Author's response to reviews

Title: Gene expression down-regulation in prostate tumor-associated stromal cells involves organ-specific genes

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Gene expression down-regulation in CD90+ prostate tumor-associated stromal cells involves potential organ-specific genes
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Dear BioMed Central Editorial Team,

Regarding our recently submitted manuscript, “Gene expression down-regulation in CD90+ prostate tumor-associated stromal cells involves potential organ-specific genes,” I wish to again thank the reviewers for their careful review and helpful commentary. Enclosed please find a revised version of the manuscript which I hope will satisfactorily address the issues and necessary corrections in review. For your convenience, extensive changes made have been highlighted in red within the manuscript.

The specific revisions incorporated in the manuscript and responses to issues raised by the reviewers are as follows:

**Reviewer 1**

**Abstract:**
1. Reviewer requested clarification regarding mention of ‘benign’ vs. ‘normal’ tissues.

We agree and have changed the Abstract text to ‘histologically normal’ rather than ‘benign’ tissues.

**Methods:**
2. Reviewer requested additional samples as validation of the current results

We agree that more samples would provide greater accuracy and precision in results. Because of the difficulty in obtaining quality data from sorted tumor samples, we have added a more detailed comparison to the whole tissue data in the Results section and Figures as validation of the differential expression we found.

See revised Figure 2, 4, 5 and Table 1 with accompanying text, changes are designated in red to assist further review.

3. Reviewer requested addition of bladder pathology in the Tissue Specimen section of the Methods.

We agree and have added the following text to the manuscript: The pathology characteristics of the tumor from which CB stromal cells were obtained were as follows: specimen 07A was a high-grade urothelial carcinoma, stage pT2N0.
4. Reviewer requested western blot control for Figure 1A.

PSA was used as a loading control and added to Figure 1A, descriptive also added to Methods section.

5. Reviewer requested CD13, and CD49a antibody source.

We agree and have added the antibody source for CD13 CD13-PE (WM15, 1:20, BD-PharMingen) to the Methods, MACS section. Data from CD49a sorted stromal cells was published previously (PMID: 16638148), text has been added to the Methods section, MACS cell isolation, to clarify this, and in the beginning of the Results section:

“The transcriptome for reactive stromal cells was determined for both CD90+ prostate stromal cells and for CD13+ reactive and normal bladder stromal cells, the transcriptome for normal prostate stromal cells was determined previously [19].”

6. Reviewer requested designation of the number of Affymetrix Genechips used.

We have added the following text to the end of the Methods, Gene expression section:

A total of 14 arrays were run for the following sample types obtained from 10 patients: 2 CD90+ prostate reactive stromal, 2 CD13+ normal bladder stromal, 1 CD13- bladder reactive stromal, and 5 whole tissue prostate cancer and 5 normal tissue from matched pairs. Additionally, 8 arrays were run for the following sample types obtained from 7 patients: 5 CD49– normal prostate stromal (published previously [19]), and 3 CD26+ prostate cancer (2 biological replicates, 1 sample run twice, Pascal et al., submitted).

Results:
7. Reviewer asked for clarification on which gene (GAPDH and/or ACTIN) was used for gene expression validation control.

For internal reference, either ACTA2 ($\alpha$-smooth muscle actin), GAPDH or RPLP0 (ribosomal protein P0) was used. Text has been added to Results and Figures to designate which housekeeping gene was used.

8. CD49+ first mentioned in the Results section – reviewer asked for addition to Methods.

This data was published previously, to clarify the source of the CD49+ NP stromal data, we have added the following text to the Results section:

“…compared to previously determined CD49a+ NP stromal cells… [19].”

9. Reviewer suggested dividing “down-regulation of organ-specific” section be further divided, i.e., Identification of potential organ-associated candidates, etc.

We agree and have made changes to the Results, see manuscript, red text.
10. Reviewer suggested adding statements of objective for experiments as emphasis.

We agree and have added objective statements to the beginning of each Results section, revisions are designated by red text within the Results section of the manuscript.

11. Reviewer requested emphasis on development genes CNTN1, SPOCK3 and MAOB.

We agree and have added further discussion in the manuscript, highlighted in red, within the Discussion section.

12. Reviewer suggested that genes identified here are not necessarily organ specific so much as they are differentially expressed by bladder and prostate, as only these two organs were analyzed here.

We agree that while the genes identified by this study are differentially expressed between the bladder and the prostate, we did not investigate whether or not they are expressed in other organs. We also agree they may therefore not necessarily be organ-specific, and have changed the title accordingly: “Gene expression down-regulation in CD90+ prostate tumor-associated stromal cells involves potential organ-specific genes.” We have also changed the text to reflect that these genes are putative rather than definitively organ-specific.


