Author's response to reviews

Title: COUP-TFI modifies CXCL12 and CXCR4 expression by activating EGF signaling and stimulates breast cancer cell migration

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Author's response to reviews:

Dear Editor,

Thank you very much for considering and handling our manuscript.

We propose this new version of our manuscript entitled “COUP-TFI modifies CXCL12 and CXCR4 expression by activating EGF signaling and stimulates breast cancer cell migration”, revised according to the reviewer's comments.

We have listed below a detailed response and explanation of the changes made according to the reviewer's remarks and underlined these changes (red colours) in the new document.

We greatly acknowledge the reviewers for their constructive suggestions and remarks and hope that the revised manuscript, including substantial new data, will now be acceptable for publication in BMC Cancer.

Yours sincerely,

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The answers to the reviewer's remarks are listed below:
Reviewer 1:

Is the question posed by the authors well defined?
The question is well defined. The authors have previously identified that the orphan nuclear receptor COUP-TFI can enhance ER transcriptional activity and proliferation in breast cancer cells. COUP-TFI has been proposed to mediate cancer progression, but the mechanisms are not known. The authors hypothesise that COUP-TFI may mediate breast cancer migration through the chemokine signalling axis. This study builds on their previous analyses and describes the effect of overexpressing COUP-TFI in the MCF-7 breast cancer cell line on the chemokine CXCL12 and its receptors CXCR4 and CXCR7. It also describes the effect of COUP-TFI on CXCL12-mediated cell migration.

We are pleased with this appraisal.

Are the methods appropriate and well described?
The methods are mostly well described. However, antibody concentrations need to be specified for both IF and western blotting.

This is done. The antibody concentrations were specified in the methods as requested (page 6 and 7, 1st and last paragraphs).

The RT-PCR section lacks detail regarding what controls were used, how the normalisation was done, the method for analysing relative expression (i.e. delta delta Ct). How many real-time PCR determinations were performed on the biological replicates?

Why was 18S used as a normaliser in tissues and GAPDH in cell lines? There is no rationalisation for the housekeeping genes chosen. This needs to be included in the methods. The authors need to present data to verify that 18S is not different between the grades and between tumours and normal tissue, otherwise, it should not be used as a normaliser in these samples.

This is done. As suggested by the reviewer, we have now introduced more details and information on the RT-PCR method and on the choice of using GAPDH and 18S for normalisation (page 7, 1st paragraph).

The most commonly used reference genes include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypoxanthine-guanine phosphoribosyl transferase (HPRT) and 18S ribosomal RNA. GAPDH was found to be appropriate for normalisation in cell lines because its expression was not affected by treatments and remained stable in control and COUP clones.

For tissues, we first verified the choice of the reference gene as an internal control and its suitability in our study. Four housekeeping genes were tested (GAPDH, HPRT1, TBP and 18S RNA). The stability of these genes across different tissues and tumor grades was assessed using geNorm algorithm (Vandesompele et al., Genome Biol. 2002, 3: research0034.1-research0034.11). This software has listed HPRT1 as the best gene but HPRT1 is expressed at
very low level in normal tissues and tumors, making it quite difficult to accurately quantify and not enough useful as an internal reference in our study. The second best gene, established by the software in the list, was the 18S RNA. This RNA is expressed similarly at relatively high levels in all tumors and made ideal positive control for our study. So, we have chosen 18S for normalisation.

It is unclear if the tissue used in this analysis was from FFPE or fresh frozen? If FFPE, how were real-time assays designed to ensure appropriate detection of these genes? This needs to be included in the methods. There is no description of what the normal tissues are- are they normal tissue adjacent to the breast, were they matched, were they from reduction mammoplasty and unmatched, etc. This needs to be included in the methods.

All samples used in this study were from fresh frozen tissues. The normal breast tissues were adjacent to the tumors but they are majority unmatched to the tumors. As suggested by the reviewer, this information was included in the methods (page 9, 3th paragraph).

I have several concerns regarding the data presentation. The below points are Major Compulsory Revisions:

Immunofluorescence:

1. Overall, the IF images are of poor quality and seem very blurry.
2. “As shown in Fig. 1, the control cells express a low level of endogenous COUP-TFI, though COUP-TFI staining is much higher in the COUP cells (Fig. 1A).” This is not obvious with the COUP-TFI antibody. Perhaps a different fluorophore or different area on the slide should be chosen to demonstrate this.
3. In Fig 1D, the down-regulation of CXCL12 in COUP clones by IF is not clear (Fig 1D) and it is difficult to see whether CXCR4 expression is actually higher in COUP cells or whether it appears higher because the cells are more confluent in the COUP image than in the control cells. This figure should be re-done showing areas of approximately equal confluence.

