Canterbury Medical Research Foundation

Project Grant

Application for 2016 PRO from Dr Rachel Purcell

Applicant

Dr Rachel Purcell
University of Otago
Surgery

rachel.purcell@otago.ac.nz
02102476749
University of Otago, 2 Riccarton Avenue PO Box 4345 8140

Application status

$88,215 approved by CMRF with reference PRO(16/07)

A. Project overview

1. Title of project

Contribution of enterotoxigenic Bacteroides fragilis to colorectal carcinogenesis

2. Short title of project

Gut bacteria and colorectal cancer

3. Project dates

Start date: 01/11/2016

Completion date: 01/10/2017

4. Media summary of research

Colorectal cancer is a common type of cancer, and while recent evidence suggests that gut bacteria may increase risk, the exact mechanisms remain elusive. Bacterial infections can cause chronic inflammation, which has been linked to cancer development. The *Bacteroides fragilis* toxin potentially alters intracellular signalling pathways that are also linked to cancer development. We will explore how chronic inflammation as a result of enterotoxigenic *B. fragilis* (ETBF) infection is associated with development of colorectal cancers, including whether genetic mutations arise as a result of this chronic inflammation. We will also investigate the role of microRNAs as a potential mechanism that regulates this process. MicroRNAs are small RNA molecules that do not code for proteins, but have recently been shown to play important roles in many cellular processes, including the development of cancer. We hope that this project will identify novel markers for the early detection of colorectal cancer.

5. Keywords

1. Bacterial toxin
2. Cell signalling
3. Colorectal Cancer
4. Gut microbiota
5. Inflammation
6. MicroRNAs

B. Proposed investigation

1. Project title

Contribution of enterotoxigenic Bacteroides fragilis to colorectal carcinogenesis

2. Health significance rationale

Colorectal cancer (CRC), the second most common cancer worldwide, is a major health problem in New Zealand(1), and South Canterbury has the highest mortality rate for CRC in the country. Research into the genetic components of CRC has highlighted that most cases (> 90%) are sporadic,

and follow a pattern one would expect from an environmental source. There is increasing evidence of a role for bacterial pathogens in the development of CRC, but a concerted effort is needed to definitively identify the pathogens involved, and the means by which they initiate CRC. This will allow the implementation of bowel cancer screening programs and the development of effective vaccines and preventative therapies.
Of particular interest to our group is the contribution of enterotoxigenic Bacteroides fragilis (ETBF) to the development of CRC. These bacterial species, which are considered directly pro-oncogenic and capable of remodelling the mucosal immune response and colonic bacterial community, have been proposed as “drivers” of CRC(2). Our group has found that ETBF is present in stool samples of CRC patients at a significantly higher frequency than in age-matched controls from the Canterbury population (Keenan et al, in press), and that ETBF is significantly associated with low-grade dysplasia and tubular adenoma from colonoscopy patients in the Canterbury region (Purcell, manuscript in preparation). ETBF produces a virulence factor called B. fragilis toxin (BFT), that induces persistent colitis in mice(3), and disrupt E-cadherin junctions, activate β-catenin signalling, and induce IL-8 secretion in colonic epithelial cells(4-8). We wish to investigate the signalling mechanisms involving IL-8, β-catenin, and E-cadherin that modulate acute to chronic inflammation in response to pathogenic bacterial infection.
MicroRNAs reportedly act as a bridge between chronic inflammation and carcinogenesis(9), and we will also study their role in modulating the balance between pro- and anti-inflammatory cytokines in CRC development.

3. Research design

Research hypothesis We hypothesize that infection with toxin-producing strains of *B. fragilis* (ETBF) induces chronic inflammation in colonic epithelial cells (CEC) by a signalling mechanism involving beta-catenin and IL-8/Stat3, and that constitutive activation of this signalling mechanism contributes to early-stage precancerous changes. We also hypothesize that microRNAs may play a role in the regulation of this process.
Background IL-8 is a potent inflammatory chemokine, and its secretion by CECs leads to downstream activation of numerous signalling pathways involved in cell survival, angiogenesis and cell migration/invasion. IL-8 signalling has also been shown to activate Stat3(10), which regulates various cell functions, including acute inflammation. However, when Stat3 is constitutively activated, it acts as an oncogene, promoting chronic inflammation and tumourigenesis(11). Recent evidence suggests that Stat3 may be involved in skewing the host response towards tolerance at the cost of immunity in *H. pylori(12)* and ETBF(13) infections. Stat3 activation has also been associated with ETBF-associated colitis(14) and colonic tumour development in mice(13).
ETBF also reportedly stimulates the secretion of IL-8 through E-cadherin disruption(5), which releases bound β-catenin and allows nuclear translocation and subsequent TCF-dependant transcription of IL-8(15), and several studies of ETBF in CECs have reported increased β-catenin pathway activation(16). β-catenin signalling is believed to have a role in modulating acute inflammation(17,18), which may be necessary for chronic inflammation, hypothesised to play a role in carcinogenesis.
The potential contribution of miRNAs to the development of CRC from inflammation related to bacterial infection has not been studied to date. MiRNAs have been shown to be involved in every aspect of carcinogenesis(9), and reportedly act as a bridge between chronic inflammation and carcinogenesis in gastric cancer related to infection with the carcinogen, *H. pylori* (19), and this may occur by modulating the intricate balance between pro- and anti-inflammatory cytokines(20).
Methods
Cell lines and bacterial strains Three colonic adenocarcinoma cell lines, HT29, SW480, and HCT116 (currently available in our laboratory) will be cultured as previously described. *B. fragilis* strain VPI13784, an enterotoxigenic strain shown to induce a persistent colitis in mice(3), will be grown in culture broth in anaerobic conditions.
Objective 1. Cell lines will be cultured in 24-well plates until confluent. Varying multiplicities of infection (MOI) of ETBF will be incubated with cell lines for 24 hours. Cells will be harvested, washed and lysed in Nonidet P-40 buffer for subsequent protein analysis. Cells will be harvested into RNAlater for subsequent nucleic acid analysis. RNA will be extracted from cells in RNAlater using RNEasy mini kit (Qiagen). RNA will be reverse transcribed using Transcriptor RT kit (Roche) and the

