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Award Number: W81XWH-09-1-0297

TITLE: Identification of Estrogen Receptor Beta Binding Sites in The Human Genomes

PRINCIPAL INVESTIGATOR: Thien Le

CONTRACTING ORGANIZATION: University of Chicago
Chicago, IL 60637

REPORT DATE: April 2012

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE April 2012		2. REPORT TYPE Annual Summary		3. DATES COVERED 1 April 2009 – 31 March 2012	
4. TITLE AND SUBTITLE Identification of Estrogen Receptor Beta Binding Sites in The Human Genomes				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-09-1-0297	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Thien Le E-Mail: lethien@uchicago.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Chicago Chicago, IL 60637				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT ERβ knock-out mice developed prostatic hyperplasia at late age, suggesting an important role of ERβ in the development of the prostate as well as prostate cancer. Here we describe a study that thoroughly investigates the genomic function of ERβ. A FLAG-tagged ERβ was stably expressed in MCF7 C4-12 cells, which allowed ERβ transcriptional activity to be studied in an ERα-independent background. In this cell model, we identified 3166 ERβ genomic binding sites. Although different from ERα genomic binding sites, these ERβ genomic binding sites had similar global distribution pattern and enriched transcription factor binding motifs to those associated with ERα genomic landscape. In conjunction with nascent RNA profiling at the time of ERβ binding events, ERβ target genes were found to be enriched in apoptotic and developmental processes. This was in agreement with our result showing that ERβ suppressed cell growth in response to E2 treatment. Interestingly, 30% of ERβ binding sites carried EBF1 (Early B-cell Factor 1) binding motif. The interaction between EBF1 and ERβ correlated with down-regulated ERβ protein stability and downstream activity. Similar phenomenon was observed between EBF1 and ERα. These results, at least to our knowledge, were the first to indicate crosstalk between EBF1 and the estrogen receptors on a large scale.					
15. SUBJECT TERMS Genome-wide mapping; estrogen receptor beta					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 20	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

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INTRODUCTION

Beside being the major target of androgens, the prostate is also influenced by estrogens. In the adult male, 17-beta-estradiol (E2), classically considered the female sex hormone, is mainly produced by adipose tissue, adrenal glands, testicles and the prostate¹. On the cellular and molecular level, E2 mainly exerts its effect via the two estrogen receptors, ER α and ER β . ER α , as a marker for breast cancer, has been heavily studied, leading to a myriad of improvement in breast cancer treatment and prevention². On the other hand, the functions of ER β remain largely elusive if not controversial. Interestingly, ER β knock-out mice were reported to develop prostatic hyperplasia at late age³. Moreover, different lines of studies have suggested that ER β played an important role in anti-proliferation⁴, immunoprotection⁵, and detoxification in the prostate⁶. Therefore, ER β strikes as an important component in prostatic normal development as well as tumorigenesis. Because ER β regulates gene expression mainly at the transcription level, here ER β genome-wide binding sites were mapped in an ER α -negative cell model using ChIP-seq technology. In conjunction with nascent RNA profiling at the time of binding events, detected by GRO-seq technology, our results provided a better knowledge of ER β target genes as well as how ER β regulates their expressions on the global scale. Ultimately, this will give rise to a better model to study this nuclear receptor.

BODY

Aim1 - To identify genome-wide ER β binding regions

The lack of a reliable antibody for ER β led to many controversies surrounding the presence and functions of this steroid receptor⁸. Due to the same reason, detecting ER β binding sites, via chromatin immunoprecipitation (ChIP) assays, was also delayed. Therefore, we proposed to generate cancer cell lines stably expressing ER β as a Flag-tagged fusion protein (Flag-ER β). Since specific and reliable antibodies raised against the Flag were broadly available, the fusion protein would be efficiently immunoprecipitated in ChIP conditions.

In order to map ER β genome-wide binding sites in a non-biased manner, we chose ChIP followed by massive parallel sequencing (ChIP-seq) as our main approach. This technology was widely proven to produce reliable data with higher resolution compared to the conventional ChIP-chip assay (ChIP followed by tiling microarray).

Also, in order to study ER β genomic functions without the interference of ER α , we wished to introduce the Flag-ER β into two well-characterized prostate cancer cell lines LNCaP, PC3 (in which ER α is undetectable), and a breast cancer cell line MCF7/C4-12 (a derivative of the MCF7 cell line which does not express ER α).