Following these remarks, we performed new IF experiments. We believe that the new pictures illustrate appropriately our conclusions that CXCL12 expression is reduced, CXCR4 expression is increased, while CXCR7 expression does not change upon COUP-TFI overexpression. In addition, together with the new western blot (Fig. 1 C, see comment #4), our data now clearly show the difference of COUP-TFI expression in our clones.

Other comments:

4. For the western in Figure 1C, I am unsure if the HA antibody or COUP antibody was used. Given my concerns with the IF results using the COUP antibody in Fig 1A, the western needs to be performed with COUP and HA antibodies.

This is done. Whole cell extracts from MCF-7 control and COUP cells were loaded on denaturing gels and the amounts of protein levels loaded were verified
with total ERK1/2 by western blots. Endogenous COUP-TFI protein was revealed with specific antibody against COUP-TFI. As expected, an increase in the expression level of endogenous COUP-TFI was detected in MCF-7 COUP compared to the control MCF-7 cells. According to the requirement of reviewer, we added these results in Fig. 1C which now shows western blots performed with COUP-TFI and HA antibodies.

5. Fig 2 CXCR7 results should be shown.

This is done as requested. The data show variability, but overall, no significant pattern.

6. “data not shown” is used throughout the manuscript. Where the authors have mentioned the data, they need to show it, particularly given that BMC Cancer does not have Figure limitations.

We are very surprised that this reviewer claims “data not shown is used throughout the manuscript”. We used only twice (and only once now). The remaining one concerns the observation that culturing our cells in different serum concentration parallels the effect of the treatment with/without EGF. This observation supports our finding that growth factor signalling can contribute to the down-regulation of CXCL12 and the induction of CXCR4 but this is not the primary focus of the publication. Here, we want to demonstrate that COUP-TFI, by modulating the cell-autonomous EGF signalling, is influencing the basal expression of CXCL12 and CXCR4. We do not believe that showing our data will clarify this message.

7. Histograms in Figure 3, Figure 4C-D, Figure 5 and Figure 6 all contain more than one comparison and the statistical tests used in these need to be corrected for multiple comparisons.

Each comparison performed in Figures 3, 4 and 5 are performed on single comparisons, either between control clone with/without treatment, or between control and COUP clones for the same experimental condition. Together with response to the comment #19, we have changed the way of presenting the significant differences, using asterisks when comparing control clones and pond when considering COUP clones.

8. How do the experiments in Fig5A address the hypothesis “that the higher expression of CXCL12 in the control clones is also involved in their lower capacity to migrate toward exogenous CXCL12”? Placing recombinant CXCL12 to both the upper chamber and the lower chamber simply removes the gradient? The authors should use siRNA to knock down the levels of COUP in either the COUP overexpressing cells or the control cells, and assess the effect on CXCL12 and migration towards the CXCL12 gradient. Additionally, they could knock down CXCL12 in the control cells to see if this has the same effect on migration as COUP overexpression. This would be a more effective demonstration of their hypothesis.

In fact, we have actually tried to knock down CXCL12 in the control and parental
cells to see if this results the same effect on migration as COUP overexpression. The problem with this approach is that, because CXCL12 is a key protein for cell survival and adhesion, its knock down using siRNA makes very difficult cell migration assays. We have therefore abandoned this track.

In addition, it is well demonstrated that the dysregulation of CXCL12 in breast tumor cells influences the invasiveness and migration of breast cancer cells in vitro and in vivo. Indeed, up-regulation of CXCR4 and down-regulation of CXCL12 mRNAs were significantly associated with lymph node metastasis. Moreover, re-expression of autocrine CXCL12 markedly reduced metastatic lung invasion in vivo (Wendt et al., Oncogene 2008, 27:1461–1471; Zhou et al., J Cancer Res Clin Oncol 2009, 135:91–102).

Utilisation of siRNA to knock down the levels of COUP is also a good point. However, it is well known that MCF-7 cell lines which have already very low migratory capacity are not a good model for reducing further their migratory capacity. Moreover a transient inhibition of COUP-TFI by siRNA is not necessarily sufficient to restore CXCL12 expression and migratory capacity. Down-regulation of CXCL12 in metastatic tumors is often due to promoter hypermethylation. But effectively, this is something that should be taken into consideration in the future work by establishing stable shRNA COUP clones not only in MCF-7 model because the low expression of endogenous COUP-TFI but also in MDA-MB-231 model because the high expression of endogenous COUP-TFI.

