Progesterone Signaling to Chromatin in Breast Cancer Cells. Two Initial Cycles of Remodeling

Guillermo P. Vicent, Roser Zaurin, Cecilia Ballaré, A. Silvina Nacht, Roni H. G. Wright, Francois Le Dily, Giancarlo Castellano, Jofre Font-Mateu and Miguel Beato

Abstract Steroid hormones control gene activity by direct interaction of their intracellular receptors with hormone responsive elements on DNA but they can also crosstalk to kinase cascades activated by signals impinging on membrane receptors. Progesterone treatment of cells in culture leads to the rapid activation of several kinases and in particular the Src/Ras/Erk/Msk1 cascade, by activating a small population of membrane-anchored progesterone receptors (PR). One to five minutes after hormone treatment, activated Erk enters the nucleus and causes the

G. P. Vicent · R. Zaurin · C. Ballaré · A. Silvina Nacht · R. H. G. Wright · F. Le Dily · G. Castellano · J. Font-Mateu · M. Beato (✉)
Chromatin and Gene Expression Lab, Gene Regulation Programme, Centre for Genomic Regulation (CRG) and UPF, Barcelona, Spain
e-mail: miguel.beato@crg.eu

G. P. Vicent
e-mail: guillermo.vicent@crg.eu

R. Zaurin
e-mail: roser.zaurin@crg.eu

C. Ballaré
e-mail: cecilia.ballare@crg.eu

A. Silvina Nacht
e-mail: silvina.nacht@crg.eu

R. H. G. Wright
e-mail: roni.wright@crg.eu

F. Le Dily
e-mail: francois.ledily@crg.eu

G. Castellano
e-mail: giancarlo.castellano@crg.eu

J. Font-Mateu
e-mail: jofre.font@crg.eu

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recruitment of the activated ternary complex of pPR, pERK and pMSK1 to target chromatin, leading to phosphoacetylation of histone H3 and displacement of an HP1$\tilde{\gamma}$ containing repressive complex. Thus, progestin activation of the Src/Ras/Erk/Msk1 cascade directly impacts chromatin. Within one minute of adding synthetic progesterone analogues to breast cancer cells, PR recruits to the target genes an ATP-dependent chromatin remodeling complex, NURF, a histone methyltransferase complex, ASCOM, which trimethylates histone H3 at lysine 4, and an activated Cyclin A/CDK2 complex, that phosphorylates histone H1 and facilitates its displacement. This first cycle of chromatin remodeling is a prerequisite for a second cycle starting 5 min after hormone addition, in which a different ATP-dependent chromatin remodeling complex, BAF, and a histone acetyltransferase, PCAF, cooperate to promote the displacement of core histones H2A and H2B, that facilitates access to the promoter of additional receptor complexes and other transcription factors necessary for gene induction. Thus, at both phases in activation of target promoters, a histone tail modification stabilizes the binding of an ATP-dependent chromatin remodeler to target promoters. These findings highlight the concept of transcription initiation as a process involving consecutive cycles of enzymatic chromatin remodeling, where each enzyme complex is necessary at a given time point and catalyzes a particular remodeling step.

Keywords Progesterone · Chromatin · MMTV · BRCA1 · Nucleosome · Progesterone responsive elements · Gene regulation

Contents

1 Introduction

The physiological action of steroid hormones in their target cells is mediated by intracellular receptors, which are members of the nuclear receptor family. The steroid hormone receptors (SHR) were originally considered as ligand-regulated transcription factors that upon binding to specific hormone regulatory element (HREs) regulate the transcription rate of their target genes. In the last years, this

simplistic view has been abandoned and replaced by a more complex vision, involving at least two subpopulations of SHR: a minor one anchored in the cell membrane via a palmitoyl residue [1], and a major one shuffling between the cytoplasm and the cell nucleus. The majority of the available data supporting this complex view have been obtained using ligands synthetic analogues of the physiological hormones, which are not efficiently metabolized in the target tissues and exhibit higher affinity for SHR than the natural ligands. The situation may be even more complex when considering the physiological hormones, which are heavily metabolized and give rise to products with the potential to interact with a larger number of receptors.