C4-12/Flag.ER β cells stably expressed Flag-tagged ER β :

Due to a variety of reasons (low viral infection efficiency, low cell growth rates, etc.), we were not able to generate the prostate cancer cells stably expressing Flag-ER β at first. However, the DNA construct of the fusion protein appeared to be integrated into the genome the MCF7/C4-12 cells at a much higher rate. The efficiency of viral infection could be ranked highest in MCF7/C4-12 cells, low in PC3 cells, and very low in LNCaP cells. Moreover, the MCF7-C4/12 cells grew at a very fast pace, which allowed generating material for the downstream assays much more efficiently. Therefore, we used the MCF7/C4-12 cells stably expressing Flag-ER β (C4-12/Flag.ER β) to optimize the condition for the ChIP-seq assay, while developing the prostate cancer cells stably expressing Flag-ER β .

Although we were able to express Flag-ER β in LNCaP and PC3 cells, the stable expression level of the ectopic protein was too low. Moreover, these cell lines appeared to quickly lose Flag-ER β expression. Meanwhile, we were advancing our analyses of ER β genomic functions using the C4-12/Flag.ER β cells. Although this cell line derived from the breast cancer cell line MCF7, the cells had gone through significant changes so that they should no longer be considered a cell model for breast cancer. However, these cells still provided sufficient cellular environment to study the biology of ER β . Therefore, we decided to carry on our project using the C4-12/Flag.ER β as our cell model.

Mapping ER β genomic binding sites

C4-12/Flag.ER β cells were serum-starved for 24 hours before treated for 1 hour with 10nM estradiol (E2). Cells treated with the same volume of vehicle (ethanol) were used as control. The cells were crosslinked and proceeded through ChIP assay, in which anti-Flag M2 antibody (Sigma-Aldrich) was used to pull down the Flag-ER β . The immunoprecipitated DNA libraries were prepared according to the Illumina ChIP-seq library prep kit. Samples were then submitted to high throughput sequencing with Solexa technology at the IGSB High-throughput Genome Analysis Core (Argonne National Laboratory, IL).

As we proceeded through the ChIP-seq assay with the C4-12/Flag.ER β cells, our data confirmed the high efficiency of the optimized ChIP-seq conditions. We were also able to identify more 3166 binding sites at high stringency (1% FDR). A few binding sites were selected at random to confirm with ChIP-qPCR (Figure 1C). The global distribution of ER β binding sites at high stringency is presented in figure 1A. Moreover, our motif analysis identified binding motifs of ERs, known ER β interactors as well as those of novel interactors (figure 1B).

The binding motif of EBF1 (Early B-cell Factor 1) was enriched in 30% ER β binding regions. Moreover, EBF1 mapping experiments revealed binding motifs of the estrogen receptors (Triggs, J., unpublished data). These evidence, at least to our knowledge, were the first to suggest a genomic interplay between EBF1 and ER β . Furthermore, EBF1 was downregulated in prostate cancer cells (Oncomine). This prompted us to perform downstream assays, described below, to further investigate such crosstalk.

Crosstalk between EBF1 and ER β

EBF1 downregulated ER β transcriptional activity

We performed a luciferase reporter assay to assess if EBF1 affected ER β transcriptional activity. Briefly, C4-12 cells were transiently transfected with a plasmid carrying a Firefly Luciferase gene preceded by three tandem estrogen response element (ERE), a Renilla Luciferase plasmid (to serve as transfection efficiency control), and an ER β plasmid. These cells were also transiently transfected with a pcDNA as vector control or an EBF1 plasmid. Upon estradiol induction, ER β was recruited to the ERE and facilitate the expression of luciferase protein, resulting in an increase of luciferase activity. However, in the presence of EBF1, this activity was significantly reduced in a ligand independent manner (Figure 2A).

Furthermore, EBF1 also downregulated transcriptional activities exerted by ER α and the two chimeric receptors in a ligand independent manner. Interestingly, constructs carrying ER β AF1 domain (full length ER β and ER β /alpha) were more affected by EBF1 comparing to those carrying ER α AF1 domain (full length ER α and ER α /beta) (Figure 2A). Such results suggested that

the association between EBF1 and the estrogen receptors involved the N-terminal regions of the estrogen receptors.

EBF1 inversely correlated with ER β protein stability

Since previous reports had been suggesting the role of the AF1 domain in the degradation of the estrogen receptors, we were interested to see if EBF1 participated in such regulation. C4-12 were transiently transfected with ER β in the presence or absence of EBF1. After cells had been serum starved for 24 hours, cell lysates were collected after 3 hours of treatment with estradiol (E2) or vehicle. Indeed, the presence of EBF1 inversely correlated with ER β protein stability while leaving ER β transcript level unchanged (data not shown) in a ligand independent manner (figure 2B). These results further suggested the role of EBF1 in the regulation of ER β on the protein levels. Similar effect of EBF1 was observed on ER α protein level (Figure 2B).

