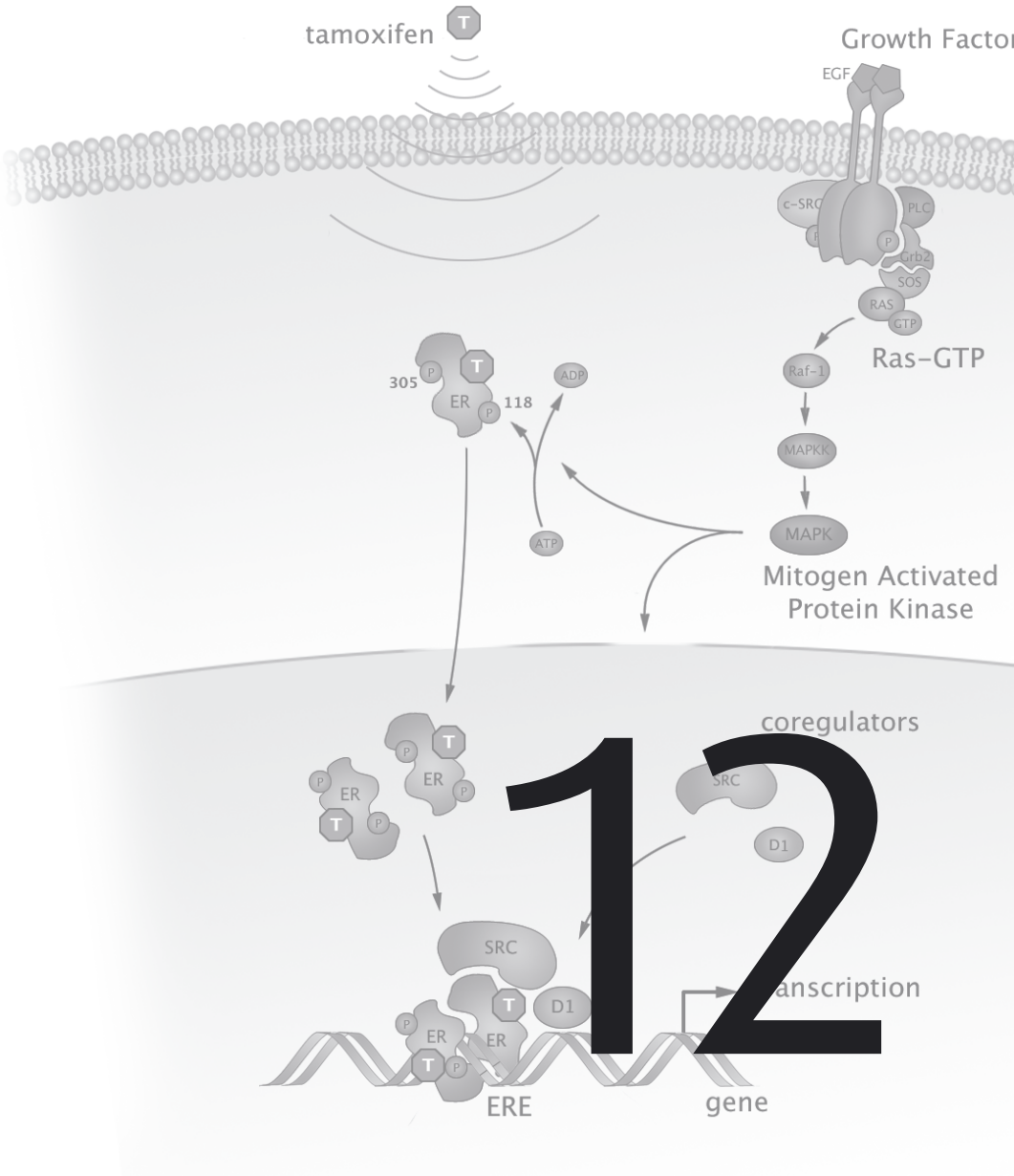
Single cell biochemistry to visualize antigen presentation and drug resistance

Griekspoor, A.C.