resultant cDNA will be used in qPCR analysis to determine changes in the levels of the IL-8 receptors, *CXCR1* and *CXCR2*, in response to co-culture with ETBF. qPCR will be carried out on a Roche LightCycler480 instrument using with appropriate house-keeping genes. Levels of IL-8 in cell culture medium will be determined using enzyme-linked immunosorbent assay (ELISA) using R&D Systems Quantikine. Cells will also be grown on coverslips and treated as above. Following incubation with bacteria, these cells will be fixed using paraformaldehyde. Immunocytochemistry (ICC) will be carried out using a primary antibody against beta-catenin, to investigate nuclear translocation (activation) occurs in response to ETBF in colonic epithelial cell lines.
The analyses from this objective will determine the optimal MOI for use in subsequent experiments. Different time-points may be used to determine the optimal incubation periods needed.
Subsequently, cell lines will be co-cultured with ETBF as described above. Inhibitors of Stat3, IL-8, CXCR1 and 2, beta-catenin, and NF-kB will be added at appropriate concentrations to the cell/bacterial co-cultures. Cells and supernatants will be harvested as outlined in the previous objective for RNA and protein analysis. Levels of IL-8 will be determined by ELISA, while protein levels of Stat3, E-cadherin, and beta-catenin will be determined by western blotting. The appropriate polyclonal antibodies, followed by goat anti-rabbit secondary antibody conjugated to horseradish peroxidase, will be visualised by enhanced chemiluminescence. RT-PCR will be carried out to determine changes in mRNA levels of *CXCR1* and *CXCR2*, *CCND1* (the gene for cyclinD1, which is transactivated by beta-catenin), and *CXCL8* (the gene coding for IL-8). ICC analysis of treated cell lines using primary antibodies to E-cadherin, beta-catenin, and Stat3 will allow visualisation of intracellular changes in the location of these proteins in response to treatment.
Outcome Our approach, which is similar to published reports regarding *H. pylori* in gastric cancer, will help to elucidate the role of beta-catenin in the induction of the pro-inflammatory cytokine, IL-8, in response to ETBF infection in colonic epithelial cells. We believe that the use of targeted inhibitors will provide evidence of an autocrine signalling loop, which may partly explain how chronic inflammation in response to ETBF, could play a role in early-stage colonic carcinogenesis. We hope to demonstrate a link between beta-catenin and Stat3 signalling in the induction of IL-8 secretion in response to ETBF.
Objective 2. To investigate the contribution of specific microRNAs to ETBF-activated pathways, we will culture our cell lines as outlined previously. Cells will be harvested following co-culture with bacteria with/without pathway inhibitors and stored in RNAlater. MicroRNA will be extracted from cells using miRNeasy microRNA extraction kit (Qiagen). qRT-PCR will be carried out using TaqMan probes (Life Technologies) to identify changes in the relative abundance of specific miRNAs in response to ETBF colonization of the cells. Any changes noted will be analysed in relation to changes in their putative mRNA targets from Objective 1. The miRNAs to be analysed include: miR-21, miR-200 and miR-205, which are involved in E-cadherin disruption and Wnt/β-catenin signalling(21,22); and miR-221 and miR-222, which have been implicated in constitutive Stat3 and NF-κB signalling in colon cancer cells(23). Additional miRNAs may be also be considered.
In order to validate any changes in miRNA levels seen, we will grow our cell cultures with bacteria as described previously, with and without the addition of *mir*Vana® miRNA inhibitors (Ambion), which allows silencing of specific miRNAs following transfection into cultured cells. RNA will be extracted from the cells, as outlined previously, and changes in the relative abundance of our genes of interest, will be measured using qRT-PCR as described above.
Outcome Specific miRNAs have already been proposed to play a role in beta-catenin and Stat3/IL-8 signalling in various cancers, and in relation to *H. pylori* and gastric cancer. We believe our approach will clarify the contribution of these miRNAs in ETBF-related activation of pathways involved in the establishment of chronic inflammation in colonic epithelial cells. This will provide further insight into early changes that may contribute to colorectal carcinogenesis. MicroRNAs also represent potential therapeutic targets that have promising applications in the clinical setting.
Statistical analyses For the in vitro study, the effect of BFT colonization on the relative abundance of mRNAs and miRNAs of interest will be analysed using the ANOVA test, with appropriate post-hoc testing. Differences between conditions will be considered statistically significant if the p-value is < 0.05. Dr John Pearson (Biostatistician, Dept. of Population Health, University of Otago, Christchurch) has been consulted in regards to this project and will provide assistance with statistical analysis, where appropriate.