EBF1 was coimmunoprecipitated with ER β

Although we wished to test if EBF1 directly interacted with ER β , the decreased ER β protein level prevented such assay. In order to go about this issue, cell lysates were collected after 3 hours of treatment with E2 or vehicle, in the presence of MG132, a proteasome inhibitor. In such condition, EBF1 was coimmunoprecipitated with ER β (Figure 2C), indicating a direct interaction. EBF1 was also coimmunoprecipitated with ER α (figure 2C).

Aim 2 – To identify genes directly regulated by ER β

Because emerging evidence had shown that trimethylation of lysine 4 on histone 3 (H3K4me3) was a reliable marker for active promoters, we first attempted to use this as the marker for actively transcribed genes with ER β binding events, hence ER β direct target genes. However, our analyses revealed that the hormone treatment time of 1 hour was too short for differential modifications to occur.

Alternatively, recent advances in high throughput sequencing technology have allowed the development of a new technology, namely global nuclear run-on followed by sequencing (GRO-seq). This technology provides the detection of nascent RNA on a global scale^{9,10}. We have successfully applied this new technology to identify ER β direct target genes.

GRO-seq

In order to capture gene regulation by ER β binding onto the chromatin, cell nuclei were extracted and collected after 1 hour of E2 or vehicle treatment (the same time point of ER β genomic binding events). Applying the GRO-seq technology, nascent RNA from these nuclei, which represented genes being actively transcribed, were extracted and processed for high throughput sequencing. The results were lists of genes being actively regulated at the time of ER β binding events. These gene lists were overlapped with the list of genes harboring ER β binding sites, revealing ER β direct target genes. Among these direct targets, 209 genes were up-regulated while 133 were down-regulated.

Gene ontology analyses showed that these ER β targets were enriched in apoptotic and developmental processes (Figure 3A). The fact that similar processes (but different genes) were found enriched in both up- and down-regulated genes suggested a switch from one pathway to another one in response to estradiol. Selected targets were chosen to confirm the assay by RT-qPCR for mRNA extracted at 2hour treatment time (Figure 3B). The presence of EBF1 also abolished the hormone responsive regulation of ER β target genes (Figure 3C).

Aim 3 - Confirmation of ER β target genes focusing on prostatic angiogenesis

We originally proposed to investigate the role of ER β in regulating genes related to angiogenesis. However, according to gene ontology analyses, this process was not enriched in ER β target genes. Therefore, we focused the confirmation of ER β target genes on those related to apoptosis and development.

Briefly, C4-12/Flag.ER β cells were starved in 5% charcoal-stripped serum for 24hr; these cells were then treated with E2 or Vehicle (ethanol) as negative control. After 4 days of treatment, cell confluency, measured by the Incucyte system (Essen BioScience), was used to determine cell proliferation. As shown in figure 4, E2 significantly suppressed C4-12/Flag.ER β cell growth. Interestingly, as EBF1 was introduced to these cells via transient transfection, cell proliferation was also suppressed. Moreover, EBF1 appeared to dominate ER β , release these cells from hormone responsiveness due to ER β (figure 4).

KEY RESEARCH ACCOMPLISHMENTS

- Identified a novel crosstalk between EBF1 and the estrogen receptors.

REPORTABLE OUTCOMES

N/A

SUMMARY

Using the C4-12/Flag.ER β cell line that we generated, we were able to map ER β genomic binding regions in an ER α -independent background. While most ER β binding regions were different than ER α binding sites, they share similar global distribution patterns and enriched transcription factor binding motifs. In order to assess the functionalities of ER β binding sites, we are applying the GRO-seq technology to profile nascent RNA being generated at time of ER β binding events. Gene ontology analyses showed that ER β target genes were enriched in apoptotic and developmental processes. This was in agreement with our cell proliferation assay as well as the hypothesis that ER β had anti-proliferative effect. Our extensive analyses of ER β binding sites also revealed an interesting crosstalk between ER β and EBF1, a crucial transcription factor regulating the process of B-cell differentiation. Further experiments suggested a direct interaction between EBF1 and ER β , resulting in decreasing ER β protein stability and downstream activity in luciferase reporter assays. Similar phenomenon was observed between EBF1 and ER α . To our knowledge, this is the first to indicate crosstalk between EBF1 and the estrogen receptors.

