Title: Effect of butyrate and cancer progression on keratin 8 expression in the colon

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Version: 2 Date: 21 September 2010

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

Thank you for the opportunity to address the careful comments made by each of the reviewers of our manuscript. We have listed our responses to the comments and changes made accordingly below, and we have highlighted changes made in the manuscript re-uploaded to identify changes in the papers itself.

Kind regards,

Bernard Corfe

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Reviewer’s report

**Title:** Effect of butyrate and cancer progression on keratin 8 expression in the colon

**Version:** 1  **Date:** 25 June 2010  
**Reviewer:** Leah Cosgrove

**Reviewer’s report:**

2. Are the methods appropriate and well described? Yes, the methods are appropriate and sound. The only issue in regards to this is the specificity of the Keratin 8 antibody. Does it cross react to any other keratins? Also with the authors data for the western blot where they aligned this to butyrate expression there appears to be a number of different bands. Is this due to cross reactivity or different phosphorylated forms of keratin. This was highlighted by the following publication These authors found the absolute levels of Keratin 8 (the non- phosphorylated forms of keratin 8 did not change with progression of disease but the phosphorlyation did. Was there a loading control for this western? Also the western ran very skewed and there were a number of different bands in some track but this was not commented on by the authors.

We thank the reviewer for highlighting this possible interpretation of the data regarding additional bands on the gel cross-reacting with the K8 antibody. We have used an antibody with claimed specificity for K8. Although we have not determined this claim exhaustively empirically ourselves we know that the antibody does not cross-react in our hands with Keratin 6, 17, 18, 19. The antibody is a well-recognised and widely used monoclonal (M20) and we have included the reference to this in the methods over and above the Abcan catalogue number.

Regarding the additional bands, we believe these are degradation products rather than phosphorylation-induced bandshifts, and we are currently evaluating mass spec data (from breast cancer material) potentially supporting this interpretation. In the Mizuuchi paper the treatment with PRL3 inhibitor which elevated phosphorylation at S73 and S431 did not induce any change in mobility of the cross-reacting keratin 8 band. We have nonetheless referenced this paper and suggested that these or other PTMs, including proteolytic cleavage, may be at the root of the appearance of lower Mw forms.
7. Do the authors clearly acknowledge any work upon which they are building, both published and unpublished? The only publication that requires citing is the publication inserted in question 2.

Please see above
Reviewer's report
Title: Effect of butyrate and cancer progression on keratin 8 expression in the colon
Version: 1 Date: 24 June 2010
Reviewer: Charles Giardina

Specific comments
1. The work described in the manuscript is technically very well done. However, the relationship between butyrate levels, keratin 8 expression and cancer development is muddled. Although this study is unlikely to lead to definitive conclusions, the authors’ views on how keratin 8 expression in normal and cancer tissue impacts colon cancer development needs to be more clearly articulated. Do lower levels of keratin 8 expression sensitize the cancer cells to apoptosis? Does lower keratin 8 expression lead to a less differentiated/more aggressive cancer? A clearer interpretation of the findings needs to be provided (starting with the abstract).

We have included an extended paragraph in the discussion to expand on the implications of altered keratin expression on prognosis, and highlighted that there are other examples of chemoprotective nutrients with a potential role in driving progression once colorectal lesions have formed.

2. With regard to the influence of butyrate on cytokeratin 8 expression, it should be clarified whether this is a direct or indirect effect of butyrate. This could be assessed by determining whether butyrate influences keratin 8 expression in colon cancer cell lines.

The experiments have been undertaken, however the data would appear superficially counter-intuitive in this paper as in vitro (in one cell line) butyrate increases keratin expression. Please see response below to Beat Schwaller’s comments, as there are several plausible explanations for this discrepancy.

3. Butyrate levels are measured at a single time point. Is there any information on the intra-individual stability of fecal butyrate levels? If so, this information should be provided and/or cited.

There are relatively few papers in this area, however we have identified some and have cited and discussed intraindividual variation and choice of butyrate measure in the discussion of limitations.
Reviewer: Beat Schwaller
Reviewer’s report:
Major Compulsory Revisions
1) First of all, the number of samples (e.g. 14 cancer patients) appears to be rather small to make any significant conclusions. Also based on the fact that authors state that “There was a wide variation in staining patterns probably because of regional heterogeneity of the tissue”.

Not clear what revision is required here by the reviewer. We have cited the small sample size as a limitation of the study. The collection of resected material is generally not challenging as attested by the large number of papers in this area, however it is much more difficult to obtain stool specimens from cancer and endoscopy subjects, and the sample set took over 2 years to obtain as part of a larger study (Corfe et al., 2009).

