The role of MYC in cellular invasion and metastasis

State of research

Cancer is an ever more important cause of mortality and morbidity worldwide. The vast majority of cancer deaths are due to the development of metastatic disease. However, the molecular mechanisms of this multistep process and its regulation are still poorly understood. In addition, we have very little understanding of the extremely variable latency of the development of metastases among patients.

New technologies allowing genome-wide analysis of gene expression have brought a wealth of new information to the area of cancer research. In the past few years, many gene expression signatures have been published that can predict the propensity of primary tumors to metastasize. Some of these signatures are currently being studied in prospective clinical trials aiming to improve the selection of patients with early stage breast cancer who would benefit from adjuvant chemotherapy after surgery and identify patients where chemotherapy would not be helpful and thereby sparing them the toxicity of such treatment [1, 2].

These poor outcome gene expression signatures are based on many different basic biological processes, samples, and gene expression platforms, and the individual gene overlap between all these signatures is very limited. However, it has been shown that four of the gene expression signatures predictive of outcome in breast cancer largely flag the same patients in spite of the minimal overlap in gene content [3].

State of personal research

During my postdoctoral studies in the laboratory of Dr. Ramaswamy, I have studied the relationship and regulation of thirteen different outcome signatures [1, 2, 4-13]. Using cell line model systems, we have demonstrated that these signatures are coordinately regulated by various oncogenic pathways. To study the effect of estrogen signaling, a well know oncogenic stimulus in estrogen receptor positive breast cancer, on the thirteen different gene expression signatures, we analyzed previously published gene expression data from the estrogen receptor (ER) positive MCF7 breast cancer cell line. In order to compare the different gene expression signatures to each other, we created two “metagenes” by separately averaging up and down-regulated genes from each multi-gene poor outcome cancer signature. The results are shown as standard deviation on plots for UP- and DOWN metagenes for each of the thirteen signatures (named s1-s13) (Fig. 1a). This

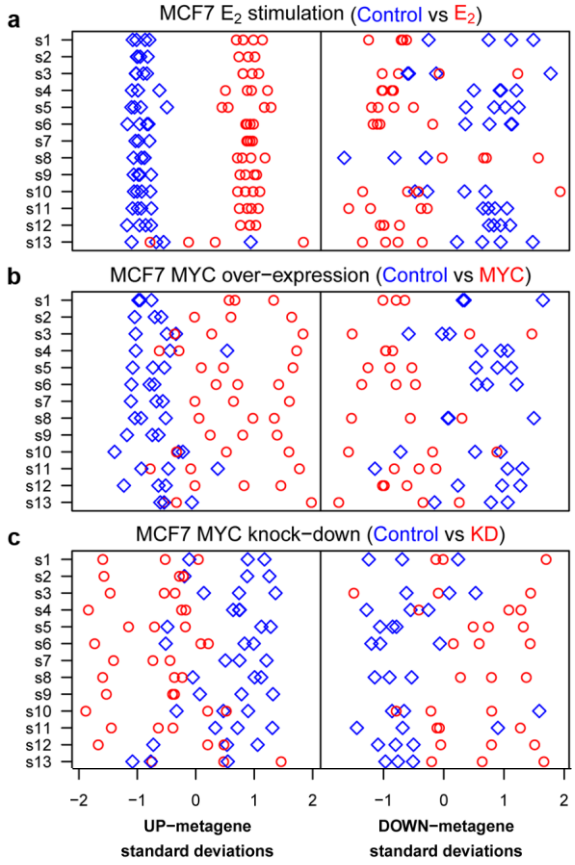

Figure 1. Molecular regulation of thirteen “poor-outcome” human cancer signatures. We studied the regulation of thirteen different signatures (s1 – s13), correlated with different biological processes associated with metastatic progression, reported to be prognostic in different tumor types in a cell culture model system using the MCF7 breast cancer cell line. To simplify analysis, we first created two “metagenes” by separately averaging up- and down-regulated genes from each multi-gene, “poor-outcome” cancer signature (s1 – s13). Plots display UP- and DOWN-metagenes derived from each signature in standard deviation units after **(a)** 17β-estradiol stimulation (red circles) vs. control (blue diamonds), **(b)** over-expression of MYC (red circles) vs. vector control (blue diamonds), and **(c)** shRNA knockdown of endogenous MYC (blue diamonds) vs. vector control (red circles) in MCF7 cells. Circles and diamonds represent independent replicates. N.B. s2, s7, and s9 are unidirectional signatures that have only up-regulated genes.

analysis showed a coordinate up-regulation of the UP-metagenes and to a lesser extent down-regulation for the DOWN-metagenes for all thirteen signatures. Estrogen starvation of MCF7 cells showed a similarly coordinate down-regulation of all thirteen signatures, as did treatment with the estrogen antagonist fulvestrant.

