Reprogramming the Chromatin Landscape: Interplay of the Estrogen and Glucocorticoid Receptors at the Genomic Level

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Abstract

Cross-talk between estrogen receptors (ER) and glucocorticoid receptors (GR) has been shown to contribute to the development and progression of breast cancer. Importantly, the ER and GR status in breast cancer cells is a significant factor in determining the outcome of the disease. However, mechanistic details defining the cellular interactions between ER and GR are poorly understood. We investigated genome-wide binding profiles for ER and GR upon coactivation and characterized the status of the chromatin landscape. We describe a novel mechanism dictating the molecular interplay between ER and GR. Upon induction, GR modulates access of ER to specific sites in the genome by reorganization of the chromatin configuration for these elements. Binding to these newly accessible sites occurs either by direct recognition of ER response elements or indirectly through interactions with other factors. The unveiling of this mechanism is important for understanding cellular interactions between ER and GR and may represent a general mechanism for cross-talk between nuclear receptors in human disease.
Here, we provide evidence that coactivation of ER and GR reprograms the chromatin landscape causing global rearrangement of SR binding in mouse mammary cells. Induction of GR facilitates selective access of ER to specific sites in the genome by maintaining an accessible configuration at these response elements. In addition, activation of ER can affect chromatin structure at estrogen-dependent GR-binding sites, resulting in a new class of GR-binding elements. Coactivation of ER and GR also leads to a loss of specific binding sites for each SR. These findings reveal that cross-talk occurs at the genome level and that activation of multiple SRs has a dramatic impact on controlling which regulatory elements are accessible to each receptor.

Materials and Methods

Cell culture conditions
For maintenance, cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, sodium pyruvate, nonessential amino acids, 2 mmol/L glutamine, and 5 μg/mL tetracycline to repress expression of the tetracycline-regulated fusion proteins (22). Cells were maintained in a humidiﬁer at 37°C and 5% CO2. Cells were plated for experiments in DMEM growth medium supplemented with 10% charcoal–dextran–treated serum with tetracycline 48 hours before conducting the experiment. Twenty hours before hormone treatment, cells were washed three times with PBS and fresh DMEM with 10% charcoal–dextran serum (no tetracycline) was added to the cells to induce expression of GFP-GR and Ch-ER.

Cell line validation
3617 and 7438 murine mammary epithelial cell lines have been described previously (17) and were constructed originally in our laboratory. All other cell lines were constructed from these two cell lines. Cells are routinely tested for GR and ER expression by Western blot analyses and ﬂuorescent microscopy and for dexamethasone and estradiol responses.

Chromatin immunoprecipitation
Chromatin immunoprecipitation was conducted via standard protocols (23), with minor modifications using the following antibodies: GR cocktail (PA-510A and PA-511A, Affinity BioReagents); sc-1004 (M-20, Santa Cruz Biotechnology); and ER cocktail (Ab-10, Labvision; sc-543, Santa Cruz Biotechnology). Cells were untreated or induced with 100 nmol/L dexamethasone (Sigma), 100 nmol/L estradiol (Sigma), or 100 nmol/L dexamethasone plus 100 nmol/L estradiol for 30 minutes. Three biologic replicates were pooled as a single replicate before generating sequencing libraries. Two replicates per treatment were sequenced.

DNaseI digestion of chromatin and size fractionation
DNaseI digestion was conducted as previously described (11, 24). Cells were untreated or induced with 100 nmol/L dexamethasone (Sigma), 100 nmol/L estradiol (Sigma), or 100 nmol/L dexamethasone plus 100 nmol/L estradiol for 30 minutes. Nuclei were isolated and digested with 100 to 120 U/mL of DNaseI (Sigma) for 3 minutes at 37°C. Digested chromatin was then incubated with 10μg/mL RNaseA (Roche) overnight at 55°C. Proteinase K (Ambion) was then added to the samples and incubated for 4 hours at 55°C. DNA was puriﬁed by phenol–chloroform extraction and fractionated using sucrose gradient centrifugation to isolate 100 to 500 bp fragments. These fragments were pooled, precipitated, and then assembled into libraries for sequencing.