4. References

1. Group, N. Z. G., Bowel cancer: Information for people at increased risk of bowel cancer. Health, N. Z. M. o., Ed. 2012.
2. Tjalsma, H.; Boleij, A.; Marchesi, J. R.; Dutilh, B. E., A bacterial driver-passenger model for colorectal cancer: beyond the usual suspects. *Nat Rev Microbiol* **2012,** *10* (8), 575-82.
3. Rhee, K. J.; Wu, S.; Wu, X.; Huso, D. L.; Karim, B.; Franco, A. A.; Rabizadeh, S.; Golub, J. E.; Mathews, L. E.; Shin, J.; Sartor, R. B.; Golenbock, D.; Hamad, A. R.; Gan, C. M.; Housseau, F.; Sears, C. L., Induction of persistent colitis by a human commensal, enterotoxigenic Bacteroides fragilis, in wild-type C57BL/6 mice. *Infect Immun* **2009,** *77* (4), 1708-18.
4. Wu, S.; Lim, K. C.; Huang, J.; Saidi, R. F.; Sears, C. L., Bacteroides fragilis enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc Natl Acad Sci U S A* **1998,** *95* (25), 14979-84.
5. Hwang, S.; Gwon, S. Y.; Kim, M. S.; Lee, S.; Rhee, K. J., Bacteroides fragilis Toxin Induces IL-8 Secretion in HT29/C1 Cells through Disruption of E-cadherin Junctions. *Immune Netw* **2013,** *13* (5), 213-7.
6. Wu, S.; Powell, J.; Mathioudakis, N.; Kane, S.; Fernandez, E.; Sears, C. L., Bacteroides fragilis enterotoxin induces intestinal epithelial cell secretion of interleukin-8 through mitogen-activated protein kinases and a tyrosine kinase-regulated nuclear factor-kappaB pathway. *Infect Immun* **2004,** *72* (10), 5832-9. 7. Sanfilippo, L.; Li, C. K.; Seth, R.; Balwin, T. J.; Menozzi, M. G.; Mahida, Y. R., Bacteroides fragilis enterotoxin induces the expression of IL-8 and transforming growth factor-beta (TGF-beta) by human colonic epithelial cells. *Clin Exp Immunol* **2000,** *119* (3), 456-63.
8. Wu, L.; Ruffing, N.; Shi, X.; Newman, W.; Soler, D.; Mackay, C. R.; Qin, S., Discrete steps in binding and signaling of interleukin-8 with its receptor. *J Biol Chem* **1996,** *271* (49), 31202-9.
9. Iorio, M. V.; Croce, C. M., MicroRNA dysregulation in cancer: diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol Med* **2012,** *4* (3), 143-59.
10. Burger, M.; Hartmann, T.; Burger, J. A.; Schraufstatter, I., KSHV-GPCR and CXCR2 transforming capacity and angiogenic responses are mediated through a JAK2-STAT3-dependent pathway. *Oncogene* **2005,** *24* (12), 2067-75.
11. Zhao, J.; Dong, Y.; Kang, W.; Go, M. Y.; Tong, J. H.; Ng, E. K.; Chiu, P. W.; Cheng, A. S.; To, K. F.; Sung, J. J.; Yu, J., Helicobacter pylori-induced STAT3 activation and signalling network in gastric cancer. *Oncoscience* **2014,** *1* (6), 468-75. 12. Kumar, S.; Kumari, N.; Mittal, R. D.; Mohindra, S.; Ghoshal, U. C., Association between pro-(IL-8) and anti-inflammatory (IL-10) cytokine variants and their serum levels and H. pylori-related gastric carcinogenesis in northern India. *Meta Gene* **2015,** *6*, 9-16.
13. Wu, S.; Rhee, K. J.; Albesiano, E.; Rabizadeh, S.; Wu, X.; Yen, H. R.; Huso, D. L.; Brancati, F. L.; Wick, E.; McAllister, F.; Housseau, F.; Pardoll, D. M.; Sears, C. L., A human colonic commensal promotes colon tumorigenesis via activation of T helper type 17 T cell responses. *Nat Med* **2009,** *15* (9), 1016-22.
14. Wick, E. C.; Rabizadeh, S.; Albesiano, E.; Wu, X.; Wu, S.; Chan, J.; Rhee, K. J.; Ortega, G.; Huso, D. L.; Pardoll, D.; Housseau, F.; Sears, C. L., Stat3 activation in murine colitis induced by enterotoxigenic Bacteroides fragilis. *Inflamm Bowel Dis* **2014,** *20* (5), 821-34.
15. Lévy, L.; Neuveut, C.; Renard, C. A.; Charneau, P.; Branchereau, S.; Gauthier, F.; Van Nhieu, J. T.; Cherqui, D.; Petit-Bertron, A. F.; Mathieu, D.; Buendia, M. A., Transcriptional activation of interleukin-8 by beta-catenin-Tcf4. *J Biol Chem* **2002,** *277* (44), 42386-93.
16. Wu, S.; Morin, P. J.; Maouyo, D.; Sears, C. L., Bacteroides fragilis enterotoxin induces c-Myc expression and cellular proliferation. *Gastroenterology* **2003,** *124* (2), 392-400.
17. Sun, J.; Hobert, M. E.; Duan, Y.; Rao, A. S.; He, T. C.; Chang, E. B.; Madara, J. L., Crosstalk between NF-kappaB and beta-catenin pathways in bacterial-colonized intestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol* **2005,** *289* (1), G129-37.
18. Duan, Y.; Liao, A. P.; Kuppireddi, S.; Ye, Z.; Ciancio, M. J.; Sun, J., beta-Catenin activity negatively regulates bacteria-induced inflammation. *Lab Invest* **2007,** *87* (6), 613-24.
19. Belair, C.; Darfeuille, F.; Staedel, C., Helicobacter pylori and gastric cancer: possible role of microRNAs in this intimate relationship. *Clin Microbiol Infect* **2009,** *15* (9), 806-12.
20. Schetter, A. J.; Heegaard, N. H.; Harris, C. C., Inflammation and cancer: interweaving microRNA, free radical, cytokine and p53 pathways. *Carcinogenesis* **2010,** *31* (1), 37-49.
21. Shi, C.; Yang, Y.; Xia, Y.; Okugawa, Y.; Yang, J.; Liang, Y.; Chen, H.; Zhang, P.; Wang, F.; Han, H.; Wu, W.; Gao, R.; Gasche, C.; Qin, H.; Ma, Y.; Goel, A., Novel evidence for an oncogenic role of microRNA-21 in colitis-associated colorectal cancer. *Gut* **2015**.
22. Gregory, P. A.; Bert, A. G.; Paterson, E. L.; Barry, S. C.; Tsykin, A.; Farshid, G.; Vadas, M. A.;

