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Chapter 6

Identification of cells with an irregular expression status in $ER\alpha$ breast cancer by single molecule RNA FISH

In collaboration with: Pernette J. Verschure, Luca Magnani
Adapted from: published as part of Magnani et al., Nature Genetics, 2017

6.1 Abstract

About 70% of diagnosed human breast cancers are estrogen receptor (ER) positive. In these tumors, growth is dependent on and stimulated by estrogen. ER-positive breast cancer treatment strategies involve selective ER modulators and downregulators (SERMs and SERDS) in premenopausal women and aromatase inhibitors (AIs) in postmenopausal women. Both treatments use different strategies to prevent endocrine signaling and cell proliferation. However, 30-40% of the endocrine therapy treated patients relapse during or after treatment and progress to an incurable metastatic disease. Gene amplification of the aromatase gene CYP19A1 has been observed in breast cancer tissue biopsies of patients that relapse from AI treatment and developed a metastatic tumor. CYP19A1 gene amplification is also noted in MCF7 breast cancer cells cultured under long-term estrogen deprived (LTED) conditions mimicking acquired resistance to AI treatment. Here we used single molecule mRNA FISH to sample single cell heterogeneity of CYP19A1 transcripts in MCF7 and LTED cells. Our findings show increased average expression level of CYP19A1 in LTED cells. A few highly CYP19A1 expressing LTED cells contribute significantly to this observed difference.

6.2 Introduction

About 70% of diagnosed human breast cancers are clinically subtyped to be estrogen receptor alpha ($ER\alpha$) positive [190]. Most of the $ER\alpha$ -positive breast cancers depend on estrogen for growth. Estrogens bind to $ER\alpha$ in order to activate transcription of ER target genes thereby stimulating cell proliferation (Figure 6.1). Targeting ER functioning represents one of the most effective approaches to treat $ER\alpha$ -positive breast cancer. Selective estrogen receptor modulators and downregulators (SERMs and SERDs) and aromatase inhibitors (AIs) in pre- and postmenopausal women, respectively are the treatment of choice [191, 192]. Tamoxifen is a SERM that competes with estrogen for $ER\alpha$ binding sites thereby preventing $ER\alpha$ -dependent gene expression. AIs disrupt estrogen signaling by blocking the conversion of testosterone to estradiol, thereby significantly reducing local estrogen biosynthesis and preventing activation of $ER\alpha$. Effectively, these treatments reduce activated $ER\alpha$ levels and constrain cell proliferation. The mechanisms by which $ER\alpha$ triggers cell proliferation are complex due to the multitude of gene targets stimulated by $ER\alpha$.

Approximately 30% of $ER\alpha$ -positive patients treated with endocrine therapy develop resistance to treatment showing relapse or cancer recurrence during or after treatment thereby inducing cancer progression leading to an incurable metastatic disease. It has been suggested that the trigger for treatment resistance is initiated by a single or a few cells circumventing the treatment effects [193]. The exact mechanisms causing treatment resistance are not fully understood; supposedly they involve transcriptional activation of counteracting regulatory pathways via altered

epigenetic regulation [194]. Robinson et al. pointed towards somatic mutations involved in ER α -activation as a mechanism involved in metastatic progression [195]. Since these mutations only occur in metastatic cancers, such ER α -activation-related mutations might be induced by endocrine therapy, which applies selective pressure [196]. Altered expression of ESR and/or CYP19A1 induces a change in ER α and aromatase levels respectively, which is expected to induce resistance and thus stimulate metastatic progression (Figure 6.1). Upon treating patients with AI, gene copy number variation (CNV) changes in the CYP19A1 locus are observed in breast cancer tissue from patients eventually relapsing from AIs treatments [193]. Moreover, such CNVs are noted in breast cancer cells that have been long term deprived from estrogen, i.e. LTED cells. These CNV changes are shown to induce increased expression levels of CYP19A1 mRNA and protein [193]. Within LTED cells, the expression of ER α target genes such as TFF1 and EGR3 was shown to be increased[193]. Taken together, these data suggest that CYP19A1 amplification underlies AI resistance development and that endocrine treatment of ER α positive breast cancer cells can drive genomic alterations within these cells. In the present study, we quantitatively determined CYP19A1 transcript levels with single molecule RNA FISH (sm-FISH) in MCF7 and LTED cells. Our setup enables the identification of individual cells or cell subpopulations that counteract the treatment effects by upregulating specific target genes.