In breast cancer cells the membrane attached SHR are part of an ill-defined complex that includes estrogen receptor alpha (ERα), progesterone receptor (PR), and possibly androgen receptor (AR), as well as members of the growth factor receptor family, such as the EGF receptor (EGFR), and likely caveolin. When activated by synthetic ligands the membrane attached receptors interact with c-SRC via de SH2 domain, in the case of ERα or via de SH3 domain in the case of PR and AR, and activate an interconnected network of kinase signaling pathways to coordinate the cell response at various levels. There are also interactions of SHRs with CDK2, PI3K/AKT, JAK/STAT, and likely other kinases. Several of these kinase pathways converge in the cell nucleus, where they act on the nuclear population of SHRs, on protein components of chromatin and on nuclear enzymes, which all together orchestrate the regulation of various gene networks by mechanisms that are not fully understood.

In the following we will describe our present knowledge of the signaling network used by the synthetic progesterone analogue R5020 in the breast cancer cell line T47D. We will place these results in the context of the genome-wide interactions of PR with nuclear genome and their consequences for gene expression. While we will mainly summarize our own experimental findings with a model promoter, the mouse mammary tumor virus (MMTV), and at the whole genome level, we will also report findings by other labs to complete the global vision and to indicate debated points.

2 Nucleosome Organization is Required for Efficient PR Binding and Gene Regulation

Elucidating the global function of a transcription factor in a particular cell type implies the identification of its binding sites in the genome of the cell. The possible role of chromatin structure in defining effective binding sites is still an unresolved question. The dominant view assumes that nucleosomes are an obstacle for transcription factor binding and that factors bind preferentially to nucleosome depleted regions, but there are reports on binding of transcription factors to nucleosomally organized sequences. Apart from the MMTV promoter that we will describe in

more detail below, a good example is p53. Using the 5′-upsream region of the p21 gene that contains two p53 binding sites, a better binding has been reported in vitro to chromatin organized sequences when compared to free DNA [2]. A genome wide study with MCF7 breast cancer cells has yielded similar results [3], suggesting that the particular topology of the nucleosomally organized p53 target sites favors DNA binding and regulation of transcription. Upon activation of p53 by DNA damage, the region around p53 binding sites becomes depleted of nucleosome reads. However, these studies detect nucleosomal occupancy of a large chromatin region, over 2 kb, and probably reflect a transition from heterochromatin to euchromatin, rather than localized changes of individual nucleosomes.

The hormone responsive region of the MMTV promoter contains a cluster of five imperfect HREs upstream of a NF1 binding site [4]. In nucleosomes assembled in vitro as well as when integrated as a single copy transgene in breast cancer cells, the HREs are precisely positioned on the surface of a histone octamer particle with a rotational orientation that exposes the major grooves of the two halves of the HRE1 palindrome and of the HRE4 half palindrome, while masking the major grooves of HRE2 and HRE3 [5]. Purified PR binds to nucleosomally organized HRE1 with similar affinity as to the same sequence in naked DNA, while it cannot access the central HREs2 and 3 in nucleosomes [5]. Access to these central HREs requires ATP-dependent remodeling of the nucleosome core particle [6], which leads to displacement of histone H2A/H2B dimers [7]. On the surface of the resulting histones H3/H4 tetramer particle we observed a synergistic binding of PR and NF1 [8]. We also found that the linker histone H1 contributes to the basal repression of the MMTV promoter but is necessary for optimal induction by promoting a nucleosome position that facilitates the synergism between PR and NF1 [9, 10]. Thus, it seems that the organization of the MMTV promoter in chromatin plays an essential role in regulating the promoter activity in response to progesterone. The question was whether this is a peculiarity of the proviral promoter or a general property of PR target genes.