Khew-Goodall, Y.; Goodall, G. J., The miR-200 family and miR-205 regulate epithelial to mesenchymal transition by targeting ZEB1 and SIP1. *Nat Cell Biol* **2008,** *10* (5), 593-601.
23. Liu, S.; Sun, X.; Wang, M.; Hou, Y.; Zhan, Y.; Jiang, Y.; Liu, Z.; Cao, X.; Chen, P.; Chen, X.; Tao, Y.; Xu, C.; Mao, J.; Cheng, C.; Li, C.; Hu, Y.; Wang, L.; Chin, Y. E.; Shi, Y.; Siebenlist, U.; Zhang, X., A microRNA 221- and 222-mediated feedback loop maintains constitutive activation of NFκB and STAT3 in colorectal cancer cells. *Gastroenterology* **2014,** *147* (4), 847-859.e11.

5. Objectives

1. We aim to elucidate the role of β-catenin signalling and IL-8/Stat3 activation in the establishment of chronic inflammation and tumour progression in response to enterotoxigenic B. fragilis in colonic epithelial cells. Optimisation of protocols, including determination of MOI of ETBF, will be carried out in the first 3 months of the project, from November 2016. Inhibition of pathways using specific inhibitors will be carried out in the cell lines over the following 6 months, and RNA and proteins will be extracted from the cells over this period. Analysis of proteins by ELISA and Western blot, and gene expression using qRT-PCR will be carried out concurrently, from February to May 2017, as will ICC to determine changes in cellular location of proteins of interest in response to pathway inhibitors.
2. We also aim to explore the regulatory role of specific miRNAs in IL-8/Stat3/beta-catenin signalling as a link between ETBF infection and chronic inflammation in the pathogenesis of colorectal cancer. Inhibition of miRNAs thought to be involved in inflammatory pathways involved in ETBF-associated inflammation will be carried out from May 2017 with analysis of effects on pathways of interest carried out by August 2017.
Statistical analysis will be ongoing during this part of the project. Final analysis and manuscript preparation will be carried out from August to October 2017.

6. About the applicant

Dr Purcell is a post-doctoral research fellow with the Department of Surgery, and has a background in molecular biology and cancer genetics. Her research is centred on the molecular mechanisms of pathogenic bacteria in colorectal carcinogenesis, and she is also interested in the epigenetic role of microRNAs involved in the progression from chronic inflammation to cancer development in the gut. The previous work on the molecular mechanisms of childhood cancer by Dr Purcell ties into the molecular aspects of the current project, with many similar techniques being proposed, such as qPCR, immunohistochemistry, protein analysis, and gene and miRNA array analysis. Her role will be to co-ordinate the project, lead experimental design, undertake key benchside research, and to carry out statistical evaluation of experimental results. In this study her tasks will include in vitro cell culture, immunocytochemistry, protein analysis, and nucleic acid analysis. The focus on the influence of bacterial pathogens on CRC, proposed in this project will allow Dr Purcell an opportunity to expand her knowledge and broaden her areas of expertise, working under the guidance of Dr Keenan. Research into the role of gut bacteria in cancer development is in its early stages and represents an exciting research field. Publication of research findings from this project in high impact journals and presentation of findings nationally and internationally, will further benefit Dr Purcell's career, and contribute to her establishment as an independent researcher.

7. Attachments

Empty

D. Other support

1. Upload files

Empty

2. Outline similarities

Grant funding from Genesis Oncology was used to screen stool samples from colorectal cancer

patients and age-matched controls in Canterbury for the presence of enterotoxigenic B. fragilis. The results of this study provide evidence that B. fragilis is involved in CRC pathogenesis, which provides the basis for the proposed study. There is no overlap between the grant proposals.
The grant proposal submitted to CSNZ is similar to the study proposed here, but it is an expanded version, which spans two years and involves an in vivo component in a mouse model.