2) Is there any reason (physiological or other) to separate into two groups (high and low butyrate), with 4 mM being the separation line? The data should be presented as a scatter plot with butyrate concentration on the x-axis and the score on the Y-axis. From this plot a regression line should be calculated.

In addition, when the 3 scores were determined for the High and Low butyrate group as shown in Table 3, none of the 6 values (3 from MS, 3 from CO) were significantly different between the groups, also shedding some doubt on the sturdiness of the results presented in Fig. 3B.

As clearly stated at the start of the paragraph describing the results, the data were split into haptile by butyrate level. There is no subliminal biological implication, this is merely a statistical way of dividing the data across the median and is commonly used.

We have undertaken a systematic analysis by scatter plot and correlations calculated for butyrate versus each K8 score at each location relative to the lesion. We have included the data in the table below. The data do not reach significance, most likely due to the very small sample number in this study. We feel that these data would not add value to the manuscript.

Regarding the apparent discrepancy between data in table 3 and figure 3, the data in table 3 refer to morphologically normal tissue at two sites in the colon; the data in Figure 3 refer to tumour tissue. One of the key points we try to suggest is that the tumour tissue is different in its response to butyrate than the
3) As reported here, the butyrate level was determined from one stool sample per patient. How representative is this value for the average butyrate level to be found in the stool of this person? It is expected that depending on the diet this value may change considerably. In another study with the involvement of some of the co-authors of this study, the tested subjects will use a high fiber diet for 8 weeks before experiments will be carried out to assess the role of butyrate (Corfe et al. (2009) BMC Cancer 9: 332). Thus, selecting just a singular point (butyrate concentration) is considered to be insufficient to make a statement about the putative role of butyrate on K8 levels, even less that “butyrate may down-regulate the expression of K8 in the cancerized colon.”

The intraindividual variation issue was also raised by another reviewer and was addressed above. A key and perhaps surprising finding of that study was that there are relatively weak relationships between diet and SCFA measures. The as-yet-unpublished findings of the Corfe et al study (a protocol paper is cited, the outcomes of the work are in analysis or are in preparation) are that there is no dose-response relationship between fibre intake and SCFA level. We have undertaken a meta-analysis of fibre interventions and shown that although there are increases in SCFA in response to fibre, there is no dose-response relationship across studies (Barker, Russell & Corfe in preparation).
Taken together these data suggest that SCFA concentrations do vary but are a more stable measure than total output (which accounts for faecal mass and hydration). As stated above we have successfully identified relationships in other studies.

4) In the Western blot shown in Fig. 4, besides a major band at around 40 kDa, many lower bands are detected by the antibody. No information is provided about the identity of these bands. Moreover, the pattern in the sample with 2.4 mM butyrate looks completely different with respect to size distribution of bands recognized by the antibody. Which signals were analyzed in this sample? No information is given how the bands were quantified: Only the upper band assuming it to be the full-length K8? All bands? What was used as the loading control for the Western blots? Without this information, Fig. 4B can’t be interpreted.

This point was also made by Dr Cosgrove (see above). We have included a discussion of the additional bands.

5) To demonstrate that butyrate directly affects K8 expression, the authors need to perform these experiments in a controlled in vitro system, e.g. using colon cancer cells. There they should show that butyrate down-regulates K8 in a butyrate-concentration dependent way. Furthermore, they could also use a reporter assay to show that the promoter region of K8 has elements that act as butyrate-dependent repressors.

We undertook a study in vitro examining the relationship between butyrate and keratin 8 expression. Our data showed an increase in expression with butyrate and indeed with other SCFA in HCT116 cells. How might this be reconciled with the in vivo finding? Firstly the data were necessarily obtained from growing cells (as is the case with most or all cell culture experiments) whereas fewer of the cells in tumour are actually in cycle. Furthermore the cells in vivo are subject to a range of contacts with each other and with substrate, microenvironmental factors known not to be adequately replicated in vitro. Finally we might choose to argue that the increase in expression with increasing butyrate in vitro in fact represents a reasonable model of the increasing expression along the crypt-villus axis where cells are exposed to increasing concentrations of butyrate over longer periods after birth from the stem cell compartment.

The regulation of the keratin promoter region through histone acetylation has been previously reported: “Prochasson P, Gunther M, Laithier M, Fossar N, Lavialle C, Brison O. Transcriptional mechanisms responsible for the overexpression of the keratin 18 gene in cells of a human colon carcinoma cell line. Exp Cell Res. 1999 Apr 10;248(1):243-59.” We feel the experiments proposed represent an enormous body of work which would justify a complete and separate manuscript (indeed our analysis of the Bak promoter and its response to butyrate formed such a paper – Chirakkal et al., 2006, Oncogene) and would not necessarily add to the information in this paper which are
concerned with the characterisation and quantification of keratin expression in the colon \textit{in vivo} and its regulation by cancer field and by butyrate.