Training progress

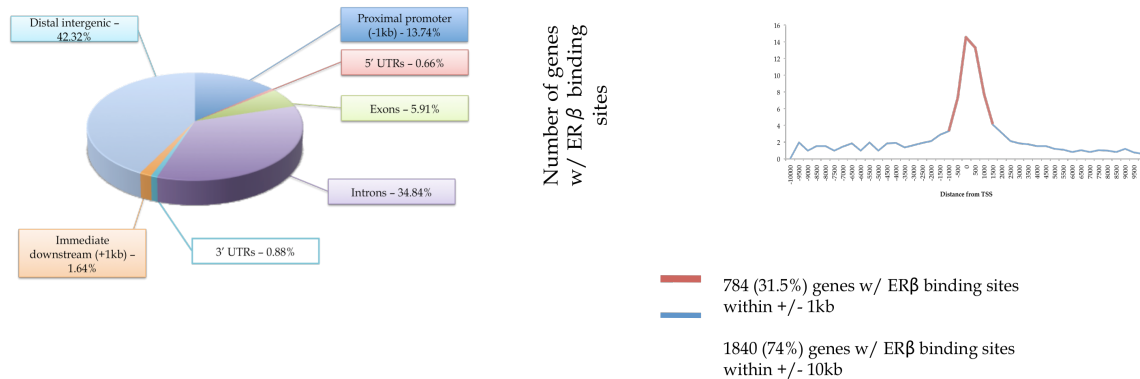
- Keystone symposium - Nuclear Receptors: Signaling, Gene Regulation and Cancer (2010).
- The PCRP IMPaCT 2011 Conference.
- GRO-seq workshop with Dr. Lee Kraus's research group (University of Texas Southwestern, 2011).
- ENDO 2011: The 93rd Annual Meeting and Expo.

REFERENCES

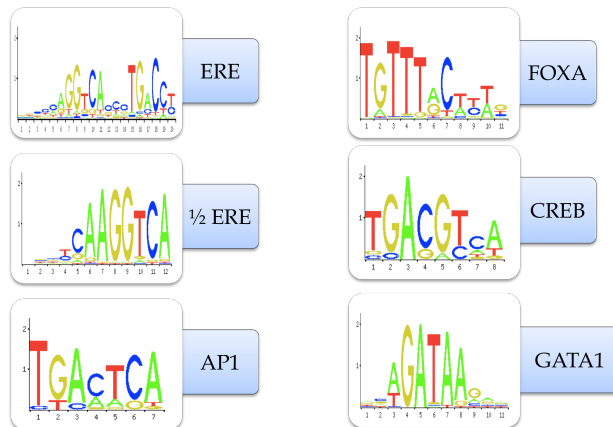
- Rickett, W.A., et al. (2007) Steroid hormones and carcinogenesis of the prostate: the role of estrogens. *Differentiation*. 75:871-882.
2. Osborne, C.K. (1998) Steroid hormone receptors in breast cancer management. *Breast Cancer Res Treat*. 51(3):227-238.
3. Zhao, C., Dahlman-Wright, K., Gustafsson, J.A. (2008) Estrogen receptor beta: an overview and update. *Nuclear Receptor Signaling*. 6: e004
4. Weihua, Z., et al. (2001) A role for estrogen receptor beta in the regulation of growth of the ventral prostate. *Proc Natl Acad Sci USA*. 98(11):6330-6335.
5. Prins, G.S., Korach, K.S. (2008) The role of estrogens and estrogen receptors in normal prostate growth and disease. *Steroids*. 73:233-244.
6. Montano, M., Jaiswal, A., Katzenellenbogen, B. (1998) Transcriptional regulation of the human quinone reductase gene by antiestrogen-liganded estrogen receptoralpha and estrogen receptor-beta. *J Biol Chem*. 273(39):25443-25449.
7. Barski, A., et al. (2007) High-resolution profiling of histone methylations in the human genome. *Cell*. 129(4): 823-37.
8. Snyder, M.A., et al. (2010) Multiple ER β antisera label in ER β knockout and null mouse tissues. *Journal of Neuroscience Methods*.
9. Core, L.J., Waterfall, J.J., Lis, J. (2008) Nascent RNA sequencing reveals widespread pausing and divergent initiation at human promoters.
10. Hah, N., et al. (2011) A rapid, extensive, and transient transcriptional response to estrogen signaling in breast cancer cells.

SUPPORTING DATA

A.



B.



C.