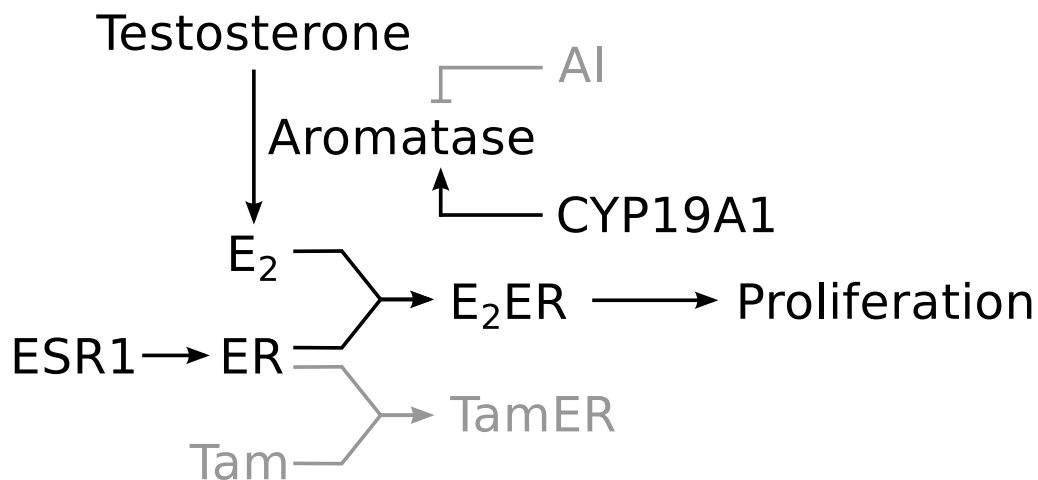


FIGURE 6.1: Schematic diagram describing ER α -stimulated proliferation in ER-positive breast cancers. Pre and postmenopausal endocrine therapy consists of ways to manipulate (estrogen) signaling. This can be achieved by lowering either the amount of estradiol (postmenopausal endocrine therapy) or the amount of ER (premenopausal endocrine therapy). Aromatase inhibitor treatment prevents the conversion of testosterone to estradiol, thereby lowering the amount of estrogen bound ER α (E_2ER) at ER target genes. Alternatively, Tamoxifen (SERM) or Fulvestrant (SERDs) competitively bind or down regulate ER thereby decreasing the amount of ER α available for estrogen (E2) binding and the amount of ER α gene (ERS), respectively. Aromatase inhibitor or SERM/SERD resistance development might be induced by an increase in CYP19A1 or ERS expression levels.

6.3 Results and discussion

To determine CYP19A1 transcript levels in LTED and MCF7 cells at the single cell level, we quantified the amount of CYP19A1 mRNA using smFISH (Figure 6.2). We observed low levels of CYP19A1 expression in both LTED cells ($n = 251$) and MCF7 non-deprived breast cancer cells ($n = 273$). In MCF7 cells no CYP19A1 transcripts were detected in around 80% of the cells, whereas in 40% of the LTED cells transcripts were lacking. On average, CYP19A1 transcript levels in MCF7 cells are significantly different from transcript levels in LTED cells ($p < 0.001$). The average amount of CYP19A1 mRNA molecules per cell that we measured was 0.24 and 1.33 in MCF7 and LTED cells, respectively (Figure 6.3). Strikingly, within the LTED cell population we observe a subpopulation with exceptionally high CYP19A1 expression showing mRNA counts of 11, 12 and 20 mRNAs per cell (Figures 6.2 and 6.3). Although these cell contribute to the significance of the result, the result remain when these cells are not considered.

