Author’s response to reviews

Title: Transcriptomic and ChIP-sequence interrogation of EGFR signaling in HER2+ breast cancer cells reveals a dynamic chromatin landscape and S100 genes as targets

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Author’s response to reviews:

Response to Reviewers’ Comments:

All changes are highlighted in Yellow in the revised manuscript.

Technical Comments:

1. We note that in the original submission file, the figures in PowerPoint were not incorporated correctly and therefore were not visible. If possible, we would advise to replace them with figures in .tiff format.

Response: We have enlarged Fonts in Figures and saved them in TIFF Format in the revised manuscript submission
Reviewer reports:

Ahmad Khalil (Reviewer 1):

This manuscript describes changes in gene expression in HER2-positive breast cancer cells (SKBR3) upon activation of EGFR. The authors use both RNA-seq and ChIP-seq to identify genes that are affected by EGF binding to EGFR and activation of downstream signaling pathways leading to changes in gene expression and chromatin modifications in a time-dependent manner. I would recommend the publication of this manuscript if the authors can address the following:

1. The authors need to clearly state how many replicates of RNA-seq and ChIP-seq were performed at each time point and the appropriate statistical analysis that were done to identify differential gene expression and changes in histone modifications.

Response: Appropriate subsections in the methods section have been updated to clearly indicate that biological replicates (n=2) were utilized to conduct RNA-seq and ChIP-seq experiments. The RNA-seq analysis method subsection has been modified to state that the statistical test for transcript abundance of biological replicates was based on the Jensen-Shannon metric of the Cuffdiff program. ChIP-seq analysis was conducted using MACS2 software. The methods subsection under “Chip-seq analysis” has been updated to indicate that q-values are calculated from p-values using the Benjamini-Hochberg method with q-value of 0.01 and p-values < 0.0005 being considered significant. The modification has been highlighted in yellow in the revised manuscript.

2. The section describing ChIP-seq data is very difficult to read and there are too many numbers. You can add a table to simplify.

Response: The section describing the ChIP-seq data has been substantially decreased and processed in order to discuss the main trends without too much details. The venn diagrams graphically demonstrate the number of peaks and overlapping peaks between the various time points. A table also been made to summarize the data (Table 2 in the revised manuscript). All changes are highlighted in yellow in Results of the revised manuscript, under subtitle “ChIP-sequence (ChIP-seq) H3K18ac and H3K27ac” (Line237- Line242, Line246-Line248, Line251-Line258).
3. The authors should cite a few more papers that discuss trastuzumab-resistance that are relevant to their findings.

Response: Total of 10 papers (references 43-53) have been cited in Discussion to discuss mechanisms for trastuzumab resistance and role of S100 genes in the revised manuscript. Please see the Discussion section in the revised manuscript (line 439-Line 468) (yellow highlighted)

4. In general, the manuscript would benefit from editing for clarity.

Response: We have edited the manuscript thoroughly. Changes to the text have been made, specifically the section describing the ChIP-seq data. We hope that the edits make the section easier to read. Please see the revised manuscript (highlighted in yellow)

5. I think this an important manuscript to the field if these concerns can be corrected.

Response: Thank you. We are very grateful to the Reviewers for your critical and helpful suggestions. We hope the revised manuscript has addressed most of your concerns.

Reviewer 2 (Reviewer 2):

REVIEWER COMMENTS FROM REPORT: This is an interesting study in which the authors aim to study alternation of gene transcription responding to EGFR in HER2+ breast cancer cells by using NGS technology. The approach is relatively robust but lacks functional validation. The overall impact is moderate.

REQUESTED REVISIONS:

A single cell line can be used for the discovery study, but functional studies should be included to validate the findings with different cell line models.

Response: Thank you. As recommended by the Reviewer, we have conducted additional experiments by using different HER2-overexpressing breast cancer cells. First, we confirmed the finding from RNA-seq that EGF activates expression of the family of S100A genes (S100A2,
S100A3, S100A5, S100A6 and S100A10) in SKBR3 by RT-qPCR. Those S100 family genes were upregulated significantly upon EGF treatment of SKBR3 cells. In addition, we also verified the same expression activation in another HER2-overexpressing breast cancer cell line, BT474. The data showed that S100A5, S100A6 and S100A10 were all upregulated in both SKBR3 and BT474 upon EGF treatment. However, induction of S100A10 was not significant. The data has been added to Figure 5 as “Figure 5D” in the revised manuscript.

