytokeratin 8,18,19+/CD45-/DAPI+ cells detected using the CellSearch® technology. Any staining for HER2 on CTCs using the CellSearch® HER2 profiling kit was considered positive. HER2 detection was calibrated through peripheral blood spiking experiments with MCF7 and SKBR3 cells. Seven samples (slides containing CTCs) were sent at the University of Regensburg (Dr K. Klein) for molecular characterization at the DNA (CGH analysis) and RNA (microarray analysis) level as part of our first pilot study.

We were able to detect more than 1 CTCs/22.5mL of blood in 6 of 81 (7%) women with preinvasive breast lesions (range 0-3 CTCs) and 15 of 101 (15%) women with early invasive BC (range 0-8 CTCs), whereas we detected > 1 CTCs/7.5mL of blood in 17 of 35 (49%) women with metastatic BC (range 0-1160 CTCs) ($p<0.001$). HER2 expression on CTCs was not associated with estrogen receptor (ER) and progesterone receptor (PR) expression or the histological grade of the primary tumor. Interestingly, in women with detectable CTCs, the ratio of HER2-positive CTCs/total CTCs was higher in HER2-positive than HER2-negative primary tumors ($p=0.023$). Despite a higher detection rate of CTCs, the ratio of HER2-positive CTCs/total CTCs was lower in metastatic BC (median 0.15, range 0-1.0) than in preinvasive (median 1.0, range 0-1.0) or early invasive BC (median 1.0, range 0.3-1.0) ($p<0.001$). Similar results were observed when only HER2-negative primary tumors were considered ($p=0.004$).

To conclude, this is the first report showing that HER2-positive CTCs can be detected in women with preinvasive breast lesions, further supporting the hypothesis that dissemination occurs early in breast tumorigenesis. HER2 expression on CTCs is more commonly observed in earlier stages of breast cancer, suggesting that HER2 plays an important role in early dissemination.

3. SNPs

This sub-study interrogates the role of the host for the presence of minimal residual disease (tumor invasion) and the development of macro-metastases (colonization) in a well-defined cohort of early breast cancer patients with and without detectable minimal residual disease (CTCs) and known clinical outcome.

For this purpose, 169 blood samples from breast cancer patients have been genotyped using the Affymetrix Genome-Wide Human SNP Arrays 6.0. We first checked the genotyping call rate and the average heterozygosity of each subject. Four samples were excluded for further statistical analyses because of a high average heterozygosity (> 0.337 %) or a low call rate (< 95 %). Of the 169 genotyped samples, 164 could be taken into account in the association studies

analysis. We kept for further analysis only those with a call rate greater than 95 % and a MAF greater than 1.53 % (the minor allele should have been found at least 5 times in the 164 samples taken into account). 720 236 SNPs passed these criteria. Only 149 of the 720 236 kept SNPs were deviating from HWE at the Bonferroni-corrected P-level.

We used the Armitage's test to estimate p values of associations with breast cancer relapsing. Two models were taken into account: a basic additive/allelic model where the genotypes dd, Dd, and DD are resolved into pairs of alleles d and d, D and d, or D and D; a genotypic model related to genotypes dd, DD, and Dd without regard to any "order" or allelic count or allelic pairing that they might have. To adjust for multiple testing with a large number of correlated markers, we also derived the empirical distribution of the chi-square statistics after 100 genome-wide permutations. Genome-wide significance was defined with an empirical p value smaller than 0.05. Nevertheless we also took into account SNPs with theorical p values smaller than 0.05 after Bonferroni correction (p value < 0.05/720 236 ≈ 6.94e-08) in order to perform haplotype analyses. An haplotype analysis with a sliding window of three neighboring SNPs was also conducted. Since this analysis is very time consuming, it was conducted only on a subset of regions of interest corresponding to genes associated to selected SNPs (with theorical p value < 0.05/720 236 ≈ 7.02e-08).

Main findings:

1) Genome-Wide Association Analysis

We identified 4 SNPs associated with low and high histological grade based on theoretical p values and after Bonferroni correction. These SNPs are associated to 4 genes: MEGF11 (multiple EGF-like-domains 11), FARP1 (pleckstrin domain protein 1), TACC2 (transforming, acidic coiled-coil containing protein 2) and TCF4 (transcription factor 4). No other associations were found.

2) Association studies focused on genes modules

The applied method is the same as described previously excepted that we performed an haplotype analysis on genes belonging to the molecular modules which describe well characterized molecular processes in breast cancer such as ER, HER2 signaling, cell cycle, immune function etc. We were able to identify, 10, 7, 17 and 13 SNPs associated with ER, HER2 status, histological grade and relapse respectively. A preliminary Ingenuity pathway analysis revealed that the 13 SNPs associated with relapse were included in only one network involved in cancer, cellular growth and proliferation.

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Mechanisms and functions of unusual estrogenic signaling by the host and the environment in breast cancer (D. Picard)

Main results obtained

1. Estrogen signaling through the membrane receptor GPR30

The membrane-bound estrogen receptor GPR30 mediates the stimulation of proliferation by estrogen of several cell lines representing different types of carcinomas independently of the presence of estrogen receptor α (ERα) or estrogen receptor β (ERβ). Our most recent results contribute to understanding its pharmacology, regulation, and biological function.

- We were hoping to recapitulate signaling by the GPCR GPR30 in the budding yeast to facilitate pharmacological analyses. Despite early positive signs, we have not been able to confirm that this may be possible. This has been put on hold for now.
- As mentioned in my last report, we have discovered together with our Italian partners that GPR30 signaling induces connective tissue growth factor (CTGF), which then stimulates the proliferation and migration of ER-negative (or most likely ER-positive) breast cancer cells. Since GPR30 signaling can also be elicited by the antiestrogen tamoxifen, these findings may be highly relevant to endocrine therapy. They argue that tamoxifen may actually make things worse, at least for a subset of (GPR30-positive) breast tumors. The paper has now been published (Pandey et al., 2009).
- With my Italian colleague Marcello Maggiolini, we have written a comprehensive review on GPR30, which is now in press (Maggiolini and Picard, 2009). It includes an extensive discussion of the current controversies and confusions that characterize the GPR30 field.

2. Mechanism and consequences of activation of ERα by cAMP signaling

For many years we have been studying how ERα is activated by other signaling pathways, notably growth factors and factors that lead to elevated intracellular cAMP. I already mentioned in previous reports that we have made significant progress towards a mechanistic understanding of this extreme form of signaling crosstalk.