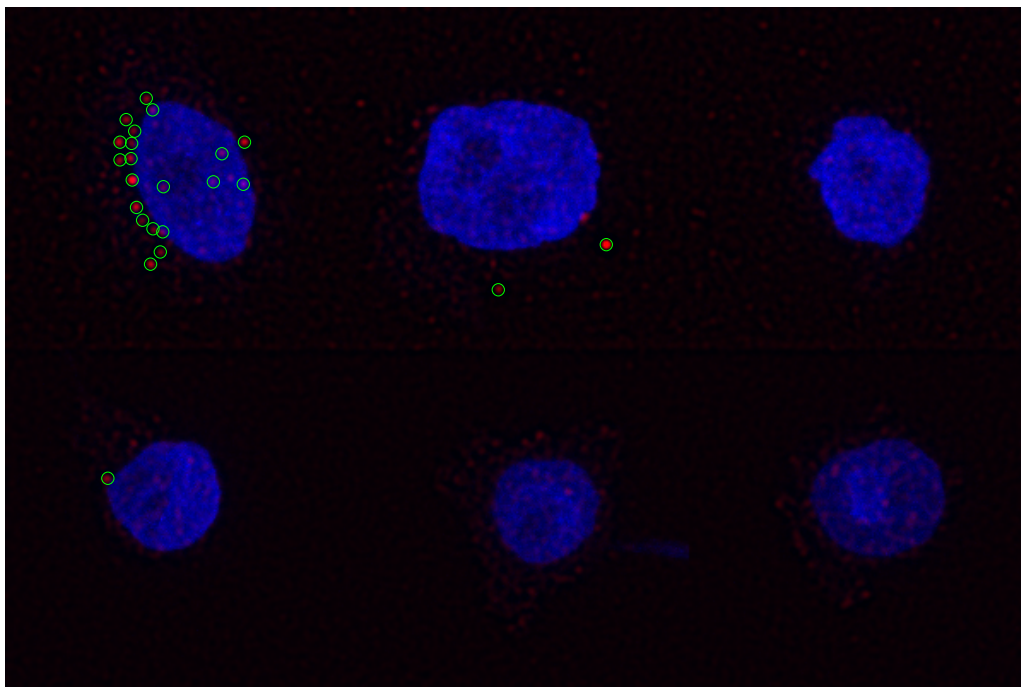


FIGURE 6.2: **Characteristic images of mRNA FISH in LTED and MCF7 cells.** The smRNA FISH signals in the LTED (top row) and MCF7(bottom row) are shown in red, and the mRNA spots, as identified by the algorithm, are marked with a green circle. The cell nucleus is stained with DAPI and shown in blue. The total volume of LTED cells in on average approximately 10% bigger than the volume of MCF7 cells.

The gene copy number of the CYP19A1 gene is increased in patients that relapse from AI treatment [193]. CYP19A1 gene duplications reported in patient-samples are also observed in LTED cells [193]. In LTED cells, this gene duplication leads to increased transcription of CYP19A1 and increased expression of ER α target genes. The data suggest that upon selective

