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Modeling breast cell cycle regulation - overcoming drug resistance

Dorit Arlt, Özgür Sahin, Ulrike Korf, Christian Loebke, Tim Beißbarth, Florian Hahne, Stefan Wiemann, Annemarie Poustka

Division of Molecular Genome Analysis, German Cancer Research Center, Im Neuenheimer Feld 580, 69120 Heidelberg, Germany

In the treatment of breast cancer in many cases intrinsic or acquired resistance against available therapeutics leads to death. According to the present biological understanding, drug resistance is caused by molecular mechanisms in the complex network of proteins that regulate cell proliferation by activation of the estrogen receptor (ER), hereby allowing the cell to bypass the classical estrogen receptor signalling pathway after inhibition of the ER [1]. The objective of this project is to elucidate the cross talk in estrogen-dependent signalling through data based mathematical models. We will use these models for the design of combinatorial therapies, thus minimizing the possibilities for obtaining anti-estrogen resistance.

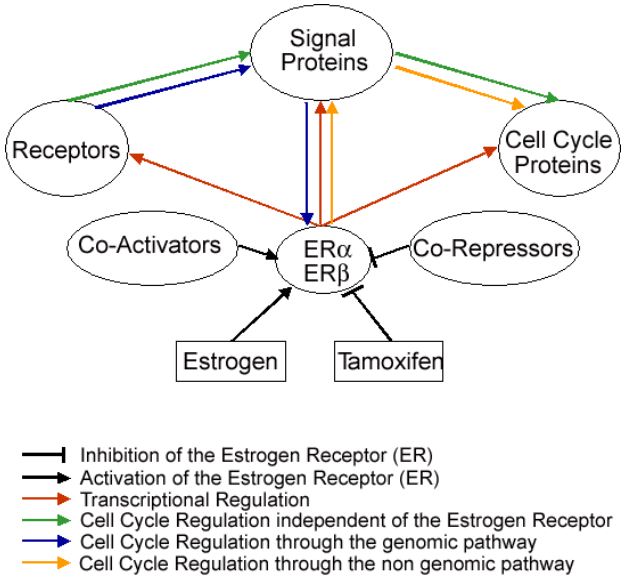

Figure 1: Schematic overview on the network of interest. Selected proteins are embedded in a systematic context with respect to the activity of estrogen receptors (ER-alpha and ER-beta).

Acquired resistance during anti-estrogen therapy is mediated by changes in different signalling pathways, that in consequence influence the estrogen receptor activity [2]. Within the complex network of genomic regulation of cell proliferation mediated by the estrogen receptor, additional target proteins exist that might support drug resistance when their expression and/or activation is deregulated. However, the mere identification of key molecules does not necessarily lead to an understanding of mechanisms. Quantitative analysis and time-resolved examinations of molecular mechanisms, leading to drug resistance, would facilitate the development of combinatorial therapies, which would consequently undermine the possibility of the tumor to bypass the anti-estrogen drug.

Selection of proteins involved in Tamoxifen resistance

Target proteins for this study were chosen according to the present understanding of estrogen receptor based cell cycle control, including proteins from genomic and non-genomic ER-dependant pathways, as well as from ER-independent signalling events (Figure 1). We have extensively screened the present literature information related to these pathways and constructed graphical models that will be used as a basis for further modeling. Additionally we utilize our functional profiling platform [3],[4] to perform a genome-wide RNAi screen, in order to identify novel players in Tamoxifen resistance (Figure 2).

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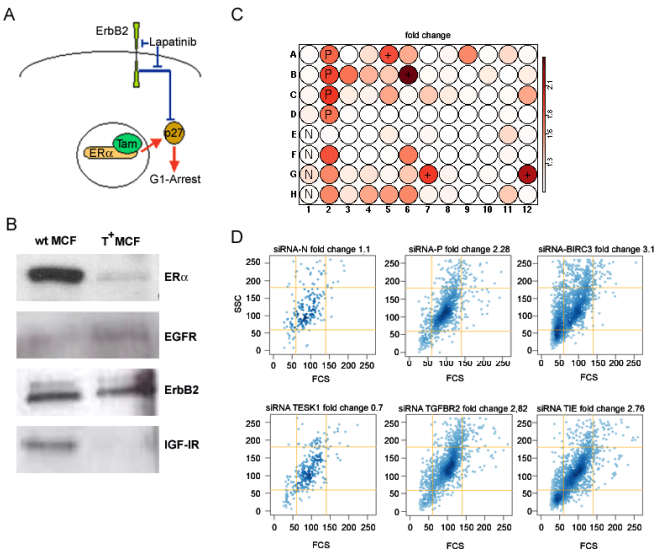