Next, we decided to look at other oncogenic stimuli and found that EGF stimulation or overexpression of ERBB2 in immortalized non-transformed mammary epithelial cells (MCF10A) showed a similar coordinate effect on signature regulation. Finally, we also studied androgen signaling in an androgen receptor dependent prostate cancer cell line (LNCaP) with the same observation of coordinate signature regulation.

Closer examination of the intersection of these various oncogenic stimuli led us to discover a core interactome of twenty genes centered on the extensively studied oncogenic transcription factor MYC. Overexpression and knockdown of MYC in cell line models showed that MYC indeed coordinately regulates these “poor outcome” signatures (Fig. 1b-c).

We then studied the effect of loss of MYC in a xenograft metastasis model. MDA-MB-231 breast cancer cells were stably transduced with shRNA against MYC and knockdown assessed by Western blotting. Surprisingly, MYC knockdown cells (MYC HP1 and HP2) did not show a decrement in cellular proliferation compared to controls (pLKO) (Fig. 2a). These cells were then injected orthotopically into mammary fat pads of immunocompromised NOD/SCID mice. We were able to show that loss of MYC (MYC HP1) leads to a significant decrease in lung metastases without affecting primary tumor size (Fig. 2b-c). In addition, we also provided evidence in an *in vitro* cell line model that MYC regulates invasion in addition to its well-described effects on proliferation, survival, and genetic stability [14].

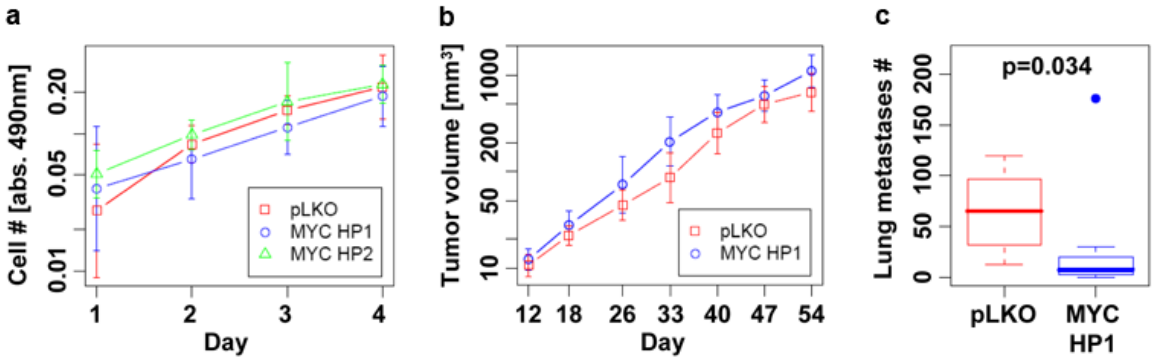

Figure 2. MDA-MB-231 primary tumorigenesis and metastasis *in vivo* with stable MYC knockdown. (a) Proliferation curves for empty vector control (pLKO), and MYC short hairpin RNA knockdown (HP1, HP2) cells after serial passage over 4 days showing absorbance as measure of cell number (N = 3, 95% CI). (b) Tumor growth of control (pLKO) and HP1 cell xenografts in NOD/SCID mice (pLKO N=9; HP1 N=7; 95% CI). (c) Boxplot of number of lung metastases per mouse in control and MYC HP1 knockdown tumor bearing mice (pLKO (N = 9), MYC HP1 (N = 7); p-value from two-sided, two-sample Wilcoxon test).

In summary, we have shown that thirteen different poor outcome signatures are coordinately regulated by the well known oncogenic transcription factor MYC. We have shown that loss of MYC in the MDA-MB-231 breast cancer cell line does not impair proliferation but has a negative effect on Matrigel invasion of these cells. And in an *in vivo* xenograft metastasis mouse model MYC knockdown cells clearly show reduced numbers of lung metastases.

