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Point of VIEW

Sensing Estrogen's Many Pathways

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ABSTRACT Estrogen receptor (ER) is an important drug target, but it has multiple signaling pathways that are difficult to dissect. A new study reports the development of a multicolor bioluminescent probe that can measure a compound's ability to modulate ER-mediated transcription and to promote an interaction between ER and Src, a key protein in a number of different cell signaling cascades. The discovery provides a new tool for quickly obtaining a more complete picture of the potential effects of a compound on estrogen signaling and could lead to more selective ER modulators with fewer side effects.

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Estrogens are molecules that are familiar to almost everyone because of their importance in female development and maintenance of women's health. Estrogen receptor (ER), the protein primarily responsible for mediating estrogen's effects, has long been a validated drug target for a number of therapeutic applications, including the relief of menopausal symptoms and the treatment and prevention of breast cancer (*1, 2*). Multiple ER-targeted drugs are available for treating breast cancer, but all have limitations due to the undesirable effects in non-target tissues. Tamoxifen, which antagonizes the proliferative effects of estrogen in breast, acts as an estrogen mimic in the uterus, stimulating proliferation and increasing the patient's risk of endometrial cancer. Most of the current models for tissue-dependent variability in the response to ER-targeted drugs focus on the multiple signaling pathways that ER can regulate (*3*). Different pathways could be playing key roles in different tissues and result in different types of responses. The challenge has been to develop rapid screening tools that would allow multiple pathways to be tested simultaneously. On page 359 of this issue, Tao and co-workers (*4*) describe a clever feat of protein engineering that results in an ER-derived construct that allows for the simultaneous determination of a compound's ability to modulate two distinct ER signaling pathways. This construct could be highly useful in developing more selective ER modulators that could then lead to a better understanding of estrogen signaling and to better drugs for women's health.

In the traditional model of estrogen action, ER acts as a ligand-regulated transcription factor. In this model, ligand binding causes a conformational change in the receptor that allows the receptor to bind to specific DNA sequences in the promoter regions of specific genes (Figure 1). Other proteins then bind to the ER and remodel chromatin to activate or repress transcription of the specific gene, depending on the promoter and the nature of the ligand bound to the ER. In this model, estrogens generally act as transcriptional activators, and compounds like tamoxifen generally act as antagonists of this activation. This mechanism has been well-studied both in terms of the structural requirements for agonist and antagonist activity as well as the nature of the accessory proteins responsible for translating ligand binding into transcriptional regulation. One key feature shared by proteins that act as coactivators of ER-mediated transcription is that they contain an ER-binding motif with the amino acid sequence LXXLL (X can be any amino acid) (*5*). One component of the probes the authors have developed has a sensor for classic ER activation by detecting ER binding to an LXXLL sequence.

It has become increasingly clear over the past few years that the classical model of ER action is only one of the pathways by which ER can regulate cellular processes. Cell signaling events such as changes in intracellular calcium and cAMP concentrations and activation of kinase signaling pathways can occur rapidly after exposure to ER ligands (*6*). The agonist/antagonist profiles of different ER ligands in this type of signaling do

not necessarily match the profiles of these compounds in classical ER signaling and could explain the mixed agonist/antagonist profiles seen in different tissues. Determining the molecular mechanisms underlying these cross-talk responses is a very active area of research, and there are many reports that ER itself is involved in cross-talk signaling with other signal transduction pathways. The mechanisms for this cross-talk signaling are still being unraveled, but one key signaling molecule that appears to interact with ER is the tyrosine kinase Src (*7*). Src has been shown to be activated after its Src homology 2 (SH2) domain associates with a specific phosphorylated tyrosine in the ligand binding domain of ER (Figure 1). The downstream consequences of ligand-induced ER activation of Src are numerous, complex, and not entirely understood, but activation can have either proliferative or antiproliferative effects through regulation of central signal transduction pathways such as the MAP kinase pathway and the PI3K/Akt pathways (*8–10*). The effects seem to depend on the type of tissue, a variability also seen with estrogen modulators.