We have approached this issue in breast cancer cells treated with progestins by performing ChIP-seq experiments with antibodies against PR and RNA polymerase II. Under the same experimental conditions we have performed global gene expression analysis and nucleosome mapping experiments (Ballaré et al. unpublished). Our data indicate that before hormone addition, there are only 844 PR binding sites (PRbs), while upon hormone treatment over 25,000 PRbs are found, most of them already detected 5 min after hormone addition and persisting for up to 6 h. Although PRbs are mainly found within 100 kb of hormone responsive genes and in introns, there is a significative enrichment within 1 kb distance from the transcription start sites. Only a small percentage of these PRbs contain the classic palindromic progesterone responsive element (PRE), while around 80% of the PRbs encompass several copies of a half PRE, often as direct repeats at variable distances. In contrast with the dominant view, the sites where PR will bind exhibit high nucleosome occupancy prior to hormone treatment, suggesting that the organization in nucleosomes favors PR binding. The nucleosomes encompassing the PRbs become sensitive to MNase digestion

upon addition of hormone correlating with the recruitment of NURF and BAF complexes. In most cases, the enhanced nuclease sensitivity is accompanied by a hormone-dependent depletion of histones H1 and H2A. PR binding to nucleosomally organized sequences and hormone induced nucleosome remodeling are more pronounced around the transcription start sites of upregulated genes, correlating with the strongest PRbs. Predicted PREs that do not bind PR do not show an enrichment of nucleosomes, supporting the notion that nucleosome occupancy is important for PR binding and hormonal gene regulation in living cells and confirming our findings with the MMTV promoter.

3 Preparing the Chromatin for Gene Regulation

A central requisite for the genomic action of hormones is their ability to facilitate access to the genetic information stored in the compacted DNA in chromatin, a process that requires extensive chromatin remodeling. Here we summarize our present knowledge of this process based on the action of synthetic progesterone analogue R5020 in the breast cancer cell line T47D.

The large majority of the PR molecules in T47D cells are shuttling between the cytoplasm and the cell nucleus, while a small fraction is attached to the cell membrane in a complex with estrogen receptor (ER) [11]. Upon hormone addition, the membrane-attached PR activates c-SRC and downstream RAS/RAF/MEK/ERK/MSK1 pathway, partly via ER, leading to ERK-mediated phosphorylation of PR and formation of complexes of pPR with the activated kinases ERK and MSK1. This activated ternary complex is targeted by PR to PRbs in chromatin [12]. Progestins also activate the PI3K/AKT pathway, the Cyclin A/CDK2 pathway, the JAK/STAT pathway, and possibly several other kinase pathways. Many of these kinases are also targeted to PR binding sites in chromatin, but except for CDK2 we do not know their targets and their function in hormonal gene regulation.

Prior to hormone induction the sites where PR will bind are organized in nucleosomes with progesterone responsive elements (PRE) partly exposed in the surface. We have shown that hormone receptors can bind to a PRE within nucleosomes provided the major groove of the palindromic sequences TGTYCt is not oriented towards the histone octamer but facing outwards [5]. This is a property that hormone receptors share with other factors that recognize relatively short sequences of DNA. On the contrary transcription factors, like NF1, that establish contacts with 10 or more DNA base pairs and completely embrace the DNA double helix cannot recognize their sites when they are wrapped around nucleosomes, no matter their rotational orientation [13].

When the activated ternary complex of pPR, pERK and pMSK1 reaches the MMTV promoter in chromatin, it binds to the exposed HRE1, leading to the MSK1-dependent phosphorylation of N-terminal tail of histone H3 at serine 10 [12]. This phosphorylation leads to the displacement of a repressive complex that is anchored at the trimethylated lysine 9 of the H3 tail via the chromodomain of

HP1γ [12]. We do not know yet the exact composition of this complex but it includes HDACs and histone demethylases. We also do not know whether acetylation of lysine 14 of the histone H3, which is observed after hormone treatment, is also required for displacement of the repressive complex.

We have identified two consecutive cycles of chromatin remodeling during the initial 5–10 min of hormone action, during which protein kinases, histone modifying enzymes and ATP-dependent chromatin remodeling complexes cooperate to facilitate access of transcription factors to previously hidden information. All these factors are recruited to the PR target sites by direct or indirect interaction with the hormone-activated PR. Although the precise order of events in individual target sites is no known, we can establish a sequence for target sites at the level of whole cell population based on the global effect of inhibiting individual steps. What follows has to be interpreted with some caution, as it may be the average result of more stochastic processes taking place at individual target sites in individual cells.