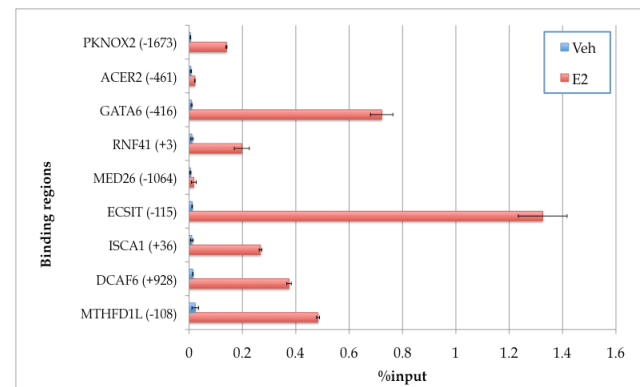


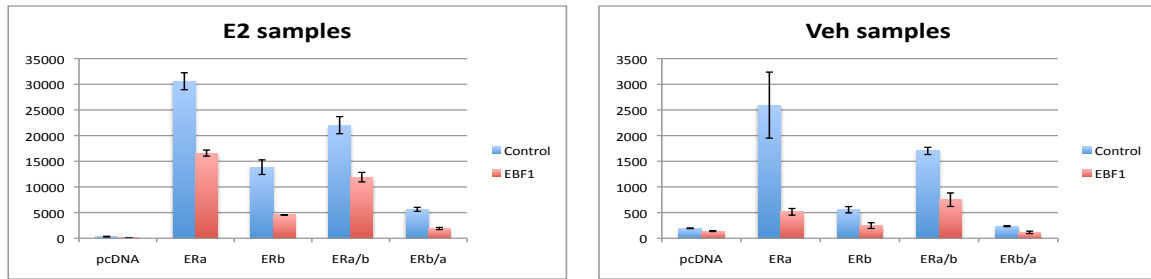
Figure 1 – Mapping ERβ binding sites in C4-12/Flag.ERβ cells.

A – Global distribution of 3166 ERβ binding regions.

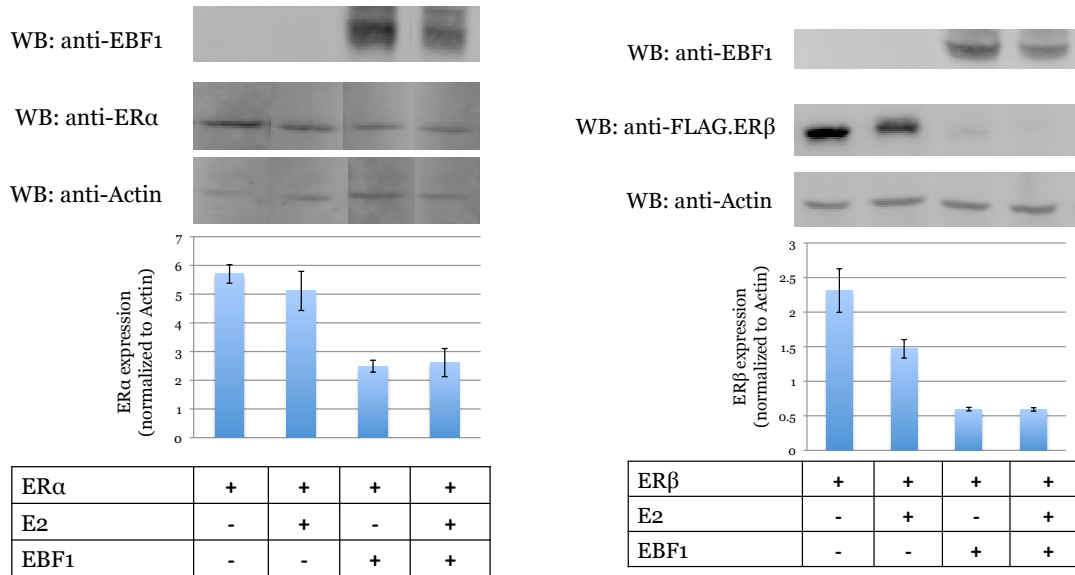
B – Enriched transcription factor binding motifs found in ERβ binding sites.

C – Confirmation of randomly selected binding sites with ChIP-qPCR.

A.



B.



C.