To examine the contribution of S100A genes in HER2-overexpressing breast cancer cells resistant to trastuzumab, we determined the levels of S100 genes in a trastuzumab resistant cell line SKBR3/100-8. SKBR3/100-8 was generated from SKBR3 through clonal selection in our laboratory. The cell line has been confirmed to exhibit acquired resistance to trastuzumab, displays an EMT phenotype and has higher levels of EGFR protein expression when compared to parental SKBR3 cells (Wu Y. et al. Mol Cancer Res. 2012 10(12):1597-606). The data from this study showed significantly higher levels of S100A2 and S100A6 in SKBR3/100-8 compared to parental SKBR3 (wild type). S100A10 also had 1.5-fold higher levels in SKBR3/100-8 compared to SKBR3. The new data has been added as Figure 6 in the revised manuscript.

Our data from ChIP-seq showed that EGF treatment induced higher expression levels of S100A genes and this coincided with an increase in H3K18ac and H3K27ac. Specifically, H3K18ac, H3K27ac levels increased dramatically at the TSS and in the S100A6 gene body within 1h and stayed elevated throughout the EGF time course. To determine whether acetylation of H3K18 and H3K27 had an effect on the expression of S100A genes in HER2-overexpressing breast cancer cells, we treated SKBR3/100-8 cells with A-485, a selective small-molecule inhibitor of P300/CBP acetylase activity, and then examined levels of S100 family genes by RT-qPCR. The data showed that inhibition of P300/CBP downregulated S100A2, S100A5 and S100A6 significantly in SKBR3/100-8. The level of S100A10 was not affected by inhibition of P300/CBP. The new data has been added as Figure 6 in the revised manuscript.

Since S100A6 showed consistent higher level in all three lines we tested, we sought to determine the role of S100A6 in HER2-overexpressing breast cancer cells and in EGF/EGFR cell signaling through siRNA knockdown experiments. First, we treated SKBR3 cells with a siRNA directed against S100A6 and then treated the cells with EGF. The data showed that knockdown of S100A6 itself did not affect cell growth, however, it did inhibit EGF-induced cell growth. The new data has been added as Figure 6 in the revised manuscript.

Next, we conducted S100A6 knockdown in trastuzumab resistant cells, SKBR/100-8, and found that downregulation of S100A6 also decreased EMT driver, Twist1. The data has been added as Figure 6 in the revised manuscript.

Description of the data has been added in the Results of revised manuscript (Yellow highlighted, Line 339- Line 375).
Daniele Lecis (Reviewer 3):

In the manuscript "Transcriptomic and ChIP-sequence interrogation of EGFR signaling in HER2+ breast cancer cells reveals a dynamic chromatin landscape and S100 genes as targets" by Nava M. and colleagues, the authors investigate the effect of EGFR stimulation on gene transcription in order to identify possible determinants of anti-Her2 therapy resistance. Starting from the notion that EGFR is upregulated in HER2+ breast cancer cells resistant to trastuzumab, the authors stimulated the HER2 positive breast cancer cell line SKBR3 with EGF in time-course experiments and performed extensive RNA-sequence and ChIP-sequence for H3K18ac and H3K27ac. With this approach, they identify clusters of genes which are activated or repressed at different timepoints. Some of these genes are discussed and their possible implication in cancer progression and resistance to therapy is described. Several mechanisms can hinder the efficacy of trastuzumab, but the authors decide to focus on the capacity of EGFR to promote the expression of genes which could affect the treatment. This work is clinically relevant and present new data that could be instrumental for future validation.

In my opinion, this work presents a few limitations that should be considered before publication:

1) Only one cell line is employed (SKBR3) for all the experiments and therefore findings can hardly be broadened to other HER2 positive cancer cells.