The first cycle takes place within 1–2 min after hormone addition and involves PR-mediated recruitment of the ATP-dependent chromatin remodeling complex NURF, the histone methylase complex ASCOM, CyclinA/CDK2 and the poly (ADP-ribose) polymerase PARP1, along with the displacement of the histone demethylases KDM5B, also known as PLU1 or JARID1B. The outcome of the coordinated action of these five enzymes is an increase in the trimethylation of lysine 4 of histone H3, which stabilizes the binding of NURF and the phosphorylation and likely the parylation of histone H1, which is displaced from the target site ([14]; Wright et al., unpublished).

The second cycle, that takes place in the subsequent 5–10 min, involves the PR-mediate recruitment of complexes with the histone acetyl transferase PCAF and the ATP-dependent chromatin remodeling complex BAF [12]. PCAF acetylates K14 of histone H3, a modification that anchors the BAF complex. The outcome of this second cycle is the ATP-dependent displacement of H2A/H2B dimers. The two cycles are connected since blocking the first cycle precludes the second. After these two cycles, previously hidden binding sites for NF1 and PR become accessible and the two factors bind synergistically to the MMTV promoter on the surface of tetramer of histone H3 and H4 [8].

A summary of the PR-interactors highlighted in the present chapter is shown in Fig. 1. Most of the partners of PR are functionally connected with chromatin either because they remodeled/modified directly the target chromatin or because they stabilized the binding of the remodeler, as described above for the MMTV promoter.

Using expression arrays and ChIPseq we found that a similar mechanism operates in a large number of cellular progesterone target genes ([14]; Ballaré et al. unpublished), although activation of other kinase pathways, such as JAK/STAT and PI3K/AKT, as well as parylation play important roles in progesterone action of different sets of genes.