E. Referees

RELATIONSHIP Colleague in a similar discipline
RELATIONSHIP Overseas colleague in a similar field

F. Curriculum vitae

1. Upload CVs

View an attachment by double clicking the icon to the left of the file name. Icons are not displayed and attachments are not accessible when this PDF is viewed in a web browser; you must open it in PDF reader software.

2016PRO-PRO_Purcell-Rachel_F1-1.pdf
334.5 KiB

2016PRO-PRO_Purcell-Rachel_F1-2.pdf
111.4 KiB

2016PRO-PRO_Purcell-Rachel_F1-3.pdf
101.6 KiB

C. Staffing and budget

		Year 1		Year 2		
Staff	**Project role**	**FTE**	**Cost**	**FTE**	**Cost**	**Total**
Applicant **Dr Rachel Purcell** **Surgery, University of Otago Christchurch** **University of Otago,2 Riccarton AvenuePO Box 4345 8140** **rachel.purcell@otago.ac.nz, 02102476749**	**Dr Purcell's role will be to co-ordinate the project, lead experimental design, undertake key benchside research, and to carry out statistical evaluation of experimental results.**	**0.8**	**$65,790**	**0**	**$0**	**$65,790**
Researcher **Dr Jacqui Keenan** **Surgery, University of Otago Christchurch** **2 Riccarton Avenue, PO Box 4345,Christchurch 8140** **jacqui.keenan@otago.ac.nz, 03-3640570**	**Dr Keenan will work alongside Rachel to ensure the proposed goals are achieved within the timeframe. In addition, she will work with Rachel on the preparation of manuscripts for publication. No salary is being requested for Dr Keenan.**	**0**	**$0**	**0**	**$0**	**$0**
Researcher **Professor Frank Frizelle** **Surgery, University of Otago Christchurch** **2 Riccarton Avenue,PO Box 4345,Christchurch 8140** **frank.frizelle@otago.ac.nz, 03-364 8174**	**Professor Frizelle is a colorectal surgeon who has a strong research interest in colorectal cancer. His role will be to take part in discussions and provide a clinical perspective on the study findings. No salary is being requested for Professor Frizelle.**	**0**	**$0**	**0**	**$0**	**$0**

		Year 1		Year 2		
Staff	**Project role**	**FTE**	**Cost**	**FTE**	**Cost**	**Total**
Total staff costs			**$65,790**		**$0**	**$65,790**
Working expenses	**Name or description**		**Cost**		**Cost**	**Total**
Other	**Cell culture: media, chemicals, serum, and plastic wear**		**$3,350**		**$0**	**$3,350**
Other	**Pathway inhibitors**		**$805**		**$0**	**$805**
Other	**RNA and microRNA extraction kits, and RNAlater storage solution (Qiagen)**		**$790**		**$0**	**$790**
Other	**qRT-PCR: plates, primers, SybrGreen Master Mix**		**$4,500**		**$0**	**$4,500**
Other	**mirVanaÂ® miRNA inhibitors x 5**		**$2,600**		**$0**	**$2,600**
Other	**Primary antibodies for WB and ICC (6 X $540) - Abcam**		**$3,260**		**$0**	**$3,260**
Other	**Fluorescently labelled secondary antibodies for ICC x 3 (Abcam)**		**$415**		**$0**	**$415**
Other	**Buffers, gels, membranes, secondary antibodies and consumables for Western Blot analysis**		**$3,750**		**$0**	**$3,750**
Other	**IL-8 ELISA kit and buffer (R+D systems)**		**$2,635**		**$0**	**$2,635**
ACC	**ACC**		**$320**		**$0**	**$320**
Total working expenses			**$22,425**		**$0**	**$22,425**
Total request to CMRF			**$88,215**		**$0**	**$88,215**
		Year 1		**Year 2**		

G. Co-funding applications

1. Financial planning

Blank

2. Contingency

Blank

3. Reporting and acknowledgement

Blank

4. Funder contact

The Canterbury Medical Research Foundation wishes to retain the right to communicate directly with your other funders on occasion. Do you agree to this?

Yes

H. Ethical approvals

1. Human

Not required

2. Animal
Not required

3. Biosafety/ERMA
Not required
Empty

5. Notes
This proposal does not require ethical approval as there is no use of human or animal tissue or samples involved.

J. Applicant agreement
By submitting this application to the Canterbury Medical Research Foundation I am agreeing to the following terms and conditions on behalf of the project team:

1. *Publicity*

   By becoming a recipient of an CMRF grant, we automatically agree to the mention of our names to news media.

   We also agree to be included in a list of CMRF funded researchers who are willing to be invited speakers for local interest groups (Rotary, U3A etc).

2. *Ethics*

   We have read the regulations of the appropriate ethics committee involved with the ethical approval of our research project and agree to abide by the principles outlined in them.

   We understand that, if successful, this grant cannot be activated until the required approvals are obtained.

3. *Grant conditions*

   We understand and agree that any grant received as a result of this application is subject to conditions regarding grants made from time to time by the Board of the Foundation and that the grant funds will not be expended for any other purpose than that described in this application without the prior consent of the Board of the Foundation.