9. “In addition, the ectopic CXCL12 added to the upper chamber prior to the migration test hampered the migration of both the control and COUP clones toward the CXCL12 gradient.” Reword. There is no gradient if you add ectopic CXCL12 to the upper and lower chambers?

This is done. We have reworded this paragraph, as suggested by the reviewer (page 12, last paragraph).

10. “As depicted in Fig. 6A, the level of CXCR4 mRNA was found to be significantly increased in the tumors compared to the healthy samples (p<0.0001) and was correlated with the tumor grade”. The authors need to perform statistical analysis to show a correlation with grade or otherwise, they should leave this statement out.

This is done. As recommended by the referee, we have performed a two sided Pearson correlation between CXCR4 expression and the tumor grades. We have found a strong correlation between CXCR4 expression and tumor grade (p-value = 0.000085, # = 0.4201 at the 95% confidence interval [0.2235; 0.5839]). We added the results of this analysis in the text to the appropriate section (page 13, 2nd paragraph).

11. Figure 6 does not support the in vitro experiments. The relationship between increased COUP expression and increasing grade is not supported (except for grade 1). Additionally, the lowest levels of CXCL12 and the highest expression of CXCR4 are observed in grade 3, tumours- where COUP levels are lowest. This
is in contradiction to the in vitro experiments. Also, CXCR7 is reduced compared
to normal tissue in all grades. The authors need to perform a correlation analysis
to determine if the relative expression of CXCR4, CXCR4 and CXCL12 in
individual tumours is associated with the relative expression of COUP.

This is done. As recommended by the referee, we have performed a two sided
Pearson correlation analysis to determine if the relative expression of CXCR4,
CXCR7 and CXCL12 in tumours is associated with the relative expression of
COUP-TFI. This analysis showed a significant correlation for CXCR4/COUP-TFI
(p-value = 0.029, # = 0.2405 at the 95% confidence interval [0.0248 ; 0.4348]),
CXCR7/COUP-TFI (p-value = 0.0042, # = 0.3129 at the 95% confidence interval
[0.1029 ; 0.4962]) and CXCL12/COUP-TFI (p-value = 0.030, # = 0.2387 at the
95% confidence interval [0.0229 ; 0.4333]). We added the results of this analysis
in the text to the appropriate section (page 13, 2nd paragraph).

12. “The expression profiles of CXCL12 and CXCR4 in breast cancer biopsies
were comparable to those obtained when we overexpressed COUP-TFI in
MCF-7 cancer cells, suggesting that our in vitro results may have clinical
relevance.” Reword. This was not shown in the clinical samples. The authors
need to perform correlation analysis to demonstrate this.

We have reworded this paragraph, as suggested by the reviewer (page 16, last
paragraph). The sentence indicates a SUGGESTION that our in-vitro results
MIGHT have a clinical relevance.

13. Why was DCIS used for this analysis? The authors’ hypothesis is that
COUP-TFI expression is associated with cancer migration/invasion- why not use
invasive breast cancer specimens (i.e.IDC)? Also, it would seem more
appropriate to analyse progressive cancer specimens ADH#DCIS#IDC rather
than increasing grades (in a non-invasive cancer subtype) to support the
hypothesis that COUP-TFI is associated with breast cancer migration/invasion.
Please explain.

We thank reviewer for this very pertinent question. In fact, all the tumor samples
that we used were invasive ductal cancer and mostly ER-positive (> 90%). This
error happened during the drafting of the manuscript and during the French /
English translation. We have now corrected this point (page 9, 2nd paragraph).

14. A major limitation of this study is that it was only performed in one cell line
and that the results in tumour tissue do not appear to replicate (with the
exception of Grade 1 DCIS- see point 11. above) the work carried out in the
MCF-7 cells. This needs to be discussed.

This is done. We added new sentences in the Discussion section of the revised
manuscript (page 15/16, 1st paragraph) to discuss this point.

Do the authors clearly acknowledge any work upon which they are building, both
published and unpublished?

Yes
Do the title and abstract accurately convey what has been found?
Yes
Is the writing acceptable?
Yes

Minor Essential Revisions:
15. SBR grade is used throughout, what does SBR stand for- this is not listed anywhere.

There are different "scoring systems" available for determining the grade of a breast cancer. One of the most common grading systems used in the United States and in Europe is SBR (Scarff-Bloom-Richardson grading system) which is based on the histologic score system. In this scoring system, there are three factors that the pathologists take into consideration: the "differentiation state" of the tumor, the nuclear features and the mitotic activity of the tumor cells. Histological grading is widely accepted as an indicator of prognosis in breast cancer. In general, SBR grade 1 is the least aggressive tumors whereas SBR grade 3 is the most aggressive tumors (grade 2 is an intermediate grade between grades 1 and 3). The abbreviation SBR is now defined (page 9, 2nd paragraph).