Response: Thank you. In response to recommendations by Reviewer 3, we have examined S100A genes in different HER2-overexpressing breast cancer cell lines. In the revised manuscript, S100A genes were examined in both SKBR3 and BT474 HER2-overexpressing breast cancer cell lines upon EGF induction. The new data are added as Figure 5D in the revised manuscript. In addition we also examined the levels of S100A genes in trastuzumab resistant breast cancer cell line, SKBR3/100-8. The SKBR3/100-8 cell line was generated from SKBR3 through clonal selection and confirmed to be resistant to trastuzumab in a previous study. The SKBR3/100-8 cells displayed an EMT phenotype, exhibited an increase in WNT3/β-catenin pathway signaling and also expressed higher EGFR levels than parental cells (reference 7). The data showed significantly higher levels of S100A2 and S100A6 in the resistance cell line (SKBR3/100-8) compared to SKBR3 cells. The data has been added as new Figure 6 in the revised manuscript. Description of results has been added in Results in the revised manuscript (Yellow highlighted, Line 339 – Line 354).

2) Genes identified as affected by EGF and discussed as crucial are not validated by qPCR and/or western blot.
Response: We have further confirmed those S100A genes (those significantly upregulated by EGF identified by mRNA-seq) in SKBR3 and BT474 cells upon EGF treatment by RT-qPCR. We also conducted RT-qPCR to examine the levels of those S100 genes in SKBR3/100-8. All data has been added to Figure 5 and Figure 6 in the revised manuscript. Results section has been updated in the revised manuscript (Yellow highlighted, Line339-Line364).

3) No functional experiment is shown to verify the involvement of the identified genes in trastuzumab resistance. This represents a drawback since the authors state several times that the aim of this study is to identify possible EGFR-mediated mechanisms of resistance to trastuzumab (Lines 48-50, 369-370, 459-462).

Response: In the revised manuscript we have verified the levels of those S100 genes in trastuzumab resistant cells, SKBR3/100-8. We have conducted the following functional experiments:

1) Confirmed upregulation of S100 genes (S100A6 and S100A2 were the most significantly upregulated) in trastuzumab resistant cell line SKBR3/100-8.

2) According to ChIP-seq data, the upregulation of S100 genes by EGF may be regulated at the level of chromatin regulation through acetylation of H3K18 and H3K27. Hence we treated SKBR3/100-8 cells with A-485, a selective small-molecule inhibitor of P300/CBP acetylase activity, and then examined levels of S100 family genes by RT-qPCR. The data showed inhibition of P300/CBP downregulated S100A2, S100A5 and S100A6 significantly in SKBR3/100-8. The level of S100A10 was not affected by inhibition of P300/CBP. The new data has been added as Figure 6 in the revised manuscript. The data suggests that S100 genes are regulated at the level of chromatin in trastuzumab resistant breast cancer cells.

3) Breast cancer cells undergoing EMT also exhibit a drug and trastuzumab resistance phenotype. We previously generated SKBR3/100-8 cells, which exhibited resistance to trastuzumab, an EMT phenotype, activation of WNT3/β-catenin signaling and transcriptional upregulation of EGFR (Wu Y. et al. Mol Cancer Res. 2012 10(12):1597-606). S100A6 has been suggested to promote EMT through β-Catenin in a pancreatic cancer cell line (Chen X et al. PLOS ONE DOI:10.1371/journal.pone.0121319 March 23, 2015). Hence we conducted siRNA knockdown of S100A6 in SKBR3/100-8 and found that expression of EMT driver, Twist1, was also significantly downregulated. Our previous study found that TGF-β/Smad3 can induce TWIST1 transcription and upregulate WNT3 resulting in activation of the β-catenin pathway in
SKBR3 (Wu Y et al. Breast Cancer Res Treat. 2017, 63(3):449-460). This study confirmed that activation of WNT3 plays significant role in trastuzumab resistance. Similarly, we have proposed in this study that, EGF mediated induction of S100 genes could be a mechanism of HER2-overexpressing breast cancer to develop resistance to trastuzumab.

4) We also tested the role of S100A6 in EGF induced cell growth and found that knockdown S100A6 in SKBR3 inhibited EGF-inducing cell growth.

The new data are presented in Figure 6 in the revised manuscript and described in Results in the revised manuscript (Yellow highlighted, Line346- Line 375).

Minor points

1) In Figure 2c, there is not enough space for the name of the identified pathway making it difficult to appreciate the findings (e.g. positive reg of…)

Response: This has been rectified with the correct figures. All fonts have been enlarged in figures.

2) In Figure 3a, the authors find accumulation of H3K18ac and H3K27ac also in genes repressed (CPNE1, MAX). If I understood correctly, these should be marks of activation. Probably this should be discussed more extensively.

