Author's response to reviews

Title: Analysis of gene expression in prostate cancer epithelial and interstitial stromal cells using laser capture microdissection

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Author's response to reviews: see over
Dear Editor,

We would like to thank you and the reviewers for helpful suggestions and comments. We have revised the manuscript according to the comments. Our point-by-point responses are given below. An additional change of name for the first author, Ms. Reese, is included, as Jennifer has married and changed her name to Jennifer L Gregg.

Sincerely yours,
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Point-by-point responses to comments:

Replies to Reviewer #1

Comment 1) Interaction of prostate stroma with adjacent benign and malignant glands is of seminal importance for normal prostate function, and tumour biology. While many proteins are differentially expressed within the stromal and epithelial compartments in the prostate, relatively little is known about the molecular pathways regulating stromal-epithelial interaction. In this manuscript Reese and colleagues use an elegant micro-array methodology after specifically selecting stroma and malignant glands form human prostate cancer by laser capture microdissection.

We thank the reviewer for insightful comments and agree that understanding the interaction of the prostate stroma with adjacent glandular epithelium is critically important for both normal prostate and tumor progression. Indeed, one of the major goals of our paper was to provide baseline information needed for future studies of this interaction. We have better emphasized this point in the revised manuscript.

Major compulsory comments:

Comment 2) While gene-expression analysis on pure cell populations represents a powerful tool to explore important pathways for stromal-epithelial pathways in both normal and malignant prostate tissue, the current manuscript is rather descriptive and does not clearly state a rationale goal for its stromal and epithelial analysis. For instance it would have been very interesting to correlate expression patterns of ‘tumour-associated’ stroma with normal stroma.

A major goal of this paper was to address the lack of information regarding pattern of gene expression in stromal tissues and, in particular, delineating those genes whose expression is enriched in stromal compared to epithelial cells. As a result, distinct gene expression patterns were identified in the two different prostate cell populations. Another goal was to determine whether some of the genes previously described as “expressed in prostate cancer” were actually expressed to a greater extent in stromal tissues than in epithelial. This distinction is important to establish potential interactions between tumor stromal and epithelial cells. We have clarified the rationale for our study both in the abstract (page 2) and background (page 3) sections.

Regrettably, no tissue from normal (healthy) prostates was available for LCM, so it was not possible to compare tumor stroma and normal stroma using the LCM microarray approach.

Comment 3) After the thorough analysis of the gene-expression data, the authors validate 6-10 interesting genes on a set of tissue specimens and cell lines. The validation strategy is however somewhat remarkable. First, the authors analyze 6 genes on LCM purified cell populations. Currently, it seems that this validation set is not independent, but rather equals the test-set. For proper validation, however, an independent tissue set is required. Second, the authors validate their gene set in a group of ten non-purified paired frozen tumour and non-neoplastic tissues, which are generally constituted of a large mix of cell types. While a very elegant way of gene discovery is used in this study, interpretation of the validation data is limited due to the contamination of various cell types.

We thank the reviewer for this opportunity to clarify the text on pages 8 and 9, where inappropriate use of the
term “validation” has now been corrected, as an independent set of LCM captured tissue was not available for analysis. However, real-time PCR was used to more precisely quantify expression differences in the test-set of LCM captured tissue, because results of microarray analyses alone have a high probability of false positives.

Then, based on the results of the microarray and real-time analyses, expression levels of the identified genes were quantified in non-purified paired tumor and non-neoplastic tissues. These experiments were not meant to validate the microarray, but rather to apply our knowledge of tumor tissue gene expression patterns to additional paired tumor and non-neoplastic samples.

Comment 4) The validation of the gene-expression profile in cell lines is interesting for future function studies, but has very limited value for representability as validation set obtained from patients’ tissues. Also, one cannot draw strong conclusions comparing normal and cancer cells since only one benign cell line was included.

We have now clarified this point on page 8. Prostate cancer cell lines were characterized, but these experiments were certainly not meant as validations of the microarray. Additionally, the single normal prostate epithelial cell line (RWPE) was used for normalization of gene expression in the several tumor cell lines analyzed by real-time PCR.

Minor essential comments:

Comment 5) Page 3, first paragraph: the text can be formulated more properly. For instance, “prostate adenocarcinoma is characterized by invasion of luminal spaces”

Text on page 3 has been corrected to read “Prostatic adenocarcinoma is characterized by invasion of the lumen and underlying stroma by malignant epithelial cells (reviewed in [2]).”

Comment 6) Page 5, top: “Areas of the slides observed to have the most abundant cell of interest were identified.”. It is not clear whether the stromal cells are derived from the same area as the malignant cells. Often stroma contains inflammatory cells; was the presence of inflammation accounted for?

We thank the reviewer for pointing out this oversight. Page 5 has been clarified to read “Stromal cells were collected from areas adjacent to glandular epithelium …”. Additionally, we have now explained in the Methods (pages 4 and 5) that visual inspection of samples used in this study revealed similar proportion of inflammatory cells, i.e., page 4 (under Tissue Acquisition) now reads “Stromal tissue of all three samples appeared to contain a similar proportion of inflammatory cells.”