16. Were the treatments performed in duplicate or triplicate? Page 7 “Total RNA from triplicate treatment groups” and page 10 “Two independent control clones and two independent COUP clones were used, and the data shown represent the pooled results.” Please clarify.

Total RNA was extracted at least in triplicate. The RT-PCR results shown in figure 1B represent the mean of the data obtained from two independent clones (control and COUP). We clarified now this point in the revised version (page 7, 1st paragraph, 1st sentence; and page 10, 2nd paragraph, 12th sentence).

17. “First, we tested the control and COUP cells for a proliferative response to CXCL12 treatment by exposing the cells to CXCL12 (200 ng/mL) for 7 days and quantifying the total cell number (Fig. 5A).” Figure 5A shows migration not proliferation, this needs to be corrected.

This is done. The Y axis title was corrected in Fig. 5A.

Figure Legends:
18. Figure 1: “real-time PCR analysis from two independent MCF-7 control and COUP clones. The results were normalized to GAPDH as the internal control and are expressed as the mean CXCL12, CXCR4, or CXCR7/GAPDH mRNA ratio ± SEM of at least three independent experiments.” Reword, this is hard to follow.

This is done. We have reworded and rewritten the sentences (page 22, Fig. 1B legend).

19. Figure 3: It is not clear what the lowercase letters are indicating in Figure 3,
this is very confusing and needs to be clarified, either in the legend or in the Figure by using only one symbol for the significant result with arms/arrows showing what the comparisons are.

We agree with the reviewer that to improve clarity, a different system should be used to illustrate significantly different results. This is done, using an asterisk for the comparison of control clones and a pound symbol for the comparison of COUP clones.

20. NT needs to be written in full with the abbreviation in brackets in the legend for Figure 6.

This is done.

21. The authors should perform chIP to show if the effect of COUP on the genes under study is direct (Discretionary Revision).

We totally agree with the reviewer that ChIP experiments could strengthen COUP-TFI action on CXCL12 and CXCR4 genes. Nevertheless, in this study, we showed that COUP-TFI action is mediated through activation of EGF/EGFR. Moreover, even if COUP-TFI interacts directly with these genes, the genomic region where COUP-TFI interacts is unknown for the moment. We therefore cannot do ChIP experiments.

Reviewer 2

The manuscript titled ‘COUP-TFI modifies CXCL12 and CXCR4 expression by activating EGF signaling and stimulates breast cancer cell migration’ showed the role of COUP-TFI in relation with E2 signaling in metastasis of breast cancer cell by regulating the CXCL12/CXCR4 axis as well as proliferation. The author’s research in this manuscript is solid and a well-designed work and proposes novel mechanism regarding the role of COUP-TFI1 in regulation of CXCL12/CXCR4 and finally correlated with the RNA level of COUP-TFI, CXCL12 and CXCR4 in clinical breast cancer tissue samples. The authors showed the increased expression of CXCR4 and decreased expression of CXCL12 at the level of promoter, mRNA transcription and protein expression in tested cell lines. The authors demonstrated the increased migration capability in COUP-TF overexpression cells was related with CXCR4/CXCL12 using chamber migration assay including CXCL12 and antagonist of it. Finally the authors correlated the altered expression of COUP-TFI, CXCR4, 7 and CXCL12 with SBR grade of breast cancer. This research article is well demonstrated and scientifically and medically significant and deserves to be published.

We are very pleased with this appraisal.

Major Compulsory Revisions

None

Minor Essential Revisions
1. Page 18 – 21, there are numbers of unidentified letters between reference journal name and year of publication in reference. Ref 2, 3, 5, 6, 9, 11, 12, 13, 15, 19, 20, 21, 22, 24, 26, 27, 28, 29, 30, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42 This is done. We corrected all references in the revised version.

2. Regarding abbreviation list of EMT in Page 17, line 3, there is no ‘EMT’ description in this manuscript. We deleted EMT in abbreviations.

3. Page 23, line 25 ‘asterisk’ should be ‘pound’. This is done. We added now a new sentence in the legend figure 5 (page 23, Fig. 5, 5th sentence) to clarify this point.

4. In Figure 5 A, the Y axis title (Relative migration) should be ‘Relative cell number’. In total agreement with the reviewer, the Y axis title was corrected now in Fig. 5A.