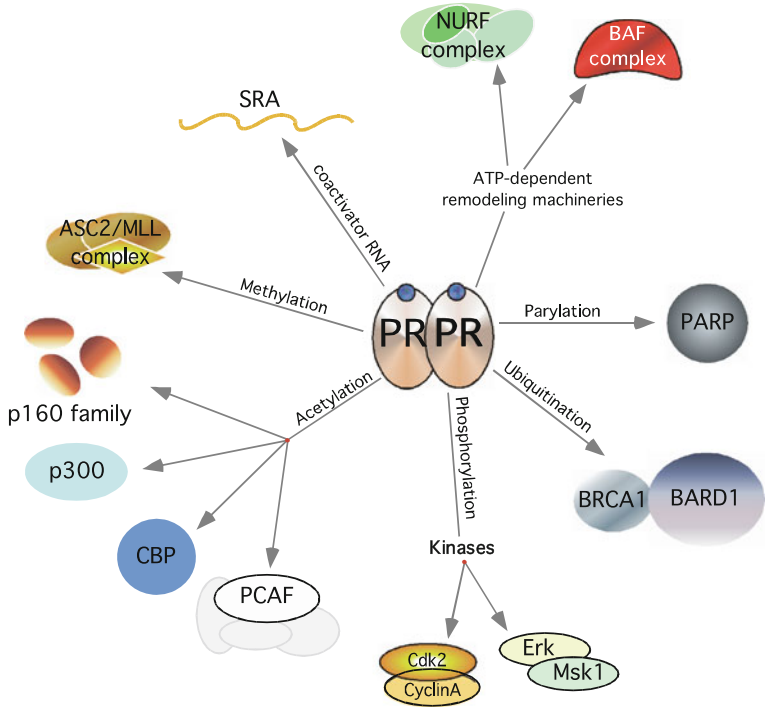
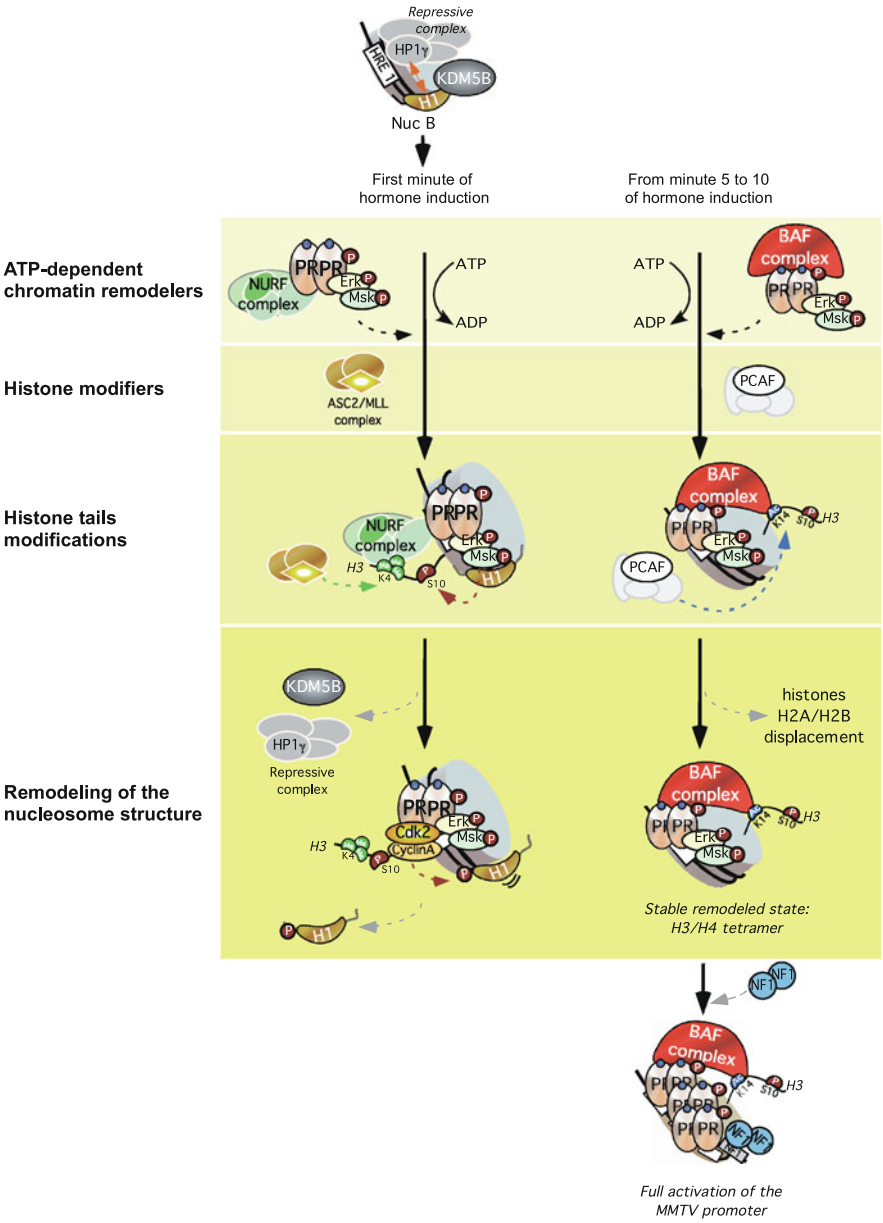


Fig. 1 PR interactors. The indicated interactors illustrate the diversity of structural and enzymatic factors that PR recruits to target promoters. The interactors are involved in different nuclear events: (1) Opening of the local chromatin structure through ATP-dependent remodeling or histone acetyltransferase (HAT) activity (BAF, NURF, p160, CBP/P300, PCAF); (2) Stabilization of chromatin remodelers by histone tail modifications (ASCOM, PCAF); (3) Downstream effectors of signaling pathways whose kinase activity impact either on chromatin or on PR itself (Erk, Msk1, Cdk2). The SRA, or steroid receptor RNA activator, serves as a specific coactivator of steroid receptors and, as such, brings a new level of complexity to nuclear receptor-mediated transcription [21]

4 BRCA1 as a Physiological Brake for Hormone Action

Germ-line mutations in the BRCA1 gene strongly increase the risk of developing breast cancer in women. One popular hypothesis to explain this tissue specificity postulates a link between BRCA1 and the action of ovarian hormones, estrogen and progesterone. Indeed it has been shown that BRCA1 counteracts the effect of estrogens and possibly progesterone in breast cancer cells [15–17]. Given the relevance of progesterone for normal mammary development and breast cancer formation, we searched for a functional relationship between BRCA1 and PR in the PR-positive breast cancer cell line T47D. We found that BRCA1 inhibits the transcriptional activity of PR by at least two mechanisms involving its E3