ChIP-Seq data analysis
Illumina Solexa genome analyzer platform was used to generate sequence reads (36-mer) and unique tags were aligned to the mouse reference genome (UCSC mm9 assembly). Hotspots, regions of enriched tags, were called using previously described methods with minor modiﬁcations (25; see Supplementary Materials and Methods for details of sequencing data analysis)

Results

Redistribution of ER and GR binding to the genome upon coactivation
Cross-talk between ER and GR plays an important role in controlling many cellular processes (18–21). However, the mechanism used by these SRs to inﬂuence each other’s actions and control speciﬁc cellular outcomes is poorly understood. Therefore, we wanted to test whether GR could dictate ER-binding genome wide. We mapped ER and GR-binding events by ChIP-Seq in cells treated with either the corticoid steroid dexamethasone, estradiol (E2), or both dexamethasone and E2. As PR itself can respond to corticoid steroids and bind to glucocorticoid response elements (GRE), it can be a challenge to study ER and GR cross-talk in cell lines containing all three SRs (26, 27). Therefore, we used a previously engineered mouse mammary cell line, which expresses GR and ER but not PR (17), to look at specific interactions between GR and ER.

We mapped 2,916 ER-binding events in the presence of E2 and 3,949 ER-bound elements upon cotreatment of cells with dexamethasone and E2 (Fig. 1 and selected examples in Fig. 2 and Supplementary Fig. S1B–S1D). Of these binding sites, 2,863 are found to overlap in both treatments (Fig. 1A, blue dots). Interestingly, 28% of ER-binding sites observed upon cotreatment of cells with dexamethasone and E2 are dexamethasone dependent (Fig. 1A, green dots). In addition, 2% of ER-binding events are lost upon cotreatment with dexamethasone and E2 (Fig. 1A, red dots). These sites are unique binding sites observed only with the single hormone (E2) treatment. Together, these results show that there is a global redistribution of ER binding to the genome when both ER and GR are induced.

To determine whether the redistribution of binding upon cotreatment of cells with dexamethasone and E2 is unique to ER or is also observed with GR, we compared the binding...
events of GR in the presence of dexamethasone and dexamethasone plus E2 (Fig. 1 and selected examples in Fig. 2 and Supplementary Fig. S1B–S1D). We observe 3,427 GR-binding sites for cells treated with dexamethasone and 3,501 GR-binding elements for cells cotreated with dexamethasone and E2. There are 3,334 peaks that overlap between the treatments (Fig. 1B, blue dots). Similar to ER, we observe a global rearrangement of GR-binding events when cells are simultaneously treated with both hormones. Five percent of GR-binding sites in cells cotreated with dexamethasone and E2 are estrogen dependent and 3% of GR-binding events observed in cells treated with dexamethasone are unique to the single hormone treatment (Fig. 1B, green and red dots, respectively).

To illustrate the overall changes in ER and GR binding across the genome upon dual hormone treatment, the ChIP-Seq data were combined and 6,487 unique chromosomal positions were identified. Sequence-read densities for each hormone treatment were obtained over a 2 kb interval for each unique peak. Supervised clustering was conducted to extract specific binding modules for ER and GR, which resulted in the identification of 9 major clusters (Fig. 1C and D and Supplementary Fig. S2A). Analysis of the binding pattern at genomic elements for each cluster was found to be similar, with majority of binding sites occurring at intron and intergenic regions (Supplementary Fig. S2B). Clusters 5 and 7 represent the basic GR-and ER-binding patterns, respectively, where GR and ER can bind to these sites upon activation with their corresponding hormone. Three different clusters (2–4) represent the unique ER-binding sites observed upon cotreatment of cells with dexamethasone and E2 in Fig. 1A, whereas the unique GR-binding sites observed in the dual hormone treatment has two identified binding modules (cluster 3, 6). Of most interest to us is the binding of ER at sites in cluster 2 (496 binding sites) and of GR at sites in cluster 6 (143 binding sites). In these modules, binding of the receptor is dependent on having the other receptor activated, in addition to the other receptor also binding at these sites. This suggests that ER and GR binding to these sites are modulated by an assisted-loading mechanism. A similar mechanism has been observed for pBox and activator protein 1 (AP1) binding. In both examples pBox and AP1 are dependent on the activation of GR for binding at specific sites (15, 17).