- Mechanism: We now know that cAMP stimulates the phosphorylation of the ERα coregulator CARM1 by protein kinase A (PKA) allowing it to bind directly to ERα in the absence of estrogen. Binding is necessary but not sufficient, suggesting that there might be additional factors. Most importantly, we have been able to correlate this mechanistic phenomenology with tamoxifen resistance. Indeed, by comparing wild-type and tamoxifen-resistant MCF7 breast cancer cells, we found that CARM1 is not only constitutively phosphorylated and binds ERα in tamoxifen-resistant cells, but this is essential for tamoxifen resistance. This argues that this type of signaling crosstalk may contribute to the tamoxifen resistance of certain breast cancers. At least for some tumors and circumstances, one can state that the presence of PKA-phosphorylated CARM1 allows ERα to be activated by tamoxifen, and thus, cells to be tamoxifen-resistant. This manuscript is now in revision for a high impact factor journal (Carascossa et al., 2010).
- Consequences: ChIPseq experiments are about to be done to gain a genome-wide understanding of where ERα goes upon activation of ERα by cAMP. ChIP experiments have been performed, optimized and validated to allow deep sequencing. In parallel, we have already repeated the transcriptome analysis with Illumina bead arrays. This was necessary since our earlier microarray analysis, in the context of a Medic project, had been

done with far more limited cDNA arrays. We expect to have the full set of data early in 2010. With that in hand, we should ultimately gain insights into the how and what of cAMP-ERα signaling.

3. microRNA regulation of estrogen signaling and microRNA transcriptome regulation

- ERα signaling being critical for a number of physiological and pathological processes, it is obvious that ERα levels must be tightly controlled. As mentioned in my last report, we performed a comprehensive and systematic assessment of all miRNAs that might regulate ERα expression by targeting the 3'UTR of its mRNA. We found that miR-22 represses ERα expression. As a consequence, miR22 overexpression diminishes ERα-mediated proliferation of MCF7 breast cancer cells (Pandey and Picard, 2009). In the course of this study, we had also identified two miRNAs that stimulate ERα responses. Since then, we have confirmed this unusual effect and have made some progress in characterizing its mechanism. We had also noticed that the mRNA for another nuclear receptor, the glucocorticoid receptor, might be a target for miR-22. Again, we have been able to confirm this hypothesis and are currently finalizing the analysis of this phenomenon. Based on our interest in miR-22 and as mentioned before, we had commissioned a conditional mouse knock-out of miR-22. Our Finnish collaborators, who are providing this service, have obtained chimeras and are in the process of breeding them. As soon as we get the animals, we will be exploring the effects of this mutation on estrogen physiology.
- miRNAs and tamoxifen resistance: using the Illumina platform, we have performed a comparative analysis of the expression patterns of all miRNAs in wild-type and tamoxifen-resistant breast cancer cells, and in particular in response to various signals (estrogen, cAMP,...). The bioinformatic analysis is still ongoing, but by comparing these data with others (e. g. transcriptome analyses of cells and breast cancer), we hope to gain insights into how various cellular behaviors are shaped by miRNAs and vice-versa.

Publications

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Lactadherin in tumor progression (A. Mariotti)

Main results obtained

Lactadherin (MFGE8) is a secreted glycoprotein that mediates cell-cell adhesion and activates signal transduction pathways promoting cell survival. This protein plays a role in developmental processes such as mammary gland branching morphogenesis, promotes angiogenesis in pathological situations, such as experimental ischemia and tumor angiogenesis, and favors melanoma growth in experimental tumorigenesis assays, through both an autocrine and a paracrine mechanism.
During the past year we have continued our studies on the role of lactadherin in the development of breast carcinomas and melanomas. A detailed analysis of gene expression microarrays of breast carcinomas, conducted incollaboration with Dr. Mauro De Lorenzi (Swiss Institute of Bioinformatics, Lausanne), revealed that high lactadherin expression is associated with low survival of breast cancer patients. Further analysis revealed that this is because lactadherin expression is higher in basal-like breast carcinomas compared to other types of breast cancer, and these tumors are particularly aggressive. We have obtained a large collection of basal-like breast carcinoma biopsies from Dr. Luca Mazzucchelli (Institute of Pathology, Locarno). Preliminary stainings performed with two different anti-lactadherin antibodies, revealed that lactadherin is expressed in the normal breast epithelium, in carcinomas in situ and in basal

like tumors. Interestingly, the staining pattern seems to vary, with lactadherin being present predominantly in the cytoplasm and membrane of the cells, but also in the nuclei of some cells in both normal glands and carcinomas in situ.
In order to understand lactadherin function in breast carcinomas, we have decided to down regulate its expression by RNA silencing in the human breast carcinoma cell lineMDA-MB-231. The derived cell population was then injected orthotopically into the fat pad of nude mice. We did not observe any difference in tumor growth between control cells and cells silenced for lactadherin expression (sh-cells). However, when the tumors were removed and analyzed, we found that tumors derived from sh-cells expressed lactadherin, suggesting that sh-cells lacking lactadherin did not proliferate or did not survive and were taken over by cells with higher levels of.
We have also started to exploit a mouse model of mammary gland carcinoma that will allow us to modulate MFGE8 expression in cells with different in vivo growth and metastatic abilities and study their properties in immunocompetent mice. The model is well described and includes 4T1 cells which are highly tumorigenic and metastatic, and two derived cell lines, 168FARN and 67NR which are tumorigenic in vivo but not, or very poorly, invasive and metastatic. Analysis of MFGE8 expression levels in these cells showed that 4T1 cells express high levels of the protein, while the other two cell lines express low levels. We have generated 168FARN and 67NR cells overexpressing MFGE8 and these cells have been injected in the fat pad of syngenic Balb/c mice. Tumor growth is currently being monitored.
In addition, we are also analyzing MFGE8 function in normal and H-Ras transformed mouse mammary epithelial cells, NMuMG and EPH-Ras respectively. NMuMG cells express low levels of the endogenous protein compared to both 4T1 and EPH-Ras cells. MFGE8 was thus overexpressed in NMuMG cells and they will be injected in nude mice. On the other hand, we have silenced MFGE8 expression in both 4T1 and EPH-Ras cells. We have observed that decreased expression of MFGE8 in 4T1 and EPH-Ras cells causes changes in cell morphology: the cells start to grow in islets characterized by tight cell-cell adhesions, and seem to acquire their original epithelial features, suggesting that they are undergoing a reversal of the “epithelial-Ras cells. We have observed that decreased expression of MFGE8 in 4T1 and EPH-Ras cells causes changes in cell morphology: the cells start to grow in islets characterized by tight cell-cell adhesions, and seem to acquire their original epithelial features, suggesting that they are undergoing a reversal of the “epithelial-mesenchymal transition” which is associated with migratory and invasive properties. EPH-Ras-sh-MFGE8 cells will be injected in the fat pad of nude mice and analyzed for their in vivo growth and metastatic properties. On the other hand, even if we were able to silence MFGE8 expression in 4T1 cells, we did not succeed in maintaining these cells in culture: after a few passages silenced cells were lost and replaced by cells expressing MFGE8, as already observed with MDA-MB-231 cells. It thus seems that lactadherin downregulation is compatible only with the presence of a signal that sustains cell proliferation or survival, like for instance activated Ras as in EPH-Ras cells. We are planning to further explore this possibility.
We have also continued with experiments aimed at understanding the function of lactadherin in melanoma cells. After overexpressing lactadherin in a panel of melanoma cell lines and performing in vivo tumorigenesis assays, we found that only one cell line (SBCl2) out of four produced larger tumors than control cells. Recently it was reported that the mouse melanoma cell line B16 engineered to overexpress MFGE8 grows in vivo faster than control cells: this result is thus in agreement with our observation with SBCl2 cells. We envisage two possibilities: on one hand it is possible that SBCl2 and B16 behave oddly compared to other melanoma cells and thus are not representative of melanomas; on the other hand it is possible that SBCl2 and B16 have some common features that distinguish them from the other melanoma cell lines that we have used so far, and they are representative of a subtype of melanoma. In an attempt to explore the latter option, we have examined the mutational status of BRaf and NRas, the two main melanoma oncogenes, in our melanoma cells. We have found that SBCl2 cells contain mutated NRas Q61K, while the other cell lines contain activated BRaf V599E. Interestingly, B16 cells contain mutated NRas. We propose that melanoma cells can be favored by lactadherin in their growth and/or survival depending on the oncogene which is at the origin of their transformation: NRas activation could cooperate with lactadherin expression in promoting melanoma development, while BRaf activation would bypass lactadherin function and thus would not synergize with it. In addition, we have examined lactadherin cellular localization in SBCl2 cells by immunostaining and confocal microscopy analysis. We have found that lactadherin is present on the surface, at the membrane, in the cytoplasm and also in the nucleus of these cells. Lactadherin nuclear localization has not been reported so far in any cell type. Future experiments will address the function of lactadherin in the nucleus of cancer cells, both melanoma and breast carcinoma.
In conclusion, our current results suggest that lactadherin may favor the development of breast carcinomas and melanomas by possibly cooperating with Ras oncogene. Future experiments will reveal if this is the case and possibly will unravel a new mode of function of this oncogene.