4. *Reporting*

   If funding is granted, we agree to provide the Canterbury Medical Research Foundation with six monthly progress reports for the full duration of the project, and a final report on the project's completion. The CMRF may use these reports, in full or in part, as it sees fit to advance the cause of medical research in Canterbury.

5. *Cost sharing*

   It is understood and agreed by the project team that any co-funding received for this project will be declared and that the Foundation reserves the right to arrange cost-sharing with other funding bodies or to reduce the amount of the award as appropriate.

6. *Declaration*

   We have read the above points in this section and agree that our project team will abide by the conditions of the agreement in respect of any grant made by the Foundation as a result of the present application. By submitting this application and assenting to these terms and conditions on behalf of the project team, I undertake that we have provided a full and factual disclosure of all relevant information and we authorise the Foundation to obtain information, including personal information in relation to the application from any other source which the Foundation thinks fit. We also authorise the Foundation to provide information, including personal information, relating to the application to referees in order for the referees to report on the application and acknowledge that to the extent that referees wish the Foundation to keep their report, and/or the fact that they have made a report, confidential then that report will not be accessible by myself or the other project team members.

Applicant acceptance
I agree

Referee review #1

Referee

Dr David Hughes

davidhughes@rcsi.ie
0035314028531
Honorary Lecturer Centre for Systems Medicine, Physiology & Medical Physics Dept Royal College of Surgeons in Ireland 31A York Street D2 Ireland
0

1. The importance and relevance of the concept and aims

There is increasing evidence of a link between bacterial dysbiosis and colorectal cancer (CRC) development. However, there is a need for much more mechanistic studies of this link, such as the study proposed here. Thus, it is a highly relevant study building on work the applicants have in press on the association of B. fragilis with CRC.

I have a few comments to address in the background sections:
The Bacteroides fragilis toxin potentially alters intracellular signalling pathways that are also linked to cancer development. -**what pathways?**

Although the work proposed is sound, interesting and worthy of funding, generally the writing could be improved (and has poor grammar in some sections, e.g., *ETBF produces a virulence factor called B. fragilis toxin (BFT), that induces persistent colitis in mice(3), and disrupt E-cadherin junctions, activate β-catenin signalling, and induce IL-8 secretion in colonic epithelial cells(4-8)*). In all, the application is a little sloppy. The objectives are not clearly stated and are confusingly described in the methods section as protocols rather than project objectives. It takes a long way into the application until one has an idea of what the researchers are planning to do – but importantly this is never very clear.

There should be more references in the first paragraph of **Health significance rationale** on the aetiology of CRC – not well explained.

Bacteroides fragilis is not written in a consistent way throughout (and a first abbreviation is not provided)

2. Your appraisal of the experimental approach and procedures

The experimental approach appears fine. However, I have a few comments:
Three colonic adenocarcinoma cell lines, HT29, SW480, and HCT116 (currently available in our laboratory) will be cultured as previously described. – Where was this previously described? What are the controls for objective 1?

As throughout application, lack of references to back up statements, e.g., 'Our approach, which *is similar to published reports regarding H. pylori in gastric cancer'....* Which published reports?

Objective 2: '*Additional miRNAs may be also be considered'* **-** Which and why or in what context? Such a sentence (as is) is uninformative.

There is no indication of the proposed study power.

3. The applicant's qualifications and ability to undertake the project and complete it within the stipulated period
The applicant, Dr Rachel Purcell, is well qualified and experienced to take on the proposed research and in the timeframe proposed.

4. Your evaluation of the budget components and their justification
The budget seems fine and is appropriate for the project.

5. Overall rating
6

6. Indicate any professional links to the applicant
No existing collaborations or professional work with the applicant.

7. Confidential comments for the CMRF Scientific Assessing Committee

Referee review #2

Referee
Dr Logan Walker

logan.walker@otago.ac.nz
03 364 0544
Mackenzie Cancer Research Group University of Otago, Christchurch 2 Riccarton Avenue PO Box 4345 Christchurch 0

1. The importance and relevance of the concept and aims
The proposal aims to better understand the molecular link between chronic inflammation and colorectal carcinogenesis in response to B. fragilis infection. It was not clear what the actual risk of colorectal cancer (CRC) was as a result of chronic inflammation. The link between gut bacteria and colorectal cancer (CRC) has, however, become an important topic for a number of groups worldwide. Such knowledge is potentially actionable in health management as it may lead to methods of cancer treatment and/or prevention. This proposal will build on data from two studies from the research team, one of which has been submitted for publication.

2. Your appraisal of the experimental approach and procedures
The experimental approaches outlined appear to address the aims of the proposal. Some of the methodological details were a little vague (eg."Varying multiplicities of infection (MOI) of ETBF will be incubated with cell lines" and "qPCR will be carried out ….with appropriate house-keeping genes."). However, I have no doubt that the research team has the required expertise to successfully carry out the proposed experimental work. It is noteworthy that the proposed experiments are similar to that used previously to explore the role of *H. Pylori* in gastric cancer. Regarding statistical analysis, it is unclear how many samples will be assessed for each assay proposed but I understand that can be difficult to predict without pilot data.

3. The applicant's qualifications and ability to undertake the project and complete it within the stipulated period
This one year study appears to be achievable. There are many experiments to be carried out over this time frame so may be little room for troubleshooting if one or more of these experiments prove challenging. The PI will be well supported by two experienced senior researchers, although it is unclear whether this experience extends to the many molecular techniques proposed.