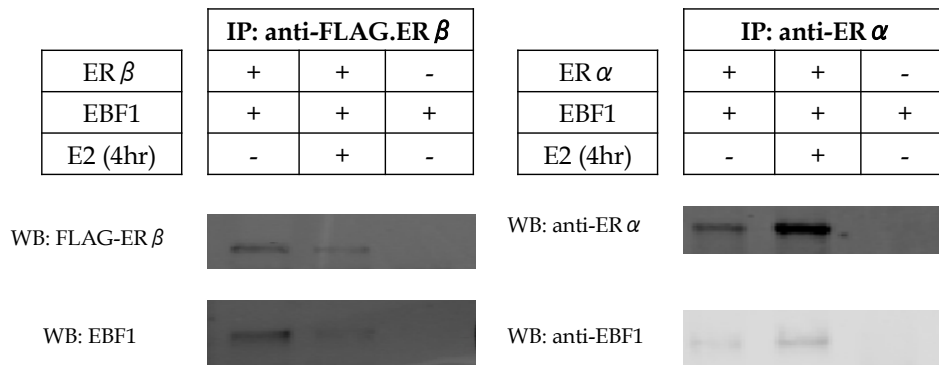


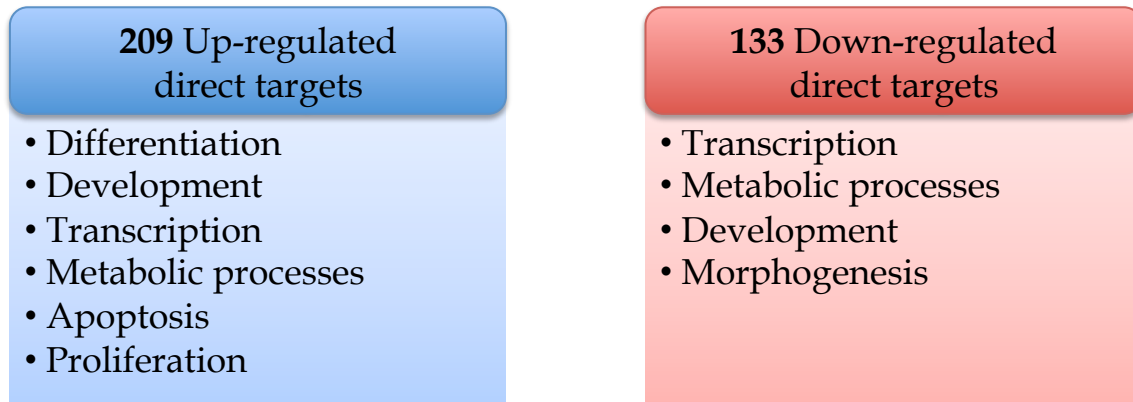
Figure 2 – Crosstalk between EBF1 and ER.

A – EBF1 suppressed ER activity in luciferase reporter assays.

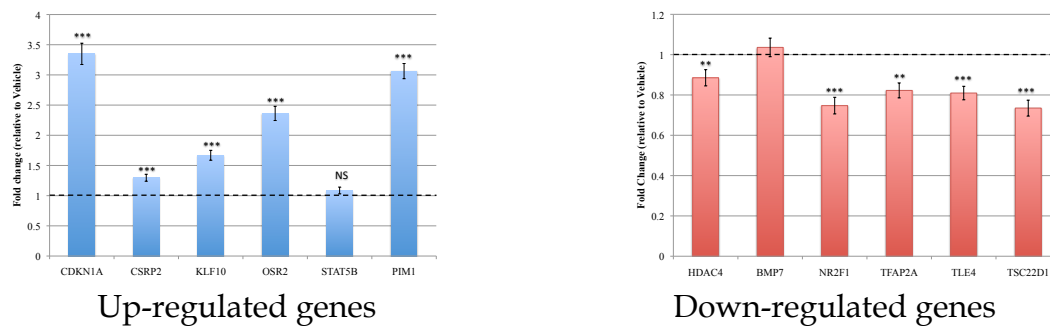
B – EBF1 correlated with decrease ERα and ERβ protein stability.

C – EBF1 was coimmunoprecipitated with ERα and ERβ.

A.



B.



C.

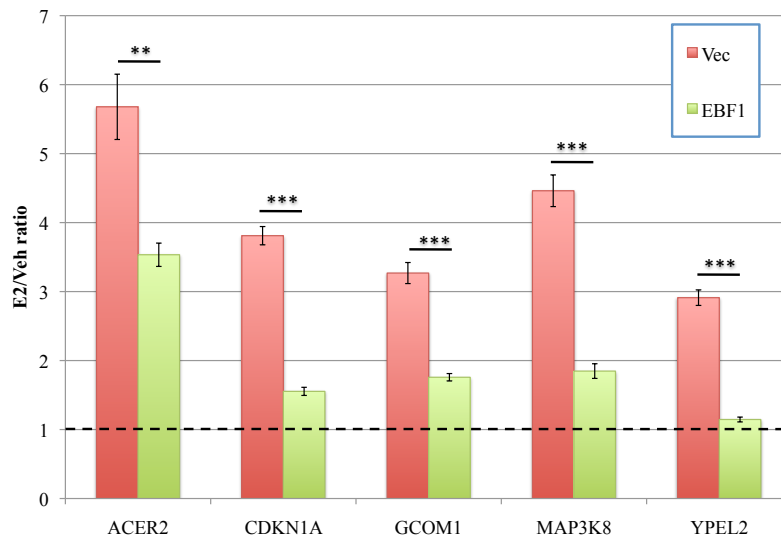


Figure 3 – ER β target genes identified by GRO-seq

A – Gene ontology analyses revealed target genes enriched in apoptosis and development.