In addition to global rearrangement of receptor binding, cotreatment of cells with dexamethasone and E2 can greatly affect expression levels at a subset of genes as shown by expression microarray analysis (Supplementary Fig. S3). A representative set of genes was confirmed by quantitative PCR (Fig. 2C). In addition, we found that many of the genes whose expression changed upon dual hormone treatment were associated with changes in either ER or GR binding within 20 kb of their transcriptional start sites (Supplementary Fig. S3C). The observation that gene expression changes upon dual hormone treatments is in agreement with previously published studies that show that cross-talk between ER and GR regulates a subset of proinflammatory genes and that corticosteroids can reverse the effect of estradiol on a small subset of genes in human leiomyoma cells (8, 9).

Changes in DNA accessibility occur at assisted-loading sites

To begin to understand the molecular mechanisms used for ER- and GR-assisted loading, we wanted to determine whether the assisted-loading sites (cluster 2 and 6) we observe with dual hormone treatments result from changes in chromatin accessibility at these sites upon hormone induction. We therefore conducted DNase-seq to map changes in DNase hypersensitive sites (DHS) between single and dual hormone treatments (11, 24).

Interestingly, ER-binding events that are assisted by GR occur at genomic locations where DNase accessibility increases upon treatment of cells with dexamethasone and E2 compared with E2 alone (Fig. 3A and B and selected example in Fig. 3C). A similar increase in hypersensitivity is observed at these sites when untreated cells are compared with cells treated with dexamethasone, indicating that these changes are dexamethasone dependent (Fig. 3A and selected example in Fig. 3C). This change is highly significant when compared with ER-binding sites that are only dependent on estrogen (Fig. 3A). This observation suggests a possible model in which GR initially binds to and recruits chromatin remodelers to these sites. GR has previously been shown to recruit SWI/SNF complexes to de novo sites, which leads to increase in accessibility at these response elements (10). In contrast, ER-binding events that are unique to the treatment of cells with only E2 (cluster 8) occur at sites that have no change in accessibility (Supplementary Fig. S4A and selected example in Supplementary Fig. S4B). In clusters 1, 3, and 4 we also observe slight increase in hypersensitivity at these sites upon cotreatment with dexamethasone and E2 (Fig. 3A, Supplementary Fig. S4A), which could account for slight increase in ER binding at these sites.