Amplification and overexpression of MYC have been known for decades to be associated with poor outcome in various cancer types. MYC has many functions associated with tumor formation and progression such as promoting cellular proliferation and survival, differentiation blockade, genetic stability and angiogenesis. All of these mechanisms could influence metastasis indirectly by increasing the number of cells with genomic instability. However, more recently several mechanisms have been described by which MYC might directly influence metastasis by regulating epithelial-mesenchymal transition (EMT) and cancer cell invasion. Our finding that MDA-MB-231 cells that lack MYC invade less and show decreased metastatic capacity, was confirmed by Chan et al. [15]. These authors show that MYC cooperates with SKP2 to recruit MIZ1 and p300 into a transcriptional complex to activate RhoA, which is necessary for migration, invasion and lung metastasis *in vivo* [15]. It has also been

shown that MYC regulates miRNA networks implicating LIN28 and let-7 leading to up-regulation of HMGA2 and the EMT-inducing transcription factor SNAIL [16, 17]. In addition, another group showed induction of miR9 by MYC leading to down-regulation of E-cadherin and also induction of EMT [18]. However, the study of our MYC deficient MDA-MB-231 cells did not show a loss of the mesenchymal phenotype of these cells or up-regulation of E-cadherin. Such a phenotype was not found in the MYC knockdown xenograft tumors either [14].

I am well-aware that MYC has been intensely studied for more than two decades [19]. During my postdoctoral work, I identified MYC as an important regulator of metastasis at least in part via regulation of cellular invasion. I intend to concentrate on elucidating the mechanisms by which MYC achieves this biological effect. As a first step to this end, I propose to study in more detail the twenty genes found to be coordinately up-regulated by different oncogenic pathways and centered on MYC. With this approach I hope to identify effectors in the MYC regulated network responsible for invasiveness that could potentially be targeted by drugs. Such findings would be important given that MYC is a transcription factor and very difficult to target by drugs given its structure. On the other hand I would like to elucidate further the network regulated by MYC responsible for invasion by analyzing other cell line pairs of control and MYC knockdown using genome wide analyses such as gene expression profiling, miRNA profiling and proteomics.

Detailed research plan

Specific aims

1. Identify the role of the twenty core interactome genes in cancer cell invasion

Several approaches will be used in order to address this question. First I want to use bio-informatics analyses and previous knowledge about these genes to prioritize the most promising candidates. A statistical analysis of mRNA expression levels based on microarray data of the twenty core interactome genes in nine different clinical datasets with more than 100 patients each [11, 12, 21-27], reveals that seven of these twenty genes are independently prognostic in two or more datasets using a false discovery rate (FDR) of <0.05 (using the Benjamini and Hochberg method): C16orf61, H2AFZ, MAD2L1, MLF1IP, ORC6L, RRM2 and TTK. A literature review on these seven genes reveals that only two of them are know MYC target genes (H2AFZ and MAD2L1). The majority of these seven genes have been implicated in transcriptional regulation, chromosome replication and DNA repair.

Having identified the most promising genes, I plan to use short hairpin RNA and lentiviral transduction to stably knock-down the function of these core interactome genes in the highly invasive MDA-MB-231 breast cancer cell line. In order to minimize the risks of off-target effects, knockdown will be performed using multiple hairpins and assessed (where possible) on the protein level. Once efficient knockdown is documented I will perform proliferation studies using life cell counting as well as BrdU incorporation. Most importantly, I want to investigate the effect of gene knockdown on the motility of these invasive breast cancer cells. Again, I propose to address the question with two different experimental systems. On one hand, I will perform traditional Boyden chamber invasion assays. However, since many of the seven genes of interest have been shown to be implicated in chromosomal replication and separation, it is to be expected that knockdown of these genes will lead to defects in proliferation. Since differences in proliferation might lead to biased results in a standard Boyden chamber assay I propose to evaluate migration and invasion also by using microchannel devices and video-microscopy allowing single cell visualization[14, 20].

To complement the knockdown studies of the seven genes of interest, I want to perform over-expression studies in minimally invasive cancer cells (MCF7) as well as non-transformed immortalized mammary epithelial cells (MCF10A, HMEC-Tert). These cell lines will then be assayed by the same methods as the knockdown cells for proliferation, migration and invasion.

While I propose to prioritize the study of the seven core interactome genes identified by statistical analysis to be of prognostic value in several human cancer data sets, I would ultimately like to also include the remainders of the twenty core interactome genes.

Finally, I plan to perform an *in silico* search for small molecules capable of enhancing or reversing the thirteen different gene expression signatures that we originally studied. The Connectivity Map is a tool

recently developed by Lamb *et al.* to pursue a systematic approach to the discovery of functional connections among diseases, genetic perturbations, and drug actions [28]. Lamb *et al.* created the first reference collection of gene-expression profiles from cultured human cancer cells treated with more than 1000 different small molecules and developed pattern-matching software to mine this data. Compounds found to reverse the gene expression signatures will then be tested for their effects on proliferation, survival and inhibition of invasion of cancer cell lines as well as for the molecular mechanisms leading to such changes. Since many of these molecules are actual drugs used in the clinic it seems possible that promising candidates could also be tested for inhibition of metastasis in a mouse xenograft model.