B – Confirmation of target genes with RT-qPCR analyses for mRNA collected at 2hr treatment time.

C – EBF1 presence abolished hormone responsive gene regulation of ER β target genes.

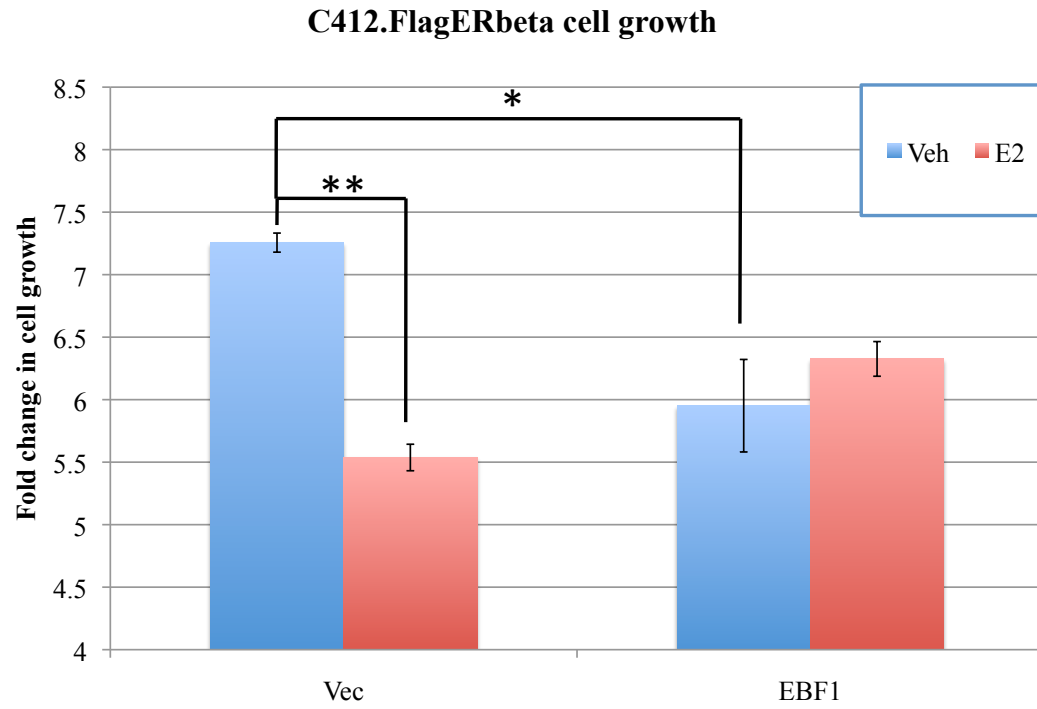


Figure 4 – C4-12/Flag.ER β cell proliferation was suppressed by E2 and, independently, by EBF1.

APPENDICES

Abstracts for past meetings

- Thien Le, Matthew Walker, Geoffrey Greene. "Genomic functions of the estrogen receptor β " (2010) Keystone symposium – Nuclear Receptors: Signaling, Gene Regulation and Cancer.

Considerable effort by numerous laboratories has resulted in an improved understanding of estrogen and SERM action mediated by the two estrogen receptors, ER α and ER β . However, many of the targets for ER β in cell physiology remain elusive. Here we propose a study that thoroughly investigates the transcriptional targets and functions of ER β .

MCF7 C4-12 cells, which do not express either ER, were engineered to stably express a FLAG-tagged ER β . In this cell model, ER β transcriptional activity can be studied in an ER α -independent manner. According to our ChIP-seq results (chromatin immunoprecipitation followed by deep sequencing with Solexa technology), <14% ER β binding sites were within proximal promoter regions of putative target genes, while most binding events occurred either in intronic (34%) or distal intergenic (42%) regions. The most prevalent binding motif was the canonical ERE. Analysis of motifs within binding sites also revealed other transcription factor sites that potentially influence ER β functions. In conjunction with active promoter, marked by H3K4me3, our results allow a global picture of ER β transcriptional activity. In a related project, using phage display and exon microarray as a novel method for screening protein-protein interactions, we have identified novel interactions between ER β and other coregulators. The combined results will allow the identification of ER β target genes as well as how ER β regulates their expressions. Ultimately, this will give rise to a better model to study this nuclear receptor.