Comment 7) Page 9, top: “As expected, the stromal genes are more highly expressed in non-neoplastic tissue than in paired tumor tissue”. Why is this expected? Was this because normal tissue obtains more stromal cells? What was the tissue composition in respect of stromal cells, inflammatory cells, pre-existent glands and cancer glands?

Although the precise tissue composition of the paired samples is not known, our collaborating pathologist Dr. C. Magi-Galluzzi informed us that there were significant amounts of tumor epithelial cells in the tumor samples of each pair. Therefore, we expected to see expression of predominantly epithelial genes, whereas normal tissue samples lacked the abundant tumor epithelial cells and instead had a larger stromal cell component. Also, as discussed above, the stromal component was expected to be heterogeneous and to include inflammatory cells. The statement regarding the expected results for paired tissue samples has been clarified. Pages 8 (bottom) and 9 (top) now reads “Overall, the expression patterns in non-neoplastic tissues from paired samples were consistent with those in stromal cells obtained from laser capture microscopy (Figure 2 Bottom). These results can be attributed to relatively fewer epithelial cells in the normal tissue samples. Thus, as a reflection of stromal cell prevalence, the stromal genes are more highly expressed in non-neoplastic tissue than in the paired tumor tissue”.

Comment 8) Page 9, middle: what is the rationale of analyzing the proteins separately in T2 and T3 tumours?

Although our LCM samples were all primarily Gleason grade 3 (with combined Gleason scores of 6 and 7), we asked whether there was any relationship between gene expression and clinical stage in the paired sample set (all Gleason score 7). The rationale for this analysis has now been specified. Page 9 (middle) now reads “Data was analyzed by clinical stage to determine whether there was any relationship between clinical stage, especially invasiveness, and gene expression.”
We thank the reviewers for the opportunity to elaborate on the significance of these IHC results. Importantly, we believe that the identification of WT1 protein in the cytoplasm appears to be specific, as both BPH and normal tissues are not stained by WT1 antibodies. Additionally, controls lacking WT1 antibodies did not stain cytoplasm of the test slides.

As shown below, we have expanded the discussion of the potential functions of cytoplasmic WT1 protein.

Page 10 now reads: “Although the exact function of cytoplasmic WT1 remains to be elucidated, WT1 can shuttle between the nucleus and cytoplasm as it contains both a nuclear localization signal and a nuclear export signal [49]. One caveat is that cytoplasmic WT1 protein could be of one specific isoform, as antibody staining cannot distinguish among the various isoforms of the WT1 protein. It is possible that cytoplasmic protein is transcriptionally inactive, indeed the phosphorylated form is thought to be retained in the cytoplasm [50, 51]. Another possibility is that the cytoplasmic function is post-transcriptional; surprisingly, it has been shown that both +KTS and -KTS isoforms can function as shuttling proteins and both associate with polyA RNPs and polysomes. [52].

Comment 10) Figure 3: it is not entirely clear what is represented in this figure.

For clarity, data in Figure 3 is now presented in tabular format. Table 5 now shows “Gene expression in tumor tissue relative to non-neoplastic tissue.” Depicted is the quantitative real-time PCR analysis of 20 paired prostate tissue samples. Gene expression levels were determined by the ddCt method after normalization using 18s rRNA primers. Expression in tumor relative to non-neoplastic tissue is depicted as “fold-change”. Listed in the table is the number of paired samples with elevated expression in tumor compared to non-neoplastic tissue (fold change Up values ≥ 2.0) and those with no change or reduced expression (No change/or Fold-change down values ≥ 2.0) in tumor relative to normal (non-neoplastic) tissue. Data is presented for each gene and clinical stage.

Comment 11) Figure 5: the non-neoplastic and BPH tissues look rather similar. How are both defined in this study?

We thank the reviewer for the opportunity to clarify the text.

Revised legend of Figure 4 (previously Figure 5): “5 samples of normal tissue from cancer-free prostates were analyzed”. Normal or non-neoplastic samples were obtained from cancer-free prostates (from normal individuals) whereas BPH samples were obtained from prostates with identified areas of benign hyperplasia. All tissues were selected and evaluated by pathologists using standard criteria, prior to creation of TMA by Creative Biolabs. Additional information about tissue composition has been added to Page 4 under Tissue Acquisition section: “Normal samples were obtained from cancer-free prostates from normal individuals. All tissues were selected and evaluated by an independent pathologist who determined Gleason grading and differentiation status”.

Replies to Reviewer #2

Comment 1) In order to make sure the microarray data, the authors re-examined expression levels of 10 representative genes as shown in the figure 2. As mentioned by the authors, one of the 10 genes (i.e. EGR1), did not show the consistent data. Although the reason of this disconcordance has been discussed in the text, additional data such as immunohistochemical data of clinical samples would be helpful to show the significance of the higher expression of EGR1 observed in the microarray analysis.

We thank the reviewer for his comments regarding the surprising EGR1 expression data, showing that expression in Gleason 7 paired tumor samples was not higher than in non-neoplastic control samples. Although microarray data suggested that EGR1 expression was higher in tumor epithelial cells than in stromal, these results were not confirmed by quantitative real-time PCR. We have not examined high-grade tumors and do not have immunohistochemical data for EGR1 expression, but have begun a new series of studies focused on EGR1 expression in prostate cancer.