4. Your evaluation of the budget components and their justification

The budget is appropriate and will cover the salary of the PI and working expenses.

5. Overall rating
7

6. Indicate any professional links to the applicant
None to declare

7. Confidential comments for the CMRF Scientific Assessing Committee

Referee review #3

Referee
Dr Cherie Blenkiron c.blenkiron@auckland.ac.nz

0

1. The importance and relevance of the concept and aims
The project looks at the potential role of specific signaling pathways in the development of CRC. As a whole the idea is interesting and it brings together multiple lines of already published evidence to better understand how these interact with one another in bacterium initiated carcinogenesis. The hypothesis is very defined, to investigate the potential cross talk between two signaling pathways wherein inflammation triggered by the bacterium in turn leads to pre-cancerous changes. The findings will be biologically interesting although the applicant does not clearly explain the relevance of this in colorectal cancer- aside from a comment about identifying novel markers for the early detection of colorectal cancer in the media summary.

2. Your appraisal of the experimental approach and procedures
The applicants propose a two step analysis using in vitro cell lines to look at the effects of bacterioides fragilis first on protein signaling pathways and then on miRNA signaling. Throughout the proposal the applicant states the hypothesis that ETBF (bacteria) ‘contributes to early-stage precancerous changes’ and that this is triggered by cross talk between b-catenin and IL-8/Stat pathways. My key query for the proposal is whether the 3 cell-lines chosen will allow this to be to be tested. The 3 cell-lines are all derived from malignant colorectal cells, on metastatic and with varying b-catenin mutation status. The use of an immortalized primary cell-line (fetal epithelial colon cells?) may be more suitable to answer the question of whether ETBF are involved in pre-cancerous changes. As a whole the experiments appear to be planned sensibly. There are minimal details provided on the inhibitors to be used and what replicates needed for each study. I query whether the simplicity of cell lines is suitable for this analysis. A larger study involving an in vivo element was submitted to Cancer Society and I feel that the addition of that aspect to this proposal would improve it greatly. The miRNA analyses are interesting to the story but somewhat feel like an add-on. The reasoning for their inclusion in the project is not strong and the data that would be generated would be correlative rather than being able to clarify the role of these in the ETBF pathways as proposed. It will generate pilot data for further combination pathway inhibitor/miRNA mimic studies.

3. The applicant’s qualifications and ability to undertake the project and complete it within the stipulated period
The team is appropriately skilled to complete the project and have all of the facilities available to them to do so successfully. The timeframe of one year may be too ambitious for the amount of work proposed, particularly with the inclusion of the miRNA aspect.

4. Your evaluation of the budget components and their justification
Some of the reagents listed appear to be slightly under costed, such as the RNA extraction kit(s) needed for all of the replicates proposed. The pathway inhibitors are not identified. The inclusion of salary at 0.8FTE for a Research Fellow to work on the project is justified as there will be a lot of hands-on work in order to complete all of the analyses proposed in the year term.

5. Overall rating
6

6. Indicate any professional links to the applicant
None

7. Confidential comments for the CMRF Scientific Assessing Committee

Annual report submitted May 2017
1. Brief summary
The first Objective of the project was to investigate whether infection with toxin-producing strains of *Bacteroides fragilis* (ETBF) induces chronic inflammation in colonic epithelial cells by a signalling mechanism involving beta-catenin and IL-8/Stat3. To this end I have cultured two colorectal cancer cell lines HT29 and HCT116. To these cell cultures, I have performed titration experiments to determine the optimal multiplicity of infection (MOI) of bacteria and the most appropriate time-points to use in subsequent experiments. The endpoints measured were the genes coding for IL-8, and IL-8 receptor (CXCR1), and these endpoints were measured using quantitative PCR. IL-8 expression was increased 4-fold in HT29 cells treated with bacteria after 3 hours while IL-8 receptor expression only increased after 24 hours in both cell lines (Figure 1). My next step is to block various molecules involved in beta-catenin and IL-8/Stat3 and repeat the experiments to determine how ETBF feeds into IL-8 activation, i.e. causes inflammation. The second Objective involves investigating the role of microRNAs in this process, and I have collected miRNAs from each set of experiments and will analyse them for changes in levels of miRNAs thought to play a role in gut inflammation.

Annual report submitted May 2017
1. Brief summary
The first Objective of the project was to investigate whether infection with toxin-producing strains of *Bacteroides fragilis* (ETBF) induces chronic inflammation in colonic epithelial cells by a signalling mechanism involving beta-catenin and IL-8/Stat3. To this end I have cultured two colorectal cancer cell lines HT29 and HCT116. To these cell cultures, I have performed titration experiments to determine the optimal multiplicity of infection (MOI) of bacteria and the most appropriate time-points to use in subsequent experiments. The endpoints measured were the genes coding for IL-8, and IL-8 receptor (CXCR1), and these endpoints were measured using quantitative PCR. IL-8 expression was increased 4-fold in HT29 cells treated with bacteria after 3 hours while IL-8 receptor expression only increased after 24 hours in both cell lines (Figure 1). My next step is to block various molecules involved in beta-catenin and IL-8/Stat3 and repeat the experiments to determine how ETBF feeds into IL-8 activation, i.e. causes inflammation. The second Objective involves investigating the role of microRNAs in this process, and I have collected miRNAs from each set of experiments and will analyse them for changes in levels of miRNAs thought to play a role in gut inflammation.