2. Study the role of MYC in invasion and migration in additional cancer cell lines

We have previously shown that loss of MYC in MDA-MB-231 breast cancer cells leads to defects in invasion and decrease of metastatic capacity in a mouse xenograft model [14]. In order to better understand the molecular mechanisms of MYC regulation of cellular invasion, I will knock down MYC in additional cancer cell lines to study the effect of loss of MYC on cellular proliferation and motility. To this end I propose to use several additional breast cancer cell lines that have previously been shown to be invasive and some of them have also been shown to be metastatic in mouse xenograft models (HCC1500, BT549, SUM159PT, MDA-MB-157). MYC will be knocked down using lentiviruses containing two different short hairpin RNA constructs [14] shown previously to be effective. The effect of MYC knockdown on proliferation and invasiveness will then be studied using assays as described above.

In order to identify MYC targets implicated in regulation of invasion I plan to perform microarrays for mRNA as well as microRNA. I will start with the cell line pairs of MDA-MB-231 cells transduced with control as well as two different MYC hairpin viruses. Once I have been able to identify further cell line pairs showing impaired invasion upon MYC knockdown, they will also be profiled and the data integrated in order to define if possible a more generalized mechanism of regulation. This approach should also allow to narrow the number of potential targets by focusing on commonly regulated genes across several cell lines. In addition, integrating the results of mRNA profiling with the results from the miRNA profiling by comparing gene expression with possible targets for indentified microRNAs should further limit potential regulators.

In addition to the transcriptional differences I would also like to search for differences at the protein level between control and MYC knockdown cell lines. To this end I propose to perform mass spectrometry analysis on cell line pairs identified to show differential invasiveness.

Candidates identified by these various approaches will then be studied for their function in migration and invasion in *in vitro* assays. Genes with promising profiles in the *in vitro* assays will be tested for their effect on metastasis formation *in vivo* by xenografting cells into immunocompromised mice.

3. Evaluate the clinical relevance of the twenty core interactome genes

In order to define the clinical relevance of the twenty core interactome genes in breast cancer, I intend to perform immunohistochemical staining of these proteins where antibodies are available on actual breast tumor sections. I propose to perform initial studies for expression of these proteins on a limited number of samples. Depending on the possibility of staining and according to results from knockdown and overexpression studies, I propose to study these genes in a larger cohort of patients and to link protein expression to clinical outcome. Access to tumor samples is given here in Lausanne through the tumor tissue bank established since 2007 by the University of Lausanne and the “Centre Pluridisciplinaire d’Oncologie”.

Potential pitfalls

I am well aware that many of my goals rely on bio-informatics analyses and statistics that I cannot perform myself. Therefore, I have already established a contact with Dr. Mauro Delorenzi from the Swiss Institute of Bioinformatics who is interested in collaborating on this project.

It is possible that the list of candidate genes implicated in regulation of invasion by MYC is too large to practically follow up on all of them. This problem could be addressed in several ways. First, a literature

search could help identify promising candidates involved with invasion and metastasis. Online network modeling tools could help to link several candidates into one or a few pathways which could then be studied in more detail. This is actually desirable since it could link MYC to pathways and signaling cascades involved in metastasis that are regulated by other molecules. Another potential problem is lack of availability of reagents for specific genes. However, here again these genes could potentially be linked into networks that contain other genes more easily studied.

In order to address the possibility of few candidates, I propose several different approaches as indicated above. In addition, I will address specific technical problems as they arise by seeking expertise and advice among the many experienced researchers in the scientific community of Lausanne (UNIL, EPFL/ISREC, LICR, SIB).

Importance

Metastasis is the primary cause of death in cancer patients. Understanding in more detail the mechanisms of the metastatic process will allow for the development of more targeted therapeutic approaches to prevent the dissemination of tumor cells. In addition, molecules involved in the metastatic process might also serve as prognostic markers guiding treatment decisions using the currently available therapeutic agents. Given that the thirteen gene expression signatures the analysis of which led to the identification of MYC as a regulator of tumor invasion are of prognostic value in actual human tumors, it seems highly likely that any findings of further downstream mechanisms will be of clinical importance. Given that MYC itself is a transcription factor and as such extremely difficult to target by drugs any downstream effectors could potentially help to circumvent this problem.

Results from these studies will be published in peer reviewed scientific journals and presented at relevant scientific conferences.

Working at a hospital and in an establishing cancer center I plan to translate findings from the laboratory to explore their importance in the clinical setting. Ultimately, I hope to design early phase clinical trials based on our laboratory findings including translational research questions within these trials on human tumor samples.

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