Previous studies have shown that induction of ER can increase DNase hypersensitivity at response elements (13). We therefore analyzed changes in DNase accessibility at
Figure 2. Examples of the effects of dual hormone treatments on ER and GR binding and on gene expression. A, genomic region illustrating assisted loading of ER by GR. Example (UCSC browser shot; ref. 40) of ER and GR ChIP-Seq in the absence of hormone or from cells treated with dexamethasone (Dex), E2, or dexamethasone and E2. ER and GR chromatin immunoprecipitation (ChIP) experiments were carried out after 30 minutes of hormone treatment. Black arrow denotes ER assisted-loading site. B, genomic region illustrating assisted loading of GR by ER. Example (UCSC browser shot) of ER and GR ChIP-Seq in the absence of hormone or from cells treated with dexamethasone, E2, or dexamethasone and E2. ER and GR ChIP experiments were carried out after 30 minutes of hormone treatment. Black arrow denotes GR assisted-loading site. C, coactivation of ER and GR effect gene expression. Expression analysis of Orm1, Orm3, 1810011010Rik, and Hes1, from cells treated with dexamethasone (diamond), E2 (square), or dexamethasone and E2 (triangle). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Figure 3. Chromatin accessibility changes at ER and GR assisted-loading sites. 
A, significant changes in hypersensitivity at ER assisted-loading sites (cluster 2). Box plot comparing changes in DNaseI hypersensitivity upon treatment of cells with dexamethasone (Dex) compared with untreated cells (top) or dual hormone treatment compared with treatment with E2 (bottom) for ER-binding modules 1, 2, and 7. Increases in hypersensitivity in ER assisted-loading sites (cluster 2) are statistically significant compared with sites that bind ER in the presence of E2. B, increases in accessibility at ER assisted-loading sites highly correlate with ER binding. Scatter plot illustrates that ER-binding sites unique to the double hormone treatment and overlapping GR-binding sites are highly correlated with increases in DNaseI accessibility upon dual hormone treatment. C, genomic region illustrating changes in DHS at ER assisted-loading site. Example (UCSC browser shot) of DHS in the absence of hormone or from cells treated with dexamethasone, E2, or dexamethasone and E2. Tracks are overlaid with ER and GR ChIP-Seq data from the same genomic site. Black arrows denote ER assisted-loading sites. D, significant changes in accessibility at GR assisted-loading sites (cluster 6). Box plot comparing changes in DNaseI hypersensitivity upon treatment of cells with E2 compared with untreated cells (top) or dual hormone treatment compared with treatment with dexamethasone (bottom) for GR-binding modules 1, 5, and 6. Increases in hypersensitivity in GR assisted-loading sites (cluster 6) are statistically significant compared with sites that bind GR in the presence of Dex. E, increases in hypersensitivity at GR assisted-loading sites highly correlate with ER binding. Scatter plot reveals that GR-binding sites unique to the double hormone treatment and overlapping ER-binding sites highly correlate with increases in DNaseI hypersensitivity upon dual hormone treatment. F, genomic region illustrating changes in DHS at GR assisted-loading site. Example (UCSC browser shot) of DHS in the absence of hormone or from cells treated with dexamethasone, E2, or dexamethasone and E2. Tracks are overlaid with ER and GR ChIP-Seq data from the same genomic site. Black arrow denotes GR assisted-loading site.
binding sites where GR loading is assisted by ER (cluster 6). We found that increase in chromatin accessibility at these sites is highly correlated with assisted loading of GR upon treatment of cells with dexamethasone and E2 compared with cells treated with dexamethasone alone (Fig. 3D and E and selected example in Fig. 3F). A similar increase in hypersensitivity is also observed at these sites when untreated cells are compared with cells treated with E2, proving that these changes are E2 dependent (Fig. 3D and selected example in Fig. 3F). However, GR-binding events unique to treatment of cells with only dexamethasone occur at sites that either have no change in accessibility or a slight decrease (Supplementary Fig. S4C and selected example in S4D). We also analyzed changes in chromatin accessibility upon treatment of cells with dexamethasone and E2 compared with cells treated with dexamethasone at other GR-binding modules. Although GR binding at cluster 3 is highly correlated with changes in DNaseI accessibility, other GR-binding modules are not dependent on changes in chromatin accessibility (Fig. 3D and Supplementary Fig. S4C). These findings indicate that both GR and ER cause changes in chromatin structure at specific response elements allowing for recruitment of the other receptor.

**DNA sequence specifies assisted-loading sites**

To determine whether ER assisted-loading sites contained unique sequences compared with other ER-binding modules, we conducted de novo motif analysis. Surprisingly, we did not observe enrichment of estrogen response elements (ERE) at assisted ER-loading sites (Fig. 4A). Instead, there is a high prevalence for GREs and (AP1)-binding motif. AP1 is a transcription factor that has been shown to interact with both ER and GR and plays a role in the recruitment of these receptors upon induction to specific sites within the genome (15, 28, 29). In comparison, motif and find individual motif occurrences (FIMO) analysis of other ER-binding modules show a higher prevalence for an ERE (Fig. 4B and Supplementary Fig. S5A) and a lower occurrence rate for GREs (Fig. 4C).

To confirm that ER binding at ER assisted-loading sites (cluster 2) is not dependent on direct binding to DNA at EREs, we constructed an ER DNA-binding domain (DBD) mutant (Fig. 5A and Supplementary Figs. S5B, S5C, and S6), which has previously been shown to abolish binding of ER to EREs but still allow tethering of ER to AP1 (30). We repeated the ChIP-Seq analysis on cells that did not express wild-type ER but did express wild-type ER but did express the DBD mutant. Mutation of the ER DBD domain did not affect ER binding at sites in clusters 1 and 2 but did affect ER binding in all other ER-binding modules (Fig. 5B). The ER DBD mutant has a 72% overlap in binding with wild-type ER at cluster 2 sites and 55% overlap at cluster 1 sites. Taken together, these results suggest that ER is recruited to cluster 2 assisted-loading sites by other factors, after GR induces chromatin remodeling at these sites.