Figure 2: A genome-wide siRNA screen to identify proteins reversing Tamoxifen resistance. A: Schematic representation of a known mechanism which causes Tamoxifen resistance. In Tamoxifen sensitive breast cancer cells the treatmant results in a G1-Arrest, due to the upregulation of p27. In response to Tamoxifen cells over-express ErbB2 and in consequence the G1 arrest is reversed by blocking p27 expression. Lapatinib treatment leads to the inhibition of ErbB2, resulting in regained resistance. B: Induction of Tamoxifen resistance. MCF7 cells were treated with increasing concentrations of Tamoxifen for 7 months. Western blot analysis reveals increasing expression levels for EGFR and ErbB2 and downregulation of ER-alpha and IGF-IR expression. C: Proof of principle for a genome-wide detachment assay with HEK293 cells. Each 96-well plate contains 16 wells for control siRNAs (A1- H2) and 80 different screening siRNAs. D: Measured effects on cell detachment by FACS counting. In the first panel the effects of control siRNAs are shown, the second panel represents two of the identified candidates (TGFBR2 for detachment and TIE for apoptosis). TESK1 is a testis specific protein, as expected the siRNA transfection has no effect on cell detachment.

We used MCF7 cells (ATCC, HTB-22), to generate a Tamoxifen-resistant cell line out of this parental strain (Figure 2B). The assay is based on upregulation of genes during Tamoxifen treatment, which allows the cells to proliferate in presence of Tamoxifen (Figure 2A). If the protein expression is down-regulated via siRNAs, this effect is annulled, resulting in non-proliferative cells. The screen is performed with 21.125 siRNAs and the measurement of reversing Tamoxifen resistance is performed by FACS counting of detached cells, as we did in a proof of principle screen with HEK293 cells (Figure 2C and 2D), or with a WST-1 viability test. The candidates out of this screen will be validated through protein quantification using Reverse Phase Protein Microarrays (Figure 3). Only proteins which show higher expression levels in Tamoxifen resistant MCF7 cells as compared to wt-cells will be considered for further systematic analysis.

Time-resolved quantification of protein abundance and phosphorylation by protein microarrays and FACS analysis

Both cell-strains, wt-MCF7 cells and tamoxifen resitant cells, are utilized to generate data for modeling cell cycle dependent changes affecting Tamoxifen resistance. Cells are serum starved and then stimulated with estrogen. Twelve time points in a 24-hour time span are measured with 3 replicates.

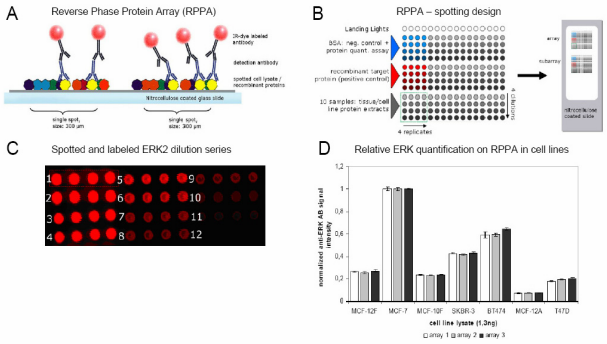

Figure 3: Protein quantification through Reverse Phase Protein Arrays (RPPAs). A: Principle of RPPAs. B: Spotting design. Cell lysate samples are spotted in dilution series and replicates. C: Antibody-labeled ERK2 dilution series on RPPA. D: Calculation of ERK expression in different breast cancer cell lines allows direct expression profile comparison

For the Reverse Phase Protein Microarrays the samples are dispensed with a four-pin Piezo-spotter on nitrocellulose coated glass Slides (Figure 3A). The lysates are spotted in multistage dilutions (Figure 3B). This allows the detection of changes in target concentrations down to femtogram of total spotted target protein (Figure 3C). Due to the spotting of several samples on the same slide, a direct comparison of expression profiles is

possible (Figure 3 D). The FACS measurements (Figure 4) are done in parallel. Here we examine changes in protein abundance through different cell cycle phases in single cells as it is shown for Cyclin D and ER alpha (Figure 4B), and complete the information we get with the Reverse Phase Protein Microarrays. Data acquired on the FACS is processed using an automated analysis pipeline that was specifically developed for high throughput flow cytometry screens utilizing robust statistical procedures to compute measures of both effect size and significance for the multi-dimensional data. Quantitative data are analysed using statistical models, gaining an inside into the protein expression levels and reliability of the measurements at different states and time points. Dynamical models describing the interaction of several players in a pathway can be fitted and used to simulate the dynamical behaviour of the system. The combination of quantitative and time-resolved measurements of relevant signal transduction molecules with mathematical modeling of the dynamic behaviour allows insights into drug action, which could not be gained using standard biological analysis of individual genes.