Publications

Role of a novel tumor suppressor identified by DATAS in melanoma progression
(V. Piguet)

Main results obtained

The main progress of this year is related to deciphering the function of BCSC-1 in the development of melanoma. We have made significant progresses in identifying some of the mechanisms of BCSC-1 function in regulating the cell cycle in melanoma using microarray approaches. We have also discovered two new proteins that interact with BCSC-1 to regulate cell functions using a Yeast-two-hybrid approach. Finally, in silico analysis (performed in collaboration with M.Delorenzi) confirmed so far several observations obtained in our dataset.
Resulting from a global genetic screen (DATAS (Differential Analysis of Transcripts with Alternative Splicing)), we have set up methods to validate the implication of specific genes in the transitions from benign lesions to tumor. This is an important platform to evaluate function of potentially significant genes identified during the genetic screen. Using DATAS, we previously identified a total of 217 sequences that are differentially expressed in metastatic melanoma and benign nevus. Out of several candidates that we screened we identified a potential novel tumor suppressor gene (BCSC-1) implicated in metastatic melanoma.
BCSC-1 (Breast cancer suppressor candidate 1) or also termed "Loss of heterozygosity 11 chromosomal region 2 gene A protein" has been suggested to have a tumor suppressor activity but the mechanisms of action and the regulation by specific isoforms of this protein are unknown. RNA expression analysis by quantitative PCR demonstrated that BCSC-1 long isoform but not its short isoform is downregulated in all patients with stage IV disease in comparison with the expression observed in benign nevus, atypical nevus or primary tumors. This screen was performed on 40 patients samples and included giant nevus cell lines from G.Ghanem (Brussels). We are further trying to validate these results by generating antibodies against BCSC-1 in order to do immunohistochemistry in tissues.
In addition, extending our qPCR studies in patient samples, in collaboration with M.Delorenzi's group, we could demonstrate that BCSC-1 expression is downregulated patients with metastatic melanoma compared to primary tumors, when using data from several datasets available on melanoma tumors. Furthermore, we generated lentiviral vectors that stably encode BCSC-1 long isoform and short isoform (F and A). Ectopic expression of BCSC-1 long isoform in melanoma cell lines using a lentivector decreases markedly the proliferation of these cells. Our results demonstrate that BCSC-1 long isoform but not its short isoform blocks potently the proliferation of melanoma cells in the G2/M phase of the cell cycle. Melanoma cell lines, as well as Hela Cells transduced with lentiviral vectors encoding BCSC-1 arrest their growth in the G2/M phase of the cell cycle. In order to tackle further the function of BCSC-1 in melanoma progression we have performed microarray studies on melanoma cells transduced with BCSC-1 long and short isoform as well as an empty vector (Illumina technology: Human WG-6 V3 BeedArray) as well as an affymetrix array at the genomics platform of NCCR genetics (P.Descombes). Strikingly while the short isoform of BCSC-1 did not show much biological activity, the long isoform of BCSC-1 modulated the expression of 365 genes. The main biological pathways that seem affected by BCSC-1 long isoform are genes involved in cell proliferation and migration. Mainly several genes in the RAS/RAF pathway and in the MITF pathway were modulated by BCSC-1 expression in melanocytes. From these results we have strong evidence that several pathways involved in melanoma progression are regulated by BCSC-1 expression in melanoma cell lines, including several targets that are in the MITF pathway, a central pathway for melanoma development.

In order to validate these studies we have performed protein expression studies on cells encoding BCSC-1 and could confirm a downregulation of phosphorylated ERK as well as a decrease in MITF expression in Mewo cells encoding stably BCSC-1. Next we evaluated the capacity of Mewo Cells expressing BCSC-1 to migrate. Several previous observations indicated that MITF can switch cells from a migration phenotype towards a proliferative state. Interestingly, while BCSC-1 expression in Mewo cells increased their capacity to migrate in a wound healing assay, in agreement with previous observations that suggested that MITF downregulation resulted in a decrease in cell proliferation but an increase in their migration capacity.
Furthermore, we have recently performed yeast-two-hybrid studies (hybrigenics, Paris) and have identified in two separate screens (using lexA and Gal-4) two putative proteins interacting with BCSC-1. Briefly, pB29-BCSC-1HA tag with LexA (N-bait-LexA-C fusion) was used to screen a cDNA library derived from human melanocytes RP1. More than $1{,}2x10^6$ interactions were analyzed and 13 clones were sequenced. A second round confirmation screen was performed with the Gal4 system. Sequence analysis of the positive clones showed two proteins interacting with full length BCSC-1, DDX16 and C2CD3 (C2 calcium-dependent domain containing). DDX16 also known as DBP2 is a RNA helicase. It was found to be a functional homolog of PRP8/CDC28 from S.Pombe that is known to be involved in the cell cycle. C2CD3 was found to be a regulator of the Hedgehog (HH) pathway; it controls the photolytic processing of Gli3, which is an inhibitor of the HH pathway. Thus, C2CD3 activates the HH pathway by inhibiting the activity of an inhibitor of HH