**API binding at ER assisted-loading sites**

To determine whether AP1 could be a potential factor for tethering ER to sites in cluster 2, we overlaid previous AP1 ChIP-Seq data with ER-binding sites in ER-binding modules 1, 2, 6, and 7 (Fig. 5C). These previous data are from a cell line derived from the same parent cell line as the cell line used in the other studies in our article (15). Interestingly, we found that there is an increase in API at these ER assisted-loading sites (cluster 2) upon treatment of cells with dexamethasone. In contrast, an increase in API binding upon treatment with
dexamethasone is not observed in clusters 1, 6, or 7 in which ER binding is not dependent on GR at these sites (Fig. 5C). To access whether AP1 is necessary for ER binding at cluster 2 sites, we overexpressed Afos, a truncated Fos protein consisting of the leucine zipper domain and a substitution of the basic region with an acidic extension that maintains dimerization with Jun but inhibits its ability to bind DNA (15). ChIP-Seq of ER and GR binding in the presence of Afos shows that the dominant-negative AP1 complex effects ER binding at sites within cluster 2 (ER assisted-loading sites) is dependent on the treatment of cells with Dex, whereas AP1 binding at sites within clusters 1, 6, and 7 (ER-binding sites in the presence of E2) are not dependent upon treatment with Dex. Afos expression disrupts ER binding at assisted-loading sites (cluster 2), whereas ER binding at cluster 7 is not affected. E, GR binding is not disrupted by Afos expression at ER assisted-loading sites (cluster 2). Shown are box plots illustrating the effect of Afos expression on the binding of GR in clusters 1 and 2. Afos expression has no effect on GR binding at assisted-loading sites (cluster 2).

Figure 5. ER binding at assisted-loading sites is dependent on AP1. A, mutation in the DNA-binding domain of ER. Diagram showing the location of two mutations made in the DNA-binding domain of ER. Mutations are E207A and G208A. B, mutations in the DNA-binding domain of ER do not affect ER recruitment to binding sites found in cluster 2 (ER assisted-loading sites). Shown are box plots of the effect of the ER DNA-binding mutant on recruitment of ER to binding sites within clusters 1, 2, 6, and 7. The ER mutant is incapable of binding directly to EREs but can still be recruited to binding sites through tethering with other factors. C, AP1 binding at sites in cluster 2 is dependent upon activation of GR. Shown are box plots of the effect of dexamethasone (Dex) on AP1 binding at sites within clusters 1, 2, 6, and 7. AP1 binding at elements within cluster 2 (ER assisted-loading sites) is dependent on the treatment of cells with Dex, whereas AP1 binding at sites within clusters 1, 6, and 7 (ER-binding sites in the presence of E2) are not dependent upon treatment with Dex. D, Afos affects ER binding at sites in cluster 2. Shown are box plots illustrating the effect of Afos expression on the binding of ER in clusters 1, 2, and 7. Afos expression disrupts ER binding at assisted-loading sites (cluster 2), whereas ER binding at cluster 7 is not affected. E, GR binding is not disrupted by Afos expression at ER assisted-loading sites (cluster 2). Shown are box plots illustrating the effect of Afos expression on the binding of GR in clusters 1 and 2. Afos expression has no effect on GR binding at assisted-loading sites (cluster 2).
Although nuclear receptors are thought to interact with the genome primarily by binding to specific DNA recognition sequences, they can also be recruited to the genome at non-canonical sites through interactions with other transcription factors (15, 31, 32). In our studies, de novo motif analysis of ER sites that are reprogrammed during dual hormone treatments implicated involvement of AP1 in mediating ER binding at these rearranged binding sites. Suppression of AP1 binding with the Afos mutant confirmed the action of AP1 at these sites. A previous study has shown that ER and GR influence each other’s activity at an AP1 response element. In this study, dexamethasone was shown to inhibit estradiol stimulation of transcription through an AP1 response element (33). Taken together, these observations suggest that AP1 plays a prominent role in mediating ER and GR responses upon coactivation. It should be noted in this context that AP1 has also been shown to be important in breast cancer progression (34–36).

Multiple mechanisms have been proposed to describe cross-talk between ER and GR (37–39). One class of models suggests that ER regulates expression or degradation of GR. These mechanisms, however, are based on long-term treatment of cells with hormones and may be the result of secondary effects (37, 38). Here, we propose a molecular mechanism for direct cross-talk between ER and GR at the genome level, whereby coactivation of two receptors leads to rapid reprogramming of chromatin structure and receptor binding (Supplementary Fig. S2A). The unveiling of this mechanism is important for understanding the molecular interplay between ER and GR and may represent a general mechanism for cross-talk between transcription factors.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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