INVESTIGATIONS INTO THE INFLUENCE OF PARATHYROID HORMONE ON GENE EXPRESSION IN THE COLORECTAL EPITHELIUM

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Abstract

There is good evidence that high dietary calcium intake reduces the incidence of colorectal cancer. The mechanism by which calcium acts is unknown. It was hypothesised that parathyroid hormone (PTH) acts on colorectal epithelium and that calcium acts by suppressing serum PTH. Real-time PCR demonstrated expression of the PTH receptor PTHR1 in normal and neoplastic colon, indicating that the colon was sensitive to PTH. Gene expression profiling using oligo-nucleotide micro-arrays was performed to identify genes;

a) Differentially expressed in response to PTH in an in-vitro model of the colorectal epithelium.

b) For which expression in normal rectal mucosa correlated with serum PTH level.

c) Differentially expressed in normal rectal mucosa in response to modulating PTH by dietary calcium supplementation.

A common finding of all three studies was regulation of the wnt signalling pathway by PTH. In the in-vivo studies, expression of genetic markers of differentiation was negatively correlated with PTH. Calcium supplementation resulted in increased expression of genetic markers of differentiation. This suggests PTH inhibits differentiation and that calcium by suppressing PTH promotes differentiation. There was also significant overlap between the genes changed by calcium, those negatively correlated with PTH and those changed by PTH in-vitro. These findings provide support for the stated hypotheses.

“Diseases can rarely be eliminated through early diagnosis or good treatment, but prevention can eliminate disease.”

Denis Burkitt

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Chapter 1

BACKGROUND

1.1: Introduction

The context of this thesis is the prevention of colorectal cancer with dietary agents. In particular, to investigate the mechanism of action of dietary calcium: This has been shown to reduce the incidence of colorectal neoplasia. This background discussion will review: current understanding of the biology of colorectal cancer and its management. The evidence relating to the protective effect of calcium will be presented. A hypothesis for the preventive action of calcium will be given, along with the evidence supporting it. Further discussion will focus on the normal colorectal epithelium: the signalling pathways that control it and the previously reported effects of calcium on it.

1.2: Colorectal cancer

1.2.1 Incidence

Colorectal cancer is the third commonest cancer worldwide with over one million new cases per year. This high incidence rate translates into a significant mortality rate, colorectal cancer being the fourth commonest cause of cancer death in the world, with over 600,000 deaths per annum (Karsa, Lignini et al. 2010). There is geographical variation in incidence and the United Kingdom (UK) is a high incidence region. In the UK the lifetime risk of developing colorectal cancer is 6%(Davies, Miller et al. 2005). As well as being a major cause of morbidity and mortality, the management of colorectal

cancer is a significant burden on healthcare budgets and therefore represents a major public health issue.

1.2.2 Age

The incidence of colorectal cancer increases with age: with a mean age at onset of sixty three (Cunningham, Atkin et al. 2010). Despite its incidence being highest in the older age group, it does occur in younger patients with around 8% of colorectal cancer occurs under the age of fifty (Edwards, Howe et al. 2002).

1.2.3 Sex

Overall the incidence of colorectal cancer is marginally higher in men than woman with an Odds ratio of 1.5. There is also a minor difference in tumour location, with the ratio of rectal to colonic cancer higher in men than women (McCashland, Brand et al. 2001).

1.2.4 Geography

There is significant variation in the incidence of colorectal cancer around the world, with up to 25 fold variation in incidence between countries (Center, Jemal et al. 2009). The areas of highest incidence are those nations considered to be „westernised', particularly those in: Europe, North America and Oceania (Australia and New Zealand). In contrast, the lowest rates of colorectal cancer are found in Asia, Africa and South America (Center, Jemal et al. 2009).

1.2.5 Predisposing factors

The development of colorectal cancer has both genetic and environmental influences. The majority of cases of colorectal cancer are sporadic, occurring in individuals with no personal risk factors other than increasing age. The

following factors have been shown to increase the risk of developing colorectal cancer.

1.2.5.1 Adenomatous polyps

A past history of a colorectal adenoma increases the risk of developing colorectal cancer by 2-4 times (Lotfi, Spencer et al. 1986). This increased risk varies depending on the nature of the adenoma. Features that are associated with the greatest increased risk are: multiplicity of adenomas (>3) and the presence of an adenoma with advanced features (Winawer, Zauber et al. 2006). The features of an advanced adenoma are: size>1cm, tubulo-villous or villous histology or the presence of high grade dysplasia (Levine and Ahnen 2006).