signaling (Gli3) suggesting a potential role for this protein in melanoma development. Interference of the HH function was shown to prevent metastasis of melanoma
During the next years, we will refine the pathways used by BCSC-1 to regulate melanoma progression with a special focus on MITF, a key player in melanoma. Taking advantage of the potential cellular partners of BCSC-1, we will evaluate which is the contribution of DDX16 and C2CD3 in melanoma progression.
We expect that molecular profiling studies of pigmented skin lesions and primary melanoma will enable us to understand the sequence of events underlying the progression of melanocytic lesions and possibly to the discovery of novel diagnostic, prognostic and therapeutic tools. Furthermore, we expect that this project will foster new collaborations since novel genes are likely to be discovered in this process. As a successful example, we have now identified BCSC-1 as a strong potential target in melanoma progression. We are trying to refine the understanding of how this gene can control melanoma progression and whether it could constitute a novel target for intervention.

Publications

Prognostic markers in melanoma: gene prognostic signatures, Braf mutation and oxidative stress (G. Ghanem)

Main results obtained

Using microarrays, we generated a 'pigmentation signature' comprising 6 genes overexpressed in melanoma metastases of patients with shorter survival [TYRP1(43x), SILV(6x), DCT(13x), OCA2(8x), TYR(6x) and MITF(3x)]. These genes are specific of melanocytes, avoiding any contribution from stromal cells. We calculated a score for the pigmentation signature (mean expression of TYRP1, SYLV, DCT, OCA2, TYR and MITF, corrected to baseline), and a score for TYRP1 (log corrected values) for each tumor sample. We found significant correlations between both scores ($p<0.001$, Spearman's rho) indicating that TYRP1 alone may represent the information provided by the signature. We also confirmed that the scores were significantly higher in the group of patients with poor survival ($p<0.001$, Mann-Whitney test). Then, we validated the microarray data on skin metastasis by evaluating TYRP1 expression at mRNA level (PCR) and protein level immunohistochemistry). We observed significant correlations between PCR and microarray data ($p=0.007$), and also between protein and mRNA expression (p00.019). Finally, we evaluated TYRP1 mRNA expression (PCR) in additional skin metastases (validation population, N=36) and we found that TYRP1 mRNA expression significantly correlated with survival (Kaplan-Meier analysis and Cox regression, $p=0.037$, HR=0.467, 95% CI=0.228-0.957). Moreover, we found that high TYRP1 score had a positive predictive value of 78% associated with a poor prognosis.
On the other hand, while examining the effect of different inhibitors under induced oxidative stress and in relation with BRAF and NRAS common mutations, we observed that the tyrosine kinase inhibitor dasatinib was highly effective at very low concentrations. Therefore, we examined 33 melanoma cell lines and observed that 11 were highly sensitive to dasatinib toxicity ($IC50<10^{-9}M$), 9 were moderately sensitive (IC50 from 10^{-8} to $10^{-6}M$) and 13 were resistant ($IC50>10^{-5}M$). Among the highly sensitive, 9 were wild type for BRAF, 6 expressed high levels of c-Kit and 7 had activated Akt pathway, while none expressed detectable levels of PDGFR. A kinome analysis revealed a profile of kinase inhibition by dasatinib in melanoma and pointed out a Cas (Crk-associated substrate) scaffolding protein family member as a critical factor in dasatinib response to the drug.
To conclude, we generated a 'pigmentation signature' of 6 genes related to melanogenesis associated with poorer clinical outcome in a high risk patient subgroup. The first ranked gene (TYRP1) may be used alone as prognostic marker, at least in skin metastases, and may be helpful where information on the primary is lacking. Interestingly, TYRP1 may be a candidate for a targeted therapy. Unexpectedly, we found that dasatinib was highly effective in melanoma cells with wild type BRAF, expressing high levels of c-Kit and/or with activated Akt pathway. As such, it appears as a promising agent for the treatment of a selected subgroup of melanoma patients.

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Unraveling heterotypic interaction in the tumor microenvironment (C. Ruegg)

Main results obtained

Endothelial cell PKB modulates cancer cell motility. We have previously identified a number of endothelial cell genes modulated by Akt, which are implicated in cell-cell communication, cell-cell attraction/repulsion, motility and differentiation. To study the effects of endothelial cell Akt activation on the behavior of juxtaposed tumor cells we have established three 3D co-culture models. Akt activation profoundly altered endothelial cell tube morphology, resulting in enlarged and less branched structures. In all the models we consistently observed that low endothelial cell Akt activity results in a tight tumor cells-endothelial cells association, while high Akt activity promotes tumor cell dissociation from the endothelial cells and invasion into the adjacent collagen gel. From these experiments we concluded that high Akt activity in endothelial cells promotes the invasion of juxtaposed tumor cells. We are now investigating individual Akt-regulated genes identified in the genetic screen for their role in this effect.

The COXIB-induced gene MAGI-1 acts as tumor suppressor in colon cancer. In collaboration with the group of Dr. Mauro Delorenzi, we have previously identified MAGI1, a scaffolding membrane protein that binds to ß-catenin, stabilizes adherens junctions and recruits PTEN to cell-cell contacts, as COXIB-induced gene in endothelial and colon cancer cells. GOF and LOF experiments in HCT 116 and SW480 colon cancer cells revealed that MAGI-1 expression promotes epithelial-like morphology, cell-cell contacts and adhesion to matrix proteins, while it inhibits cell migration, invasion, and Wnt-signaling and suppresses tumor growth in vivo. Conversely, MAGI-1 silencing promotes cell aggregate formation and anchorage-independent growth. Gene expression analysis in colon cancer, however, did not reveal loss of MAGI1 during progression. We propose that MAGI1 acts as conditional tumor suppressor possibly involved in mediating some of the cancer-protective activity of COXIBs. We are now finalizing experiments in view of a publication submitted early 2010.