This Figure has been revised and reference to CD49/PELO removed.

**Figures:**

14. Figure 1A, validation of CD13+ by FACS

Add further discussion on the validation of bladder. Regarding the further validation of the bladder data, at this point we are unable to assess it to the same degree as we have the prostate data. We have several prior papers with extensive analysis of the various prostate cell types and their expression profiles, whereas to date we have two prior papers discussing the differences between bladder and prostate stroma (Goo *et al.*, 2005 and 2008). We have added text to the Discussion noting the need for further more complete analysis of the bladder cell types (in red).

15. Figure 1B, signal intensities refer to what? 3 samples, 2 intensities, Raw, unadjusted values?

Figure 1B was revised as a graph rather than the previous database format for clarification. Additionally, text was added to the Results section as well:

“The data were reported as robust multi-array average (RMA) [40] normalized Affymetrix signal intensities implemented in the in-house analysis pipeline SBEAMS [41] or as its logarithmic value: \( X = \log_2(\text{Cancer normalized intensity}/\text{Normal normalized intensity}) \).”
16. Figure 4, ACTIN control levels for all samples examined?

ACTA2 was examined for all samples however only the result for the CP1/NP1 was shown.

17. Table 1 shows decrease in ACTIN in CP vs NP but no difference when used as control in Figure 4 RT-PCR results, please explain.

The expression level of ACTA2 is in the abundant class, the RT-PCR in Figure 4 was used to determine presence or absence of the organ-specific genes. At 35 cycles, no difference was seen for CP vs NP ACTA2 levels as the reaction had reached plateau.

18. Figure 4, relabel, in text C not mentioned, format is confusing – GAPDH and not ACTIN used as control? Need actin control for all samples not just 1 sample pair, signal intensities correspond to what? Similar questions for other figures where intensities are designated.

ACTIN was used as a control, the text has been amended accordingly.

Minor Revisions:
19. Present address, corresponds to which authors?

Clarified on Title page

20. 27 prostate samples used for this study? Not entirely accounted for?

Revised text to accurately describe sample numbers. See Revision item 6.

21. Clinical sample designations are difficult to follow, suggest simplifying as TS1, TS2.

These codes were used to allow us to link our data to pathology descriptions. In order to minimize confusion we have revised their usage within the text.

22. Methods, Gene Expression section, authors write “samples showing no evidence of RNA degradation were used” would suggest adding RNA integrity numbers

For this study, we did not determine RNA integrity numbers. Instead, RNA quality was determined using the Agilent 2100 Bioanalyzer according to manufacturer’s protocol. RNA quality was determined through visual inspection of the electropherogram. RNA was considered of sufficient quality by presence of 18S and 28S ribosomal peaks, the absence of smaller well-defined peaks between the two ribosomes, and the baseline between 29 seconds and the 18S ribosome being relatively flat and free of small rounded peaks corresponding to smaller RNA molecules. Text was added to reflect this:

“For this study, we did not determine RNA integrity numbers. Instead, RNA quality was determined using the Agilent 2100 Bioanalyzer according to manufacturer’s protocol. RNA quality was determined through visual inspection of the electropherogram. RNA was considered of sufficient quality by presence of 18S and 28S ribosomal peaks, the absence of smaller well-defined peaks between the two ribosomes, and the baseline between 29 seconds and the 18S ribosome being relatively flat and free of small rounded peaks corresponding to smaller RNA molecules. Text was added to reflect this:”
23. Suggest that FACS section be added after MACS instead of as part of Tissue Section

We agree and have added a separate FACS section.

24. Section beginning “Comparison to the whole transcriptome datasets” since the PENK gene is not represented until Fig 3 it is confusing in current written format.

We agree and have moved this discussion.

25. Figure 1 legend is mislabeled. Data includes Western and Affymetrix results, not just Western. Figure 4 legend is confusing and Figure 4C is not mentioned in text. Legend for Figure 5 and Table 1 is missing. Reference 29 is incomplete.

We have revised the legends for these figures and tables and fixed the reference.

Reviewer 2

1. Authors not presented title for Table 1

2. Reviewer noted the absence of a legend for Figure 5

Revised as requested.

We greatly appreciate the thorough review of our manuscript. The constructive suggestions helped us to greatly improve and focus our work for this revised manuscript.

The authors indicate no potential conflicts of interest.

Sincerely,

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