Response: Although it was very interesting to observe H3K18ac or H3K27ac at repressed genes (Figures 3A, 4A, and S2) discovering the underlying mechanism(s) would certainly require ChIP-seq for P300/CBP (the lysine acetylases responsible for H3K18ac/H3K27ac).

In the “Enriched motifs” subsection of the Results we have stated that “Even though they were not the most significantly enriched at all clusters, every cluster contained binding sites for transcription factors known to be downstream of EGFR signaling (Figures 4B and S3). One possibility is that EGFR signaling promotes the recruitment of P300/CBP to every regulated gene regardless of whether there is consequential transcription.” (Highlighted in green in Results, Line303- Line 307). However, as mentioned in the discussion section “chromatin regulation does not account for all of the fluctuations in gene expression observed in EGF treated SKBR3 cells. To that end, other groups have demonstrated that microRNAs play a role in modulating mRNA’s in EGF treated MCF10A cells [9,13]. Nonetheless, studying the role of microRNAs in EGF treated SKBR3 cells using NGS technologies remains unexplored and might provide key differences of post-transcriptional regulation of gene expression.” (Highlighted in green in Discussion, Line482- Line 487)
3) The authors study the EGF-dependent gene expression also at very late timepoints, contrarily to what reported in literature (lines 136-137). Nevertheless, it should be considered that at late timepoints, the effect observed could be due to the activation of other pathways activated in turn (e.g. by cytokines or soluble factors released in an EGFR-dependent manner) and not merely specific to EGF stimulation.

Response: That could be true. Our ChIP-seq data offers insight into the chromatin landscape of all genes in regards to the enrichment of H3K18ac and H3K27ac status. Even though the modulation of these modifications and of actual transcript abundance at later time points in the investigation is complicated by other cytokines and growth factors, at the very least, we know the state of H3K18ac and H3K27ac at 1h post-EGF treatment. In some cases, genes like the S100 family reach their peak height in expression at 24h post-EGF treatment but have already gained H3K18ac and H3K27ac at 1h post-EGF treatment and have subtle upward trends in gene expression (Figure 5A and 5B). Moreover, some S100 genes already have an increase in pol2 within 20 minutes of EGF treatment (Figure 5C).

4) The importance of p21 (lines 210-214) and S100 genes (lines 357-362) is speculated but not experimentally tested.

Response: We examined p21 by using Western blot with protein lysis from SKBR3±EGF, however, the p21 levels were undetectable in those cells. Also due to the time limited for resubmission we have not been able to confirm if S100 genes are direct EGFR signaling transcriptional targets. We agree with Reviewer 3’ comments that those are important questions and future studies need to be conducted. We are planning to study those questions in our Laboratory soon.

5) In general, the manuscript lists a number of genes affected by EGF stimulation. Probably it would be possible to summarize everything in a table and reduce the text, hence making the paper easier to read.

Response: We have made a table to summarize genes mentioned in the manuscript as Table 1. In addition, Tables S1 and S2 listed all genes modulated by EGF treatment.
6) It would be extremely interesting, but I understand it is not the aim of this work, to compare the effect of EGF with the other ligands which stimulate EGFR (e.g. TGFα). What do the authors expect?

Response: Thank you. This is a very interesting and thoughtful question. EGFR is known to bind to at least seven different ligands, included TGFα. The ligand binding allows EGFR to dimerize with other HER family receptors and activating signaling pathways. We believe that other binding ligands are able to activate EGFR and turn on cell signaling as well. However, different binding ligands may mediate different downstream cell signaling events. In fact crosstalk between receptors and cell signaling also could stimulate EGFR indirectly. For example we previously demonstrated trastuzumab resistant SKBR3 cells (SKBR3-100/8) overexpressed WNT3 when compared to the parental cell line. The upregulation of WNT3 in SKBR3/100-8 transcriptionally upregulated EGFR through activation of the β-catenin pathway (reference 7). We also found that TGF-β is able to sustain the upregulation of WNT3 through Smad3 and TWIST in HER2-overexpressing breast cancer cells that would continuously promote the upregulation of EGFR (reference 49). Nonetheless, we agree with Reviewer 3, that it would be interesting to explore the role of EGF with other ligands and in particular how these interactions lead to drug resistance. Our laboratory will continue to investigate such mechanisms.