CYR61 suppresses EMT in normal breast epithelial cells, while it promotes it in breast cancer cells. We have previously identified CYR61 as metastasis-promoting gene associated with poor prognosis in breast cancer. We set to study the effect of CYR61 on epithelial-to-mesenchymal transition (EMT) in normal and transformed murine mammary epithelial cells. The EMT-promoting cytokine TGFß induced transient CYR61 expression in the normal murine mammary gland epithelial cells NMuMG. CYR61 overexpression, however prevented TGFß-induced EMT in a call autonomous manner. CYR61 did not suppress TGFß signaling nor Snail expression, but prevented TGFß-induced expression of N-cadherin and loss of E-cadherin. In contrast in the tumorigenic cell line EpRas (Ras transformed EpH4) CYR61 promoted EMT. In these cells CYR61 induced genes of the TGFß pathway (Smads2/3, TGFßRII), TGFß targets (HMGA2, PAI1 and FN1) as well as the EMT-promoting transcriptional repressors Smail, Slug and Twist. Pharmacological inhibition of the Ras-MAPK pathway suppressed CYR61-induced EMT in EpRas cells. From these experiments we conclude that CYR61 has a bimodal effect on EMT depending on the cellular context. Current experiments are focusing on the molecular mechanism by which CYR61 modulates EMT. In addition we have generated Rip1CYR61Rip1Tag2 mice to investigate the effect of CYR61 on tumor progression in vivo. Furthermore, in collaboration C. Sotiriou and DIAGNOPLEX, we are investigating the prognostic and predictive values of a CYR61 signature in breast cancer. Additional collaborations are planned to study the cross talk between CYR61 and other oncogenes or tumor suppressor genes, and the mechanisms controlling CYR61 expression.

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Leukemia-host interactions: interplay between leukemic cells, marrow immune and mesenchymal stem cells (P.Martiat)

Main results obtained

1. Immunomodulatory effects of mesenchymal stem cells

Mesenchymal stromal cells (MSC) possess immunomodulatory properties and are successfully used in vivo for controlling graft-versus-host disease (GVHD). Different immune cells are targeted by MSC involving cell contact-dependent or independent mechanisms. However, these effects, especially on T-cells, are not fully understood yet. We investigated this on purified T-cells from adult peripheral and cord blood and the impact of cell to cell ratio in determining MSC activities, using T-cell proliferation in response to different stimuli. We found that MSC induced a dose- and contact-dependent inhibition of T-cell proliferation. Naive (cord blood) T-cells were less sensitive to MSC inhibition than peripheral blood cells. At low concentration, MSC stimulated T-cell proliferation rather than inhibiting it. The stimulation of T-cell proliferation was contact independent. MSC-derived IL-6 was the major cytokine responsible for this effect. These results demonstrate the crucial importance of MSC/T-cell ratio to either support or inhibit T-cell proliferation, and the critical role of IL-6. We then evaluated the inhibitory activity of MSC on purified T-cell proliferation at various MSC/T-cell ratios, concentrating on the requirement for direct contact, the impact of paracrine mediators and MSC conditioned media (CM), recombinant IL10 and anti-IL10 neutralizing antibodies. We found that a cross-talk between MSC and T-cells is crucial for activating MSC immunosuppressive functions and the release of inhibitory factors. We were able to show that IL-10 can act as an activator of the MSC immunosuppressive function.

2. Regulation of Foxp3 and human regulatory T-cells.

How regulatory T cells mediate dominant tolerance is a subject of considerable debate at the moment. We investigated the micro-RNA (miR) signature of human Tregs, using CD4-positive, CD25-positive non-activated T-cells originating from cord blood of uninfected newborns. A signature composed of five miRs (21, 31, 125a, 181c and 374) was identified, of which two were considerably under-expressed (miR31 and miR125a). We identified a functional binding site for miR31 in the 3' UTR of the Foxp3 mRNA. We performed co-transfection experiments in Hela and 293T cells, and could demonstrate a direct regulatory effect of miR31, mediated via this site. Further experiments await the development of lentiviral vectors, which is currently in progress. MiRNA31 was found to be involved in the regulation of Foxp3 gene expression but other mechanisms must contribute to the level of expression. Studying the potential involvement of the Ets family of transcription factors we found that Ets1 and Ets2 play a positive role in the regulation of Foxp3 expression.

3. Pre-clinical studies to optimize the generation of fully functional APCs

We have previously investigated the role of different maturating agents on functional aspects of mature dendritic cells (DC) by comparing different maturation stimuli for their ability to reproducibly generate stable mature DC secreting high amounts of bioactive IL-12. We could confirm that DC, having already produced high amounts of IL-12p70 during the maturation step, are still capable of secreting IL-12p70 after in vivo administration at the time of interaction with the targeted T cell. but only when poly (I:C) is used as the maturating agent.

A finding with important practical implications is the fact that DC generated in bags and then elutriated secreted much less bioactive IL-12 than DC's generated in plates. We found the activation status of Erk to be markedly decreased in DCs generated in plates, with is consistent with an increase in IL-12 secretion. Preliminary results of the micro-array analysis give a hint that plates favor a pathway leading to activation of TH1 T-cells.

4. Effects of global Tregs removal on in vitro generation of anti-WT1 responses

Experiments using WT1 protein loaded APCs and T-cells from a small number of WT1 positive-patients, with or without aspecific depletion of T-Regs, strongly suggest that specific responses are drastically amplified after Tregs depletion. This finding justifies our to focus on the molecular and cellular study of Tregs, in the context of the global topic "Host tumor interactions".

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Analysis of PROX1 role in colon and small cell lung cancers (Tatiana Petrova)

Main results obtained

The main goal of our project is to investigate the contribution of transcription factor PROX1 to colon and lung tumorigenesis.

1. PROX1 DNA binding sites in colon cancer cell line have been identified by genome-wide ChIP-chip approach. The preliminary bioinformatics analyses indicate that PROX1 ChIP sites are enriched in TCF/LEF binding sites, i.e. transcription factors implicated in colon cancer development, suggesting that PROX1 may contribute to the regulation of TCF/ß-catenin transcriptional program in colon cancer.
2. To study this hypothesis, several luciferase reporter constructs containing PROX1 genomic sites have been produced and tested in transient transfection assays in colon cancer (SW480, DLD-1) and non-colon cancer cell lines. In addition, we have investigated the ability of PROX1 to interact with its target DNA using electrophoretic mobility shift assay. The data obtained suggest that PROX1 ability to bind DNA requires the presence of TCF/ß-catenin complexes and that PROX1 acts as a sequence-specfic co-repressor of TCF mediated transcription in colon cancer but not other cell types. To substantiate these findings and to identify target genes that are co-regulated by PROX1 and Wnt signalling in colon cancer, we plan to compare gene expression profiles following ß-catenin or PROX1 siRNA knockdown.

3. The orthotopic model of colon cancer has been established in the laboratory. The studies of the role of PROX1 in tumor invasion and metastasis using this model are underway, using the model in which the expression of PROX1 is inducibly suppressed by PROX1 shRNA.
4. We have established a small cell lung cancer cell line that overexpresses PROX1 and plan to study whether this overexpression affects tumor growth in an vivo model.