2. Photographs
View an attachment by double clicking the icon to the left of the file name. Icons are not displayed and attachments are not accessible when this PDF is viewed in a web browser; you must open it in PDF reader software.

HT29_HCT116 14_04_17.docx
19.0 KiB

Annual report submitted Mar 2018

1. Brief summary

The first Objective of the project was to investigate whether infection with toxin-producing strains of *Bacteroides fragilis* (ETBF) induces chronic inflammation in colonic epithelial cells by a signalling mechanism involving beta-catenin and IL-8/Stat3. To this end I have cultured two colorectal cancer cell lines HT29 and HCT116. To these cell cultures, I have performed titration experiments to determine the optimal multiplicity of infection (MOI) of bacteria and the most appropriate time-points to use in subsequent experiments. The endpoints measured were the genes coding for IL-8, and IL-8 receptor (CXCR1), and these endpoints were measured using quantitative PCR. IL-8 expression was increased more than 2-fold in both HT29 and HCT116 cells treated with bacteria or purified bacterial toxin after 3 hours while IL-8 receptor expression only increased after 24 hours in both cell lines. The next step was to block various molecules involved in beta-catenin and IL-8/Stat3 and repeat the experiments to determine how ETBF feeds into IL-8 activation, i.e. causes inflammation. This was done by pre-treating the cell lines with small-molecule inhibitors of Beta-catenin and Stat3, prior to treatment with ETBF. Levels of the IL-8 gene and protein were then measured. Our results showed no difference in IL-8 expression when we blocked Beta-catenin activity, but we found that when we blocked Stat3 activity, neither ETBF nor its toxin was able to elicit an inflammatory response. In other words by blocking the Stat3 pathway, we could negate the effect of ETBF on expression of IL-8. This was seen looking at both the gene levels, using qPCR, and the protein levels using an ELISA assay.

The second Objective involves investigating the role of microRNAs in this process. We had some difficulties out of our control in this regard. All of the reagents needed for miRNA experiments were lost due to a freezer failure. These reagents were replaced in January 2018, and I have just finished these experiments. I looked at six different miRNAs in cells that were treated/untreated with ETBF or its toxin. Out of these, two miRNAs: miR-205 and miR-220A were significantly elevated in response to treatment with ETBF. Further analysis showed that by blocking Beta-catenin, ETBF was unable to cause an increase in miR-205 expression. Further experiments will need to be done to validate these miRNA findings and I hope to have this completed by mid-2018. This work adds novel information to the published data regarding the mechanism of action of ETBF in causing inflammation in colorectal cells. I hope to have a manuscript prepared for publication later in 2018.

Final report

1. Report for the Scientific Assessing Committee

View an attachment by double clicking the icon to the left of the file name. Icons are not displayed and attachments are not accessible when this PDF is viewed in a web browser; you must open it in PDF reader software.

Final report_CMRF_2019 (002).pdf
88.5 KiB

2. Brief summary

The first Objective of the project was to investigate whether infection with toxin-producing strains of *Enterotoxigenic Bacteroides fragilis* (ETBF) induces chronic inflammation in colonic epithelial cells by a signalling mechanism involving beta-catenin and IL-8/Stat3. To this end I have cultured two colorectal cancer cell lines HT29 and HCT116 with ETBF and measured changes in gene and protein expression of IL-8. I found that IL-8 expression was increased more than 2-fold in both HT29 and HCT116 cells treated with bacteria or purified bacterial toxin. The next step was to block various molecules involved in beta-catenin and IL-8/Stat3 signalling pathways and repeat the experiments to determine how ETBF feeds into IL-8 activation, i.e. causes inflammation. I pre-treated the cell lines with small-molecule inhibitors of Beta-catenin and Stat3, prior to treatment with ETBF and again measured IL-8 gene and protein expression. I found that when I blocked Stat3 activity, neither ETBF nor its toxin was able to elicit an inflammatory response. In other words by blocking the Stat3 pathway, we could negate the effect of ETBF on expression of IL-8.

I then used Western Blotting and Immunofluorescence techniques to investigate whether loss of cell adhesion through disruption of E-cadherin was through Stat3 signalling. I found that ETBF disrupted E-cadherin but not in a Stat3-dependent manner. This shows that ETBF disrupts cell adhesion and elicits an inflammatory response through different signalling mechanisms.

The second Objective involves investigating the role of microRNAs in this process. I looked at six different miRNAs in cells that were treated/untreated with ETBF or its toxin. Out of these, two miRNAs: miR-205 and miR-220A were significantly elevated in response to treatment with ETBF. Further analysis showed that by blocking Beta-catenin, ETBF was unable to cause an increase in miR-205 expression. Further experiments will need to be done to validate these miRNA findings and I hope to have this completed by mid-2019. This work adds novel information to the published data regarding the mechanism of action of ETBF in causing inflammation in colorectal cells. A manuscript detailing these findings is currently being prepared for publication.

3. Photographs

View an attachment by double clicking the icon to the left of the file name. Icons are not displayed and attachments are not accessible when this PDF is viewed in a web browser; you must open it in PDF reader software.

Figure 1A.pdf
25.6 KiB

Figure 2.pdf
1.5 MiB

Figure 3.pdf
1.1 MiB

4. Feedback