Summary and Discussion

Drug Resistance



Summary & Discussion Drug Resistance

The estrogen receptor is member of the nuclear hormone receptor superfamily, and plays an important role in the development and maintenance of sexual reproductive tissues (reviewed in **Chapter 8**). Not surprisingly, this receptor is essential for tumor maintenance in many tumors derived from these tissues as well. Therefore, estrogen receptor-positive breast cancer patients are frequently treated with tamoxifen, a potent and widely used anti-estrogen. Resistance to these drugs forms a major problem in the clinic. The recurrences in tamoxifen-treated, resistant patients arise even after years of treatment have passed. We therefore aim to develop an assay in which resistant cases can be identified beforehand, providing physicians with a rationale to decide which available anti-estrogen to apply.

In **Chapter 9** we describe how we developed a sensitive assay that allowed us to directly visualize conformational changes within the estrogen receptor α (ERα), and study the mechanism by which the receptor can become drug-resistant. We coupled the human estrogen receptor to two color variants of the green fluorescent protein, YFP (yellow) at its N- and CFP (cyan) at its C-terminus, and created a stable cell line with this chimeric receptor. Next, we performed Fluorescence Resonance Energy Transfer (FRET) measurements on single nuclei. Whereas no detectable FRET change could be detected after the addition of the natural ligand estradiol, a rapid increase in FRET was observed after administration of anti-estrogens, indicating that the receptor had undergone a conformational change which represented the inactive state. Using this assay we studied the effects of different factors that had previously been associated with anti-estrogen resistance.

Our experiments revealed that phosphorylation of serine-305 in the hinge region of the ERα by Protein Kinase A (PKA) induced resistance to tamoxifen. Tamoxifen still bound the receptor, but then failed to induce the inactive conformation, invoking ERα-dependent transactivation instead. In clinical samples we found that downregulation of a negative regulator of PKA, PKA-RIα, was associated with tamoxifen-resistance in breast tumors prior to treatment. Enforced downregulation of this inhibitory subunit of PKA using RNAi kept the receptor in its active conformation and impairs tamoxifen action, ultimately resulting in tamoxifen resistant cell growth. Activation of PKA thus converts tamoxifen from an estrogen receptor inhibitor into an activator, a situation highly unwanted in breast cancer

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but observed in the clinic when tumors shrink following tamoxifen withdrawal. Importantly, this form of resistance was not observed for a different anti-estrogen, Fulvestrant, that is also available in the clinic.

The development of clinical assays that can *a priori* determine resistance to tamoxifen could therefore lead to pronounced benefits for patients as they can be treated with alternative, more stringent anti-estrogens. We are currently testing the use of an antibody that specifically recognises a phosphorylated serine-305 of ERα. Positive staining of tumor material with this antibody indicate elevated levels of PKA activity, and hence tamoxifen resistance. These patients can then be treated with Fulvestrant instead.

Our FRET methodology already showed that it could effectively discriminate between two different anti-estrogens, tamoxifen and Fulvestrant. Both ERα antagonists differ in the extent of the observed FRET response after receptor binding, and in their resistance to modification of the receptor by cellular signalling pathways. X-ray crystallography studies on the Ligand Binding Domain (LBD) of the receptor had already revealed that the receptor adopted slightly different conformations upon binding with various anti-estrogens, explaining the differences in observed FRET responses. However, these studies uncovered little information on how these structural differences could be translated into patterns of anti-estrogen resistance. Therefore, in **Chapter 10** we again used FRET analysis to now characterise a larger set of different anti-estrogens, and profile their requirements for resistance.

We show that the nine anti-estrogens we tested can be divided into 7 groups based on the requirement for PKA and/or Mitogen Activated Protein Kinase (MAPK) modification of ERα to confer resistance. At the sensitive end of the spectrum we find tamoxifen, for which only a single modification is sufficient to render the receptor resistance. At the other end we find ICI-164,384, which does not even show resistance after combined activation of both PKA and MAPK pathways. The results from our FRET analysis are in line with those obtained by conventional methods for determining ER mediated transcriptional activation. Importantly, resistance as determined by FRET correlated directly with growth of ERα positive breast cancer cells treated with the different anti-estrogens. FRET analysis is a rapid and direct way to demonstrate sensitivity to anti-estrogens in ER positive cells. The resulting resistance profile from this study allows us to rank the different available anti-estrogens based on their stringency, and provides a rationale for matching patients and anti-estrogen for adequate treatment.