Publications

Study of bone marrow-derived peripheral blood cells in human cancer progression: evaluation of their potential use for prognostic, diagnostic and therapeutic means. (Gian Carlo Alghisi)

Main results obtained

In contrast to their well-accepted role in sustaining tumor angiogenesis at tumor sites, little is known on the mechanisms by which tumor-mobilized myelo-monocytic cells are educated to acquire proangiogenic properties. In particular it remains unclear whether this education might already occur early during their differentiation from hematopoietic progenitors or whether it only occurs late at tumor sites though differentiation of already mobilized mature CD11b+ monocytes. We have addressed this question through experimental studies in vitro, in vivo and correlative analyses in cancer patients. We show that breast cancer cell conditioned medium promotes the differentiation of human CD34+ hematopoietic progenitors into pro-angiogenic CD11b+ cells. To confirm that tumorigenic, but not normal, mammary epithelial cells can induce pro-angiogenic properties on CD11b+ cells during their differentiation, we tested conditioned medium from normal human mammary epithelial cells (HMEC) and the oncogenic HMEC-derived tumorigenic cell line Wnt-1HMEC obtained by forced Wnt-1 expression and show that only CD11b+ cells isolated from cultures supplemented with supernatants from tumorigenic cell line induced sprouting. Next, we tested four angiogenic cytokines secreted by MDA-MD231 cells: IL-8 , IL-1b, VEGF and PlGF for their putative education function. Only CD11b+ cells generated from CD34+ cultured in presence of PlGF induced endothelial sprouting. To confirm the role of PlGF in the acquisition of pro-angiogenic properties of CD11b+ cells, inhibitor of its receptor (anti-Flt-1 mAb KM1732) or PlGF itself (sVEGFR-1/Fc trap) were tested during the culture of CD34+ cells exposed to MDA-MB-231 supernatant or PlGF. For both inhibitions, generated CD11b+ cells did not induce endothelial sprouting. Next, we addressed the question whether CD11b+ cells circulating in the peripheral blood of breast cancer patient have pro-angiogenic properties compared to healthy donors; and whether this might correlate with PlGF level. Blood CD11b+ isolated from early-diagnosed breast cancer patients versus healthy donors were tested for their pro-angiogenic capacities in vitro, and in parallel plasma PlGF was measured. Only CD11b+ cells isolated from breast cancer patients induced sprouting. PlGF levels were significantly elevated in plasma samples derived from breast cancer patients compared to healthy donors. Finally, to directly test for the role of PlGF in tumor growth in vivo, we reduced PlGF expression in experimental breast cancer (4T1 tumors) using a shRNA approach. Significant tumor growth delay was observed in PlGF-silenced 4T1 tumor bearing mice. Plasma PlGF levels and frequency of leukocytes infiltrating the tumors were considerably reduced compared to mice bearing control tumors. Power Doppler analysis revealed that blood perfusion in PlGF-KD mice was strongly reduced compared to control tumors. Taken together data generated at this time revealed a new function of PlGF in the acquisition of pro-angiogenic properties by CD11b+ cells during their differentiation fromCD34+ progenitor cells.

Publications

Alghisi GC, Ponsonnet L, Rüegg C. The integrin antagonist cilengitide activates alphaVbeta3, disrupts VE-cadherin localization at cell junctions and enhances permeability in endothelial cells. PLoS One. 2009;4:e4449. Epub 2009 Rüegg C, Alghisi GC. Vascular integrins: therapeutic and imaging targets of tumor angiogenesis. Recent Results Cancer Res. 2010;180:83-101

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Tumor-host interactions in cancer progression and metastasis (Ivan Stamenkovic)

Main results obtained

Transcriptome analysis of reactive stroma associated with invasive tumor growth in a mouse model of multistep prostate carcinogenesis revealed upregulation of several genes that reflect extracellular matrix (ECM) turnover and inflammation. Comparison with existing databases revealed signatures composed of about 100 genes that are associated with poor prognosis in breast and prostate cancer. Among these genes eleven were found to be shared as poor prognosis markers between breast and prostate cancer.

We undertook the study of the functional role of these genes, starting with securin. Mammalian securin was initially identified as an oncogene but the mechanisms underlying its oncogenic properties remain controversial.

> As an anaphase inhibitor, loss rather than overexpression of securin may be expected to favour tumor progression by allowing aberrant chromosome segregation resulting in aneuploidy. Contrary to such expectations, securin deletion in cells results only in transient chromosomal instability, which is supported by the observation that securin-deficient mice do not develop tumors at a higher frequency than their wild type counterparts and suggests the existence of compensatory mechanisms for its chromosome segregation regulating function. Similarly, securin overexpression may be expected to block cell proliferation, yet it increases proliferation and tumorigenicity of NIH3T3 and HEK293 cells. Moreover, securin is not only highly expressed in primary and metastatic tumor cells, but is a predictor of poor outcome in a variety of malignancies, consistent with functions that diverge from anaphase inhibition.
>
> In addition to separase, securin is reported to interact with the transcription factor SP1, the regulatory subunit of DNA-PK, Ku70, the MAP kinase MEK1 and protein phosphatase 2A (PP2A), supporting a role in transcription, DNA repair and proliferation, and consistent with its nuclear localization. However, securin can also display predominantly cytoplasmic distribution in some malignant cell types, including the T cell lymphoma Jurkat, pituitary adenomas and a variety of carcinomas. Unlike its nuclear interactions and functions, its physiological partners and role in the cytoplasm remain unknown. Biophysical analysis of securin indicates that it belongs to the family of "intrinsically unstructured proteins", whose flexibility provides the ability to overcome steric restrictions, enabling larger surface interactions. Interestingly, unfolded protein regions are frequently found in oncogenes and signaling proteins, allowing them to bind multiple partners with high specificity.

Based on these observations, we sought to better characterize the cytoplasmic localization of securin, identify its potential cytoplasmic protein partners and address its possible extranuclear functions.

Securin was detected in only a fraction of normal glandular epithelial breast and prostate cells where it adopted an apical orientation in the perinuclear region. In contrast, its increased expression in invasive and metastatic cells of a broad range of malignancies was diffuse and both nuclear and cytoplasmic. In cultured breast carcinoma MDA-MB-231 cells, cytoplasmic securin expression outlined the biosynthetic/secretory pathway, and colocalized with markers specific for the endoplasmic reticulum (Calnexin, Bip), the *cis*-Golgi (GM130, Giantin) and *trans*-Golgi (TGN46) networks, as well as early endosomes (EEA1). Strong anti-securin antibody reactivity was also detected beneath the plasma membrane and cell fractionation experiments confirmed securin presence in the membrane, cytoplasm, and nucleus.

Securin association with the GA was further investigated during drug-induced reversible Golgi disassembly. Despite Golgi dispersion throughout the cytoplasm following Brefeldin A (BFA) or nocodazole treatment, securin colocalization with the Golgi markers remained intact. Following washout of both drugs, the Golgi stacks reassembled in the pericentriolar region and securin colocalized with giantin as it did prior to treatment. To determine whether securin has a role in the structural maintenance of the GA, we assessed the efficiency of Golgi reassembly in cells depleted of securin after nocodazole or BFA treatment. Transient securin downregulation was achieved using two different oligonucleotides, one targeting the coding region and the other the 5' untranslated sequence. An oligonucleotide targeting the vsv-g was used as a control. Reduction of securin expression was almost complete as early as 24 h following transfection with either oligonucleotide and remained so for more than 72 h. *Cis*- and *trans*-Golgi compartments reassembled efficiently in both control and securin-depleted cells, indicating that, consistent with the prediction of its amino acid sequence, securin is not a structural constituent of the GA.