1.2.5.2 Genetic predisposition

Genetic influences play a part in the development of up to a third of cases of colorectal cancer (Dove-Edwin, Sasieni et al. 2005). The presence of genetic predisposition is identified by the presence of a family history of colorectal cancer. The extent of family history is helpful in stratifying patients into different levels of risk. In around 2-3% of patients with colorectal cancer there is a clear genetically inherited syndrome with high penetrance. The two main genetic syndromes predisposing to colorectal cancer are hereditary non-polyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP). Both of these are inherited in an autosomal dominant fashion. Though relatively uncommon, these genetic syndromes provide understanding of the molecular biology of sporadic cancers.

1.2.5.2.1 Hereditary non-polyposis colorectal cancer (HNPCC)

HNPCC is the commonest inherited condition and is caused by mutations in the DNA mismatch repair genes (MLH1, MSH2, MSH6 and PMS2). Carriers of the mutation have a lifetime risk of colorectal cancer of 40-80% and increased risk of extra-colonic malignancies (Vasen, Taal et al. 1995; Dunlop, Farrington et al. 1997). Cancers from patients with HNPCC invariably demonstrate micro-satellite instability (MSI) in tumour DNA. This is the result of the development of deficient DNA mismatch repair and a „mutator phenotype' which is associated with a specific mutational spectrum in tumour DNA.

1.2.5.2.2 Familial adenomatous polyposis (FAP)

FAP is less common than HNPCC but has a distinctive phenotype with the development of hundreds of polyps throughout the colon and a 100% lifetime risk of colorectal cancer (Bulow 1989). Mutations in the adenomatous polyposis coli (APC) gene are responsible for FAP and are identified in over 60% of families (Wallis, Morton et al. 1999). The Wnt signalling pathway is regulated by APC and de-regulation of the Wnt pathway is found in the polyps of FAP patients and also sporadic colorectal cancers (Kinzler and Vogelstein 1996).

1.2.5.2.3 Other genetic conditions

There are also other rarer dominantly inherited genetic conditions which predispose to colorectal cancer including Peutz-Jehgers syndrome (PJS) and juvenile polyposis (JPS). Mutations in STK11 have been identified in PJS and in SMAD4 and BMPR1A in JPS (Dunlop 2002). More recently, (Mut-Y Homolog) MYH-associated polyposis which is inherited in an autosomal

recessive fashion was identified (Sampson, Dolwani et al. 2003). More common are the patients with one or more relatives affected with colorectal cancer but without any of the syndromes described above. These individuals constitute 10- 15% of patients with colorectal cancer and have an increased risk 2-5x that of the general population (Dunlop 2002).

1.2.5.3 Inflammatory bowel disease

Patients diagnosed with Ulcerative colitis or Crohn's colitis have an increase risk of developing colorectal cancer. The risk of colorectal cancer increases with duration of colitis. With colorectal cancer rates of 2%, 8% and 18% after 10, 20 and 30 years respectively (Eaden, Abrams et al. 2001).

1.2.5.4 Environmental factors

A number of risk factors have been identified, these include: red meat, high-fat diet, inadequate intake of fibre, obesity, sedentary lifestyle, diabetes mellitus, smoking, and high alcohol consumption (Cunningham, Atkin et al. 2010).

1.2.6 Pathology of colorectal cancer

Colorectal cancers are most commonly of the adenocarcinoma type, arising from the epithelium of the large intestine. There is a recognised pattern of tumour location around the colon with two thirds of cancers arising distal to the splenic flexure (Ponz de Leon, Marino et al. 2004).

1.2.6.1 Adenoma-carcinoma sequence

The widely accepted model by which colorectal cancer develops is the adenoma-carcinoma sequence. In this model, the development of cancer progresses from normal colorectal mucosa via the intermediate stage of the adenoma to a cancer. There is considerable evidence to support this model

of development of colorectal cancer. That cancers arise from adenomas was first proposed in the 1920's (Lockhart-Mummery and Dukes 1928). This was based on a number of observations; firstly the development of cancer in patients with previous polyps, the presence of polyps at the site where cancers develop and the presence of malignant change in adenomas and residual adenomatous elements in cancers (Lockhart-Mummery and Dukes