One striking feature of our resistance profile is the presence of multiple phosphorylation sites on ERα that can confer resistance to anti-estrogens. Numerous literature reports show that phosphorylation is by far the only modification that is associated with drug resistance. Especially a specific region near the so-called hinge domain turns out to be a hotspot for post-translational modifications that regulate activation and/or degradation of the receptor. The already mentioned phosphorylation of serine-305 of ERα that confers tamoxifen resistance is one example, but also acetylation, sumoylation, and ubiquitinylation of the same region have been reported. It remains to be determined how these modifications interrelate, and whether only one or more are required to induce anti-estrogen resistance. In this light it is also interesting to note that this region is not present in ERα's sister molecule, the close homologue estrogen receptor β (ERβ).

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The same is true for a number of other sites for post-translational modifications. Despite a high degree of similarity, ERβ shows a different tissue distribution than ERα, and plays a more growth suppressive rather than stimulating role. Its role in drug resistance is still largely unknown, and a similar FRET assay as used for ERα is currently being developed in our lab. This allows us to compare the mechanisms of inactivation by anti-estrogens and sensitivity to drug resistance between the two receptors.

A question that remains is how phosphorylation induces resistance, and can even convert an ERα antagonist like tamoxifen into an agonist. In **Chapter 11** we obtained more insights in the mechanism behind this phenomenon. After binding of its natural ligand estradiol, the estrogen receptor binds to specific sites on the DNA, called Estrogen Responsive Elements (EREs). At the same time a conformational change in the Ligand Binding Domain of the receptor recruits a number of cofactors that are essential for transcription to occur. It is this step that is affected by anti-estrogens. To understand how phosphorylation of ERα allows transcription to occur in the presence of anti-estrogens, we studied the interaction with

Steroid Receptor Cofactor 1 (SRC-1). We show via fluorescence recovery after photobleaching (FRAP), and mammalian 2-hybrid assays that phosphorylation of serine-305 by PKA does not affect the binding affinity of SRC-1 to ERα. Interestingly, our results show that even in the presence of anti-estrogens, SRC-1 can still interact with the receptor. Many of the SRC-1 recruitment assays described in literature have been performed using the Ligand Binding Domain only, and these suggest that SRC-1 is unable to bind in the presence of anti-estrogens. While this might be correct for the LBD, our data show that this is not true for binding through other regions within ERα. Still, the affinity of this LBD-independent binding of SRC-1 is unaffected by PKA stimulation, and thus cannot account for the observed resistance to tamoxifen after phosphorylation of serine-305. We therefore decided to study the interaction between SRC-1 and ERα in more detail.

Confocal FRET microscopy between ERα-CFP and SRC-1-YFP revealed an altered orientation between the two proteins after PKA stimulation and tamoxifen treatment. This change in orientation was indeed found to be dependent on serine-305 phosphorylation. In addition, immuno-fluorescence studies showed successful recruitment of RNA polymerase II in the presence of tamoxifen, which strongly suggests that this altered orientation is transcriptionally active. We propose a model, in which tamoxifen resistance is not explained by altered protein-affinities, but rather by a change in the orientation of the SRC-1/ERα complex after serine 305 phosphorylation. This model provides direct mechanistical insights in the details of tamoxifen resistance.

A number of questions still remain. For instance, does our model also apply to other cofactors like SRC-2 and 3, and what is their relative contribution? Neither do we have explained why the amount of gene transcription under tamoxifen conditions is only a fraction of that induced by the same resistant ERα treated with estradiol. Despite the fact that polymerase II is recruited in both cases, there still must be significant differences in the mechanism by which both induce actual transcription, something that needs further investigation.

It is clear however, that in a situation where protein-protein orientation rather than interaction becomes crucial, advanced microscopic techniques provide the only way to understanding the complex cascade of events leading to anti-estrogen resistance.