Despite not being a structural component of the GA, securin colocalization with Golgi markers was maintained during drug-induced GA disassembly. We therefore sought to identify securin interactors that may be responsible for its association with the GA and implicate it in functions related to vesicle transport and secretion. Using an anti-securin antibody, 63 proteins were pulled down with endogenous securin from MDA-MB-231 cell lysates depleted of nuclei. Mass-spectroscopic identification of the proteins revealed both known securin partners, including separase (by far the most highly represented interactor as reflected by the number of peptides identified), Ku86, a partner of Ku70, PP2A, and an array of novel candidate interactors. Several of these, including calnuc (NUCB1), clathrin heavy chain (CLH1), kinectin 1 (KTN1), sec23A and syntaxin 7 (STX7) play a functional role in the biosynthetic/secretory pathway. Additional cytoplasmic interactors included proteins implicated in microtubule organization (MAP4, GCP2, GCP3, GCP4).

Seven candidate interactors (NUCB1, KTN1, CLH1, MAP4, sec23A and STX7) were selected for validation in co-immunoprecipitation experiments with endogenous securin from MDA-MB-231 cell lysates. All six proteins co-immunoprecipitated with securin.

The location of the interactions between securin and its partners was addressed using confocal microscopy. Securin and separase were found to colocalize in the nucleus, beneath the plasma membrane, and along the biosynthetic/secretory pathway. Similar to securin, separase colocalized with *cis*- and *trans*-Golgi markers. Securin colocalization with calnuc was restricted to the GA consistent with the previously reported *cis*- and medial-Golgi localization of calnuc. Despite an abundant amount of clathrin at the plasma-membrane, colocalization with securin was predominantly observed in the perinuclear region, presumably in the TGN, where clathrin forms the coat of newly budding vesicles.

To gain additional insight into the functional implications of the identified interactions, we constructed an extended protein interaction network by complementing the results of our pull-down experiment with previously published data deposited in public databases. Analysis of functional families into which the extended-network interactors were grouped revealed that some of the most statistically significant Gene Ontology (GO) terms were *"Vesicle-mediated transport", "Endocytosis", "Clathrin coat of trans-Golgi network vesicle"* and *"Trans-Golgi network transport vesicle membrane"*, *"Microtubule nucleation", "Spindle", "Centrosome"* along with *"DNA replication", "Cell cycle"* and *"DNA repair, and replication fork"*. The diversity of its interaction network suggests that securin may be implicated in a much broader range of cellular processes than recognized thus far, one of which appears to be the regulation of vesicle trafficking and possibly secretion.

To probe the putative functional role of securin in the GA and vesicle trafficking, we investigated the effect of securin depletion on the morphology and function of the ER, GA and endosomes. Endoplasmic reticulum and *cis*-Golgi compartments remained unaltered upon securin depletion as assessed by anti-calnexin, anti-Bip and anti-KDEL antibody staining of the ER and anti-GM130 antibody staining of the *cis*-Golgi network.

Contrary to the ER and *cis*-Golgi network, the medial- and *trans*-Golgi compartments as well as cargo vesicles positive for mannose-6-phosphate receptor underwent robust swelling, in 40-50% of cells depleted of securin. Analysis of EEA1-positive early endosomes uncovered giant vesicles located predominantly in the perinuclear region in 50-60% of cells. Similar effects were observed on late endosomes and lysosomes. To exclude off-target effects the same experiment was performed on cells transfected with conservatively mutated securin cDNA, which generates transcripts that cannot be targeted by the selected siRNA oligonucleotide while encoding the amino acid sequence of the wild-type protein. Expression of non-siRNA-degradable securin in cells depleted of its endogenous counterpart prevented GA swelling and giant endosome formation, further indicating that the observed effects were specifically due to securin depletion.

Given the role of securin in cell cycle control and the profound morphological changes of the GA during mitosis, we investigated whether the effects of securin depletion are cell cycle-dependent. Consistent with observations reported by others, downregulation of securin 72 h following siRNA treatment was accompanied by an increased number of cells with multi-lobed nuclei and in the G2/M phase, a slight decrease in proliferation but no increase in apoptosis. We next investigated whether the swollen GA and giant endosomes were restricted to cells arrested in mitosis by performing double-immunofluorescence staining using the anti-phospho-Histone H3 antibody (a marker of mitosis) in combination with Golgi and endosomal markers. No significant difference in phospho-Histone H3 staining was observed between control and securin-depleted cells, indicating that the latter were not arrested in mitosis. Moreover, swollen Golgi stacks and giant endosomes appeared irrespective of cell cycle status, and were also observed in securin-depleted, serum-starved, G0-synchronized cells. Taken together, these findings suggest that the effects of securin depletion on GA and endosome swelling are uncoupled from the cell cycle.

In view of its effect on TGN swelling, we asked whether securin depletion affects protein secretion by transfecting cells with a plasmid expressing horse radish peroxidase carrying a basolateral sorting signal (ss-HRP). The activity of HRP released into the culture medium and of that retained in cells was measured by chemiluminescence in MDA-MB-231, Hela and HEK293T cells. Securin depletion led to a significant reduction of HRP secretion paralleled by a corresponding increase in intracellular levels in all three cell lines analyzed. Thus, swelling of the medial- and *trans*-Golgi compartments caused by securin depletion affects protein secretion.

We next assessed the functional significance of endosomal swelling in response to securin depletion given that early endosomes are the initial sorting station of cargoes destined for degradation and recycling. Following engagement by its receptor, Alexa594-labeled EGF was efficiently endocytosed in securin-depleted cells and detected in giant EEA1-positive endosomes at 20 min following uptake. Control cells efficiently delivered EGF-Alexa594 from early to late endosomes/lysosomes at 1.5 h, and almost completely cleared it from late endosomes/lysosomes by 5h. By contrast, cells depleted of securin displayed delayed delivery of the EGF-Alexa594 from early to late endosomes/lysosomes and impaired clearing of the ligand from the latter. Remarkably, while EGF-Alexa594 fluorescence was no longer detectable in vsvg-siRNA-treated control cells 12 h following uptake, it was still present in securin-depleted cells. Similarly, FACS analysis of Alexa488-labeled transferrin revealed significantly stronger fluorescence intensity in securin-depleted cells compared to the control counterparts that remained so for more than 12 h following uptake. Thus, tracking of receptors destined for degradation or recycling revealed a delay in both pathways in cells depleted of securin.