Repressive complex
HP1γ
HRE 1
KDM5B
H1
Nuc B
First minute of hormone induction
From minute 5 to 10 of hormone induction
ATP-dependent chromatin remodelers
Histone modifiers
Histone tails modifications
Remodeling of the nucleosome structure
NURF complex
PRPR
Erk
Msk
ATP
ADP
BAF complex
PCAF
ASC2/MLL complex
H3
K4
S10
KDM5B
HP1γ
Repressive complex
Cdk2
CyclinA
histones H2A/H2B displacement
Stable remodeled state: H3/H4 tetramer
NF1
Full activation of the MMTV promoter

◀ **Fig. 2** Initial steps in PR activation of the MMTV promoter. One minute after hormone addition the activated complexes of pPR, pErk, pMsk1 with either the NURF complex or the methyltransferase ASCOM complex are recruited to the promoter, which is occuppied by a repressive complex containing HP1γ and KDM5B, among other factors. Msk1 phosporylates H3 at serine 10 promoting the displacement of the repressive complex. The combined action of the ASCOM complex and the displaced KDM5B increases histone H3 in K4 trimethylation, stabilizing NURF at the promoter. NURF remodeling facilitates access of the Cdk2-CyclinA kinase, which phosphorylates histone H1 and promotes its displacement, which also requires activation of PARP1. This first cycle of chromatin remodeling is a prerequisite for a second cycle starting 5 min after hormone addition; activated PR complexes bind BAF and PCAF and recruit them to the target chromatin. The BAF complex, stabilized by PCAF-dependent H3K14 acetylation, catalyzes ATP-dependent H2A/H2B displacement. Opening of the nucleosome core particle facilitates NF1 binding generating a stable $(H3/H4)_2$ platform that exposes previously hidden HREs for the recruitment of additional PR and BAF complexes, facilitating interaction with other coactivators and assemble of the transcription initiation complex. At both phases in activation of the promoter, a histone tail modification stabilizes the binding of an ATP-dependent chromatin remodeling complex to the target promoter followed by a nucleosome remodeling step. Thus, transcription initiation is a complex process including connected cycles of enzymatic chromatin remodeling, where each enzyme complex is crucial at a given time point

ubiquitin ligase activity. First, BRCA1 has a direct effect on the cellular level of PR and, hence, on the extent of PR recruitment to target promoters through the promotion of ligand-independent and -dependent degradation of PR [18]. We demonstrated by in vitro and in vivo assays that BRCA1/BARD1 might be the main E3 ubiquitin ligase responsible for the ubiquitination and degradation of PR in the absence of hormone. Second, following hormone treatment the BRCA1/BARD1 complex is recruited via interaction with PR to the hormone-responsive regions of PR target genes and affects the local levels of monoubiquitinated histone H2A, contributing to the epigenetic silencing of these promoters [18]. This connection between BRCA1/BARD1 and progesterone receptor activity may contribute to explain the particular tissue specificity of BRCA1-related tumours. Given the relationship of BRCA1 with DNA repair, it is interesting to note that breast cancers with mutations in BRCA1 or 2 are particularly responsive to chemotherapies involving inhibition of PARP1, which is also involved in DNA repair [19, 20].

5 Conclusions

Our focus on the initial events of the hormone signaling to chromatin has unraveled an unexpected complexity that integrates several kinases, tumor suppressor genes, histone modifying enzymes and ATP-dependent chromatin remodeling complexes in a coordinate sequence of enzymatic activities aimed at controlling the extent of hormone action and the preparation of the chromatin for

access of the RNA polymerase II and other basic factors of the transcriptional machinery. A schematic model that attempts to include our present knowledge of these multiples steps is shown in Fig. 2. We are aware that this scheme represents only a part of the numerous steps involved in gene regulation and that many more PR partners will have to be incorporated in the model, even if we limit ourselves to the very initial 10 min of hormone action.

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