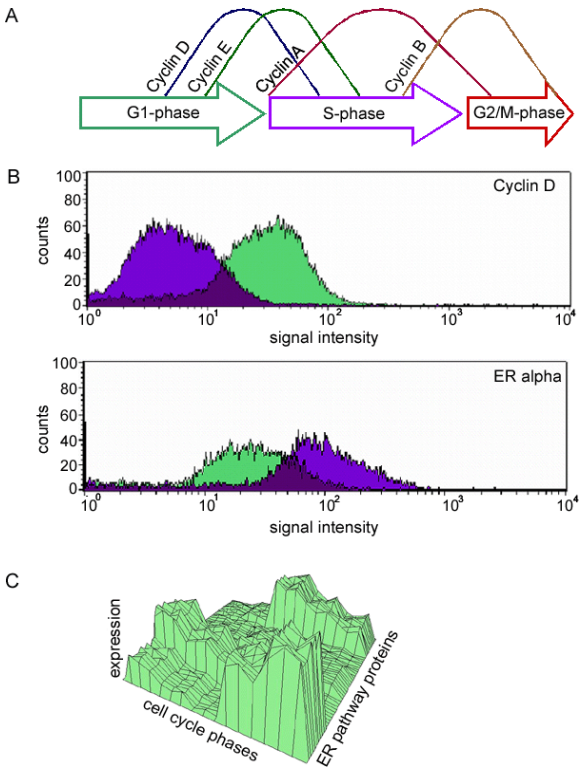

Figure 4: Cell cycle dependent changes in protein abundance. A: Scheme of Cyclin expression in different cell cycle phases. B: FACS measurement of Cyclin D and ER alpha expression in G1-phase (green) and S-phase (blue). C: Example of a cell cycle dependent protein expression profile considering ER pathway proteins.

Modeling of anti-estrogen resistance

In this study, two main questions need to be answered: 1. Where are the cross-talk events between the known pathways and the novel candidate proteins that allows to circumvent the effect of Tamoxifen, and 2. What is the dynamical behaviour of this network, allowing this effect. We have constructed a model of the known pathway components (e.g. Figure 1) that will serve as a basis for this analysis. The time-resolved quantification of abundance and phosphorylation by protein microarrays and FACS analysis will allow us to identify cell cycle dependent changes. Analysis of these data will start with statistical modeling and fitting of robust models that allow us to summarize the protein expression profiles and time-dependent effects. Through exploratory analysis using visualisation, clustering and pattern recognition approaches, we aim to place the novel components and links into the picture adding to our current understanding of these pathways. Dynamical models can then be generated and fitted to the time-course data. These will be used in simulation studies to predict effects of perturbations with siRNAs. The predictions can then be tested and will help us to establish validated models. These models will integrate the experimental data of the different experiments and will provide an insight into Tamoxifen resistance. On this basis we will hypothesize possible combinatorial therapies that impede the cells to bypass the effect of Tamoxifen.

References

1. Ali, S., and Coombes, R.C. (2002). Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer *2*, 101-112.
2. Ring, A., and Dowsett, M. (2004). Mechanisms of tamoxifen resistance. Endocr Relat Cancer *11*, 643-658.
3. Arlt, D., Huber, W., Liebel, U., Schmidt, C., Majety, M., Sauermann, M., Rosenfelder, H., Bechtel, S., Mehrle, A., Bannasch, D., Schupp, I., Seiler, M., Simpson, J.C., Hahne, F., Moosmayer, P., Ruschhaupt, M., Guilleaume,

B., Wellenreuther, R., Pepperkok, R., Sultmann, H., Poustka, A., and Wiemann, S. (2005). Functional profiling: from microarrays via cell-based assays to novel tumor relevant modulators of the cell cycle. Cancer Res *65*, 7733-7742.

4. Wiemann, S., Arlt, D., Huber, W., Wellenreuther, R., Schleeger, S., Mehrle, A., Bechtel, S., Sauermann, M., Korf, U., Pepperkok, R., Sultmann, H., and Poustka, A. (2004). From ORFeome to biology: a functional genomics pipeline. Genome Res *14*, 2136-2144.