Taken together, our observations provide several lines of evidence that implicate securin in the regulation of the secretory and endocytic pathways; it is physically associated with the GA and endosomes; its depletion strongly alters

TGN and endosomal morphology and function; and it interacts with several proteins that are functionally linked to secretion and endocytosis. Calnuc, clathrin heavy chain 1, kinectin 1, sec23A and syntaxin 7 all play a role in different compartments of the secretory pathway. Calnuc is an abundant *cis*-Golgi calcium-binding protein suggested to regulate calcium release required for Golgi dynamics. It also binds the a subunit of heterotrimeric G proteins that regulate vesicle trafficking in the secretory pathway. Clathrin is the major coat protein of membrane-bound vesicles that transfer macromolecules in the secretory and endocytic pathways between donor and acceptor compartments. Kinectin 1 is the principal receptor for the motor protein kinesin and is required for kinesin-driven motility of transport vesicles. Sec23A is a component of the COPII cargo adaptor complex that links cargo sorting to target recognition and tethering. It is conceivable that by virtue of its structural plasticity, securin may provide a scaffold for the recruitment and assembly of divergent molecules required for the function of specific events in vesicular trafficking and/or secretion.
It is noteworthy that securin was discovered in pituitary tumors where hormonal secretion is aberrant, and that securin itself has been shown to be secreted in human pituitary adenomas. Moreover, pituitary glands of transgenic mice engineered to overexpress securin display focal hyperplasia with a prominent GA and an increased number of secretory vesicles. Perturbed secretion, be it of hormones or cytokines may have major consequences on cell growth and function and provide a potential mechanism whereby securin overexpression promotes tumor growth and progression. Our study provides evidence of the complex nature of securin interactions that supports a role in multiple events relevant to both normal cell physiology and tumor development. Identification of hitherto unrecognized interactors and a role in vesicular trafficking sheds new light on securin that will help elucidate the full spectrum of its functions. Understanding these functions will constitute a major goal of the present project.

Publications

4. Financial status

Table 3 presents the balance of the MEDIC account as kept by the Lausanne Cancer Center (CePO) over 2009.

Table 3 status of the MEDIC accounts

Comptes MEDIC 2009		**en CHF**			**en €**
Débit	Crédit	Libellé	Débit	Crédit	Libellé
	1134958.46	Précédente clôture		46590.48	Précédente clôture
	434680	Medic Fondation Rech. Méd.		371432	DON MEDIC FONDATION
155720		Don Medic - C. Ruegg	500		Rbt Piccard Meeting MEDIC
84000		Don Medic - T. Petrova	76500		Prof. Ghanem - 50 % 2009
51500		Don Medic - GC Alghisi	113436.09		Prof. Martiat - 50 % 2009
41500		Prof. Stamenkovic - 50 % 09	180004.09		Dr. Sotiriou - 50 % 2009
86860		Prof. Picard - 50 % 2009		180.11	Interet & frais 1er trim. 09
60120		Dr.Delorenzi ISREC - 50 % 09	5.54		Interet & frais 1er trim. 09
45147.5		Prof. Romero - 50 % 2009	2.85		INTÉRÊTS ET FRAIS 2TRIM 09
	1104.48	Interet & frais 1er trim. 09		371432	DON MEDIC FONDATION
5.86		Interet & frais 1er trim. 09	76500		Pr G.Ghanem,Instit JulesBordet
120332.5		Don Medic A. Mariotti 1er.	113436.02		Pr Martiat,Instit JulesBordet
	298.63	INTÉRÊTS ET FRAIS 2TRIM 09	180004.02		Pr Sotiriou,Instit JulesBordet
4.01		INTÉRÊTS ET FRAIS 2TRIM 09	4.06		INTÉRÊTS ET FRAIS 3TRIM 09
86860		Prof D Picard Uni Geneve	430.79		Sotiriou C-Institut JuleBordet
41500		Prof Stamenkovic-Hospices	525.15		Ghanem G-Institut JulesBordet
66864.5		Prof Romero Instit Ludwig	517.6		Desmedt C-Institut JulesBordet
60120		Pof Delorenzi bioinformat.	3.81		INTÉRÊTS 4E TRIM 09
120332.5		Don - Medic - Dr A Mariotti			
84000		Don - Medic - Prof T Petrova			
51500		Don - Medic - Dr C Alghisi			
155720		Don - Medic - Prof C. Ruegg			
	434680	DON - Medic Fondation			
330006		Prof C.Sotirou,Instit JulesBo			
287691.88		Prof C.Sotirou,Instit JulesBo			
5.98		INTÉRÊTS ET FRAIS 3TRIM 09			
	96.56	INTÉRÊTS ET FRAIS 3TRIM 09			
6282.4		Hotel de la Paix			
2481		Hotel de la Paix			
3.99		INTÉRÊTS 4E TRIM 09			
	21.83	INTÉRÊTS 4E TRIM 09			
1938558.12	2005839.96		741870.02	789634.59	
	67281.84	Solde au 31.12.2009		47764.57	Solde au 31.12.2009

The data indicates that the accounts have ceased to accumulate reserves: the sums spent are close to the budgets received. The available reserves are largely sufficient to allow for unforeseen ad hoc support to projects in case of need. The expenses of the individual groups have been globally evaluated and accepted. Running cost (the MEDIC day, administrative and accounting support and financial compensation for application reviewers) will increase somewhat but this will remain within the available budget foreseen.

5. Outloook

The way the MEDIC foundation supported consortium 'Tumor-host interaction' has evolved shows that high quality research can be supported in an approach that is not competitive in the sense of the usual research grant providing institutions (National Research Foundation, Swiss Cancer League). A high standard has been attained through auto-evaluation, internal review within the consortium and external peer review of new applications. The research program has favored the development of new interactions and allowed research directions that the individual groups alone would not have made so easily. External peer review of the consortium is an essential step towards a stable and scientifically valid modus operandi. It is therefore imperative that an independent external Scientific Advisory Board is created, which can evaluate the overall performance of the groups and of the consortium as a whole. To this end board members will evaluate the annual report and attend the annual MEDIC symposium. The board members can also function as reviewers or as jury in case of lack of consensus of external reviews of new applications. The trustees have confirmed their satisfaction with the choices made and the structures developed and have confirmed their intention to continue to support the consortium to the extent of the possible at the present level.
The Foundation does not seek a high profile but more explicit visibility of MEDIC through its research support would be desirable. An important element is here the obligation of investigators supported by MEDIC to specifically mention MEDIC support in their publications. Another approach will be the creation of a 'MEDIC prize', salary support and a small running budget for a particularly promising young clinician scientist. A call for applications will go out during the course of 2010. Received applications will be reviewed and the prize will go to the application with the highest appraisal. The call will be open, applications not necessarily limited to the central theme of the consortium. The first MEDIC prize will be given in the second semester of 2010.

Fred T Bosman
Lausanne
October 2010