Author's response to reviews

Title: Bladder inflammatory transcriptome in response to tachykinins: Neurokinin 1 receptor-dependent genes and transcription regulatory elements.

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Author's response to reviews: see over
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**Reviewer #1: Dr. Anthony Atala.**

The authors would like to thank Dr. Atala for all the time and effort he dedicated to this manuscript in order to make it better suited for a clinical oriented journal. The constructive comments are well taken and will definitive improve the quality of this manuscript.

General comments: The authors are in debt for the kind comments from Dr. Atala and will work even harder to deserve them.

**Comment # 1.** This is an excellent molecular investigation, but I doubt there are many (if any) individuals in the general urologic community, especially clinical urologists, who will understand the manuscript.

**Answer:** Dr. Atala is correct and the revised manuscript now incorporates the urologic importance of performing the study.

The following statements were introduced in the revised manuscript regarding the general urologic community:

A. .... most of the studies on transcription regulation in urology are related with oncology which makes it difficult to further illustrate the clinical relevance of our findings

B. It has to be taken into consideration that the approach of this study permitted the generation of a new testable hypothesis rather than the more traditional hypothesis-driven research. Indeed, the major question being answered by this work is what TREs can be
therapeutically targeted for reducing the influence of tachykinins in bladder disorders?

C. The overall hypothesis is that genes sharing the same TREs can be associated in a molecular network that will represent key pharmacological targets for modulating of the influence of tachykinins in bladder diseases.

Comment #2. The Introduction and Discussion sections are much too long and reflective of a review type article. Both sections could easily be cut in half and focused largely on the urologic importance of performing the study and the translational nature of the findings.

Answer: We agree and significantly reduced both the Introduction and Discussion sections and tried to focus largely on the urologic importance of the study and the translational nature of the findings.

Comment #3. The study would have been greatly strengthened if there were some information concerning the status of the bladder(s) from these animals. Histological or physiological data from the same bladders on which the molecular studies were performed would have been very helpful in understanding the importance of the findings.

Answer: Dr. Atala is correct. We added to the revised manuscript histological data from the same bladders on which the molecular studies were performed (new Figures 1 and 2).

Comment #4. What experimentally testable hypothesis results from this study? The authors have already nicely demonstrated the role of the NK1 receptor to antigen induced cystitis, so how does this new level of molecular analysis further impact mechanistic understanding of the disease process? That is, are any of the identified targets subject to pharmacological modulation in vivo or in vitro to establish their importance? That is, what is the therapeutic target that would result from this work? How would this information transfer to the clinic?

Answer: We can not thank Dr. Atala enough for requesting the intimate scope of this manuscript. We modified the whole manuscript in order to answer these pertinent questions.

Our introduction now reads:

it cannot be excluded that peripheral tachykinins may be involved in pathophysiologic afferent signaling associated with detrusor
overactivity [28]. However, despite promising effects in animal models, there seems to be no published clinical study showing that NK-receptor antagonists are an effective treatment of pain [31] or overactive bladder disease [28]. Therefore, in order to search for therapeutic targets that could block the tachykinin system, we set forth to determine the regulatory network downstream of NK1 receptor activation.

The synthesis of our answer to Dr. Atala is also in the conclusion: This work indicates an overriding participation of NK₁ receptors in bladder inflammation, provides a working model for the involvement of transcription regulators such as NF-kappaB, and Nkx-2.5, and evokes testable hypotheses regarding a role for tachykinins in the urinary tract pathology. It remains to be determined whether the control of Nkx-2.5 activity by gene silencing or double mutant negative blockers will ameliorate the clinical manifestations of cystitis.

Reviewer #2. Dr. Pedro Vera
The authors appreciate the kind words from Dr. Vera, an expert in this field. We also appreciate the synopsis that he wrote which place this work in perspective.

Minor Essential Revisions #1. Although TRE are first described in the introduction, a more detailed description of what they are and their significance would be helpful to the reader.

Answer: This is a very important concern that we tried to address by adding the following statements to the introduction:

.....despite promising effects in animal models, there seems to be no published clinical study showing that NK-receptor antagonists are an effective treatment of pain [31] or overactive bladder disease [28]. In addition, despite the known existence of NK₂ receptors in the human detrusor, the NK₂ receptor antagonist does not block the non-cholinergic contraction in unstable human bladder [32].

Therefore, in order to search for putative therapeutic targets that could be manipulated to reduce the influence of the tachykinin system, we set forth to determine the regulatory network downstream of NK1 receptor activation. This network is composed of genes and the transcriptional regulatory elements (TREs) that are putative binding sites for the transcription factors. In this way, we could define not only genes downstream of NK1 receptor activation but also the regulators of their expression. This is based on the fact that when active
transcription factors associate with TREs of their target genes, they can function to specifically repress (down-regulate) or induce (up-regulate) synthesis of the corresponding RNA. The overall hypothesis is that genes sharing the same TREs can be associated in a molecular network that would represent key pharmacological targets for modulating the influence of tachykinins in bladder diseases.

For this purpose, we used a combination of cDNA arrays and in silico analysis of TREs, as described previously [33]. cDNA array analysis defined the interactome of NK1-dependent genes by querying a web-based entry tool developed by Ingenuity Systems Inc [34]. Next, we uploaded the sequence of NK1-dependent genes into PAINT software and the respective TREs were identified using MATCH® tool in the TRANSFAC Professional database. Genes and TREs were assembled in regulatory networks and selected TREs were confirmed by EMSA.

**Minor Essential Revisions #2.** Pg. 11. Section b. the link under reference [55] does not appear to be working.  
**Answer:** We apologize for that but it seems that Clontech has changed the link. A new link [http://www.clontech.com/images/pt/PT3140-1.pdf](http://www.clontech.com/images/pt/PT3140-1.pdf) was added to the revised manuscript and we contacted Clontech for the authorization regarding presenting the list of all genes as supplemental material, if Dr. Vera thinks it is fundamental.

**Minor Essential Revisions #3.** Pg. 12. section f. “...gene should be upregulated (ratio between antigen- and saline-treated <3.0)...” This should probably read: “..saline treated >3.0...”.
**Answer:** Thank you so much for finding this important error.

**Minor Essential Revisions #4** Pg. 14. describing the control group (0 hours) the authors mention the bladders were removed without instillation. However, was the catheter still inserted? If not, please state this explicitly, since catheter insertion is likely to cause some irritation.
**Answer:** That is a very good point. The text was modified to better describe the methods, now it reads:

“an additional group, urinary bladders were removed without insertion of the catheter or fluid instillation”

**Minor Essential Revisions #5** Pg. 35 References. For ref. #52, please list the full reference, not the manuscript number. In addition, this
paper was published in 2006, not 2005 as listed. 
**Answer:** Thank you so much for catching the error. When we first drafted this manuscript, ref 52 was in press. The revised manuscript now has the proper reference (33).