A significant challenge in trying to incorporate the type of cross-talk signaling represented by the ER-Src relationship into the discovery of new ER modulators has been the lack of a straightforward screening approach. Numerous assays have been established for screening compounds for their effects on classic ER signaling, mostly focused on measuring the effect of a compound on the ability of ER to associate with a peptide containing an LXXLL nuclear receptor-binding motif (*11–13*). The interaction between the SH2 domains of Src with ER is not strong enough for a similar type of screen, but the authors developed a different kind of screen based on protein complementation. On the basis of substantial previous work using protein complementation to measure protein−protein interactions, the authors developed a fusion protein containing two halves of a click beetle luciferase split by the SH2 domain of Src fused to the ligand binding domain of ER through a flexible linker. If a ligand causes an association of the SH2 domain and the ER ligand binding domain, the two halves of the luciferase will be brought together and will generate red luminescence when the luciferin substrate is added. This probe was tested against a series of compounds, and it was determined that 4-hydroxytamoxifen and other antagonists of classic ER signaling could cause the association to occur. The authors then made another probe with a different luciferase that generated green luminescence instead of red. This luciferase was split by the ER ligand binding domain fused through a linker to a peptide containing an LXXLL sequence. The two halves of the luciferase could be brought together by agonists of classic ER signaling such as estradiol that induced the association between ER and the LXXLL motif.

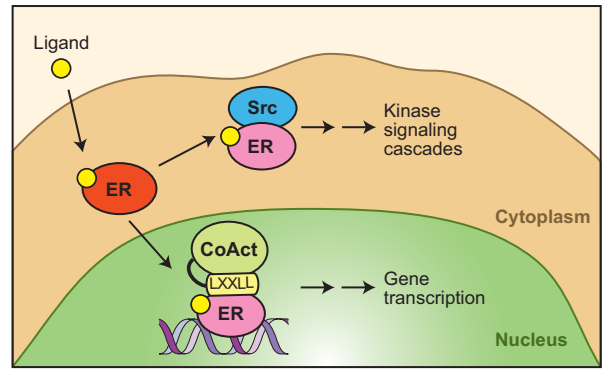

Figure 1. In this simplified diagram, ER binds to ligand and then activates the transcription of specific genes through binding to coactivator proteins (CoAct) containing the amino acid sequence LXXLL. Ligand-bound ER can also bind and activate Src in the cytoplasm to activate a number of kinase signaling cascades. In this issue, Tao and co-workers (*4*) report a fusion protein sensor that can detect whether a compound can activate either or both of these pathways.

The authors then took these two different probes—one that measured classic ER signaling and one that measured a component of cross-talk signaling—and integrated them into one probe. This probe was designed to have both N-terminal halves of the green- and red-emitting luciferases compete for a single C-terminal half-common to both versions of the enzyme. The Src SH2 domain, the LXXLL-containing peptide, and the ER ligand binding domain separated the luciferase halves in such a way that the red luciferase was formed if the compound promoted ER−Src interactions and the green luciferase was formed if the ER−LXXLL interaction was promoted. The authors then tested this dual-colored single-molecule probe with various compounds and found that it could detect induction of either association simultaneously, even for compounds that induced both ER−LXXLL and ER−Src interactions equally. The reporter was robust enough to allow for its immobilization on nitrocellulose strips at subfemtomole amounts while still retaining its ligand sensitivity.

This construct allows for the intriguing possibility of screening compounds for their ability to modulate both classic and cross-talk ER signaling quickly and in a high-throughput manner, but a few unanswered questions must be resolved before this approach can be validated. One of the interesting features of this probe is that 4-hydroxytamoxifen stimulated the ER−Src interaction much more strongly than estradiol did, even though estradiol can promote Src activation by ER in cell-based studies (*8*). The authors acknowledge this and point out that while 4-hydroxytamoxifen and estradiol both could stimulate ER−Src interactions equally as well, the conformation of the ER bound to 4-hydroxytamoxifen is better at promoting the interaction in their

particular probe. It would be interesting to see if this construct behaves similarly in other cell lines or if the relative estradiol/tamoxifen sensitivity might be different. The other key question about the probe is the possible role of other proteins in facilitating the ER−Src interaction in cells. One particular protein, known as MNAR, has been shown to be necessary for Src activation by ER and is thought to act as a tethering protein by binding to both ER and Src and then allowing them to interact (*14*). Whether the tethering caused by MNAR complexation is similar in nature to the tethering caused by being part of a fusion protein is an interesting question raised by this novel and possibly very useful tool for studying estrogen signaling.

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