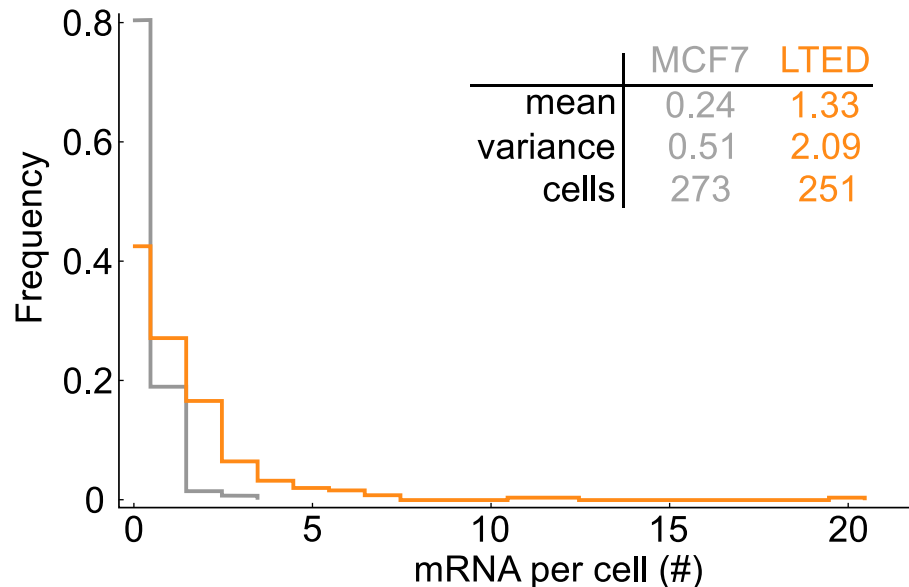


FIGURE 6.3: **Distribution of the number of CYP19A1 mRNA molecules in MCF7 ($n = 273$) and LTED ($n = 251$) cells.** The smRNA FISH distributions show that CYP19A1 mRNA is more abundant in LTED cells compared to MCF7 cells. Some LTED cells express up to 20 CYP19A1 mRNA molecules. LTED cells express 5.6 times more CYP19A1 mRNA than MCF7 cells (1.33 vs 0.24). The mean, variance and number of cells measured for both conditions are given in the inset.

pressure (cancer) cells adapt their phenotype by mobilizing alternative routes to restore cell proliferation and thereby circumventing treatment effects [193].

Our single cell measurements reveal that CYP19A1 expression is almost absent in MCF7 cells. In contrast, the CYP19A1 mRNA expression in LTED cells is increased more than five-fold compared with the MCF7 parental cells. On average, 1.33 mRNA molecules per cell are detected in the LTED cells. These smRNA FISH transcript measurements qualitatively correspond to the transcription measurements observed with RT-qPCR [193]. Interestingly, our single cell approach indicates that a subset of cells contributes significantly to the observed increase in average transcript levels. Since we were unable to perform DNA and mRNA FISH simultaneously, we cannot link this subset directly to (specific levels of) gene amplification in LTED cells. However, our results do provided evidence of increased mRNA expression due to amplification of the CYP19A1 locus in LTED cells.

6.4 Materials and methods

6.4.1 Cell cultures

MCF7 breast cancer cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Estrogen-deprived MCF7-derived LTED breast cancer

cell lines were maintained in phenol-red-free DMEM containing 10% charcoal-stripped fetal calf serum(SFCS). Both media were supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 0.1 mg/mL β -Estradiol (Sigma) was added routinely to MCF7 cell cultures.

6.4.2 smRNA FISH

We used the protocol for adherent mammalian cell lines for custom Stellaris FISH probes. Hybridization was performed overnight and no anti-fade was used for imaging. The sequence of the CAL Fluor Red 590-tagged probes targeting the CYP19A1 mRNA can be found in Table X. We used a Nikon Ti-E scanning laser confocal inverted microscope A1 to generate fluorescent images. Excitation at 561 nm and a 595-50 nm filter was used for detection of the CYP19A1 mRNA. An automated spot count algorithm determined the number of mRNA. To obtain a more reliable spot count from our detection algorithm, we included 30 images with in total 4402 mRNA signals. Differences in the transcript numbers between MCF7 cells and LTED cells were analyzed using a Mann-Whitney U test in Mathematica 10.2 (Wolfram Research, Inc.).