- Thien Le, Geoffrey Greene. "Early B-cell Factor 1 regulated estrogen receptor β activity on a genomic scale" (2011) The PCRIP IMPaCT 2011 Conference.

Considerable effort by numerous laboratories has resulted in an improved understanding of estrogen and SERM action mediated by the two estrogen receptors, ER α and ER β . However, many of the targets for ER β in cell physiology remain elusive. Interestingly, ER β knock-out mice developed prostatic hyperplasia at late age, suggesting an important role of ER β in the development of the prostate as well as prostate cancer. Here we describe a study that thoroughly investigates the transcriptional targets and functions of ER β . MCF7 C4-12 cells, which do not express either ER, were engineered to stably express a FLAG-tagged ER β . In this cell model, ER β transcriptional activity can be studied in an ER α -independent background. According to our ChIP-seq results (chromatin immunoprecipitation followed by deep sequencing with Solexa technology), <15% ER β binding sites were within proximal promoter regions of putative target genes. The majority of binding events occurred either in intronic (45%) or intergenic (36%) regions. While the most prevalent binding motif was the canonical ERE, ~30% of ER β binding regions also carried the binding motif of

EBFs (Early B-cell Factor 1). This result suggests that EBF1 might potentiate or attenuate ER transcriptional activities on a global scale. In our luciferase reporter assay, EBF1 down-regulated both ER α and ER β activities. Interestingly, ER constructs carrying the AF1 domain of ER β (full length ER β and ER β / α chimera) were more affected compared to those carrying ER α AF1 domain (full length ER α and ER α / β chimera). Moreover, the ectopic expression of EBF1 correlated with reduced ER β protein stability, which might have explained the suppressed transcriptional response in luciferase reporter assay. These results, at least to our knowledge, are the first to indicate crosstalk between EBF and ER transcriptional activities on a large scale. The roles of other proteins in the EBF family (i.e. EBF2, EBF3, EBF4) in influencing ER β activity are under active investigation. Moreover, in conjunction with gene expression profiling, still to be completed, our results should reveal a global picture of ER β transcriptional activity, as well as the role of EBF proteins in this process.

- Thien Le, Geoffrey Greene. "Early B-cell Factor 1 regulated estrogen receptor β activity on a genomic scale" (2011) ENDO 2011: The 93rd Annual Meeting and Expo.

Considerable effort by numerous laboratories has resulted in an improved understanding of estrogen and SERM action mediated by the two estrogen receptors, ER α and ER β . However, many of the targets for ER β in cell physiology remain elusive. Here we describe a study that thoroughly investigates the transcriptional targets and functions of ER β . MCF7 C4-12 cells, which do not express either ER, were engineered to stably express a FLAG-tagged ER β . In this cell model, ER β transcriptional activity can be studied in an ER α -independent background. According to our ChIP-seq results (chromatin immunoprecipitation followed by deep sequencing with Solexa technology), <15% ER β binding sites were within proximal promoter regions of putative target genes. The majority of binding events occurred either in intronic (45%) or intergenic (36%) regions. While the most prevalent binding motif was the canonical ERE, ~30% of ER β binding regions also carried the binding motif of EBFs (Early B-cell Factor 1). This result suggests that EBF1 might potentiate or attenuate ER transcriptional activities on a global scale. V5-tagged EBF1 was co-immunoprecipitated with FLAG-tagged ER β , demonstrating a direct interaction in a ligand independent manner. Moreover, in our luciferase reporter assay, EBF1 down-regulated both ER α and ER β activities. Interestingly, ER constructs carrying the AF1 domain of ER β (full length ER β and ER β / α chimera) were more affected compared to those carrying ER α AF1 domain (full length ER α and ER α / β chimera). These results, at least to our knowledge, are the first to indicate crosstalk between EBF and ER transcriptional activities on a large scale. Because EBF2, EBF3, and EBF4 transcripts were found to be abundant in MCF7 C4-12 cells expressing FLAG-tagged ER β (EBF3 > EBF2/EBF4 > EBF1), the roles of these proteins in influencing ER β activity are under active investigation. Moreover, in conjunction with gene expression profiling, still to be completed, our results should reveal a

global picture of ER β transcriptional activity, as well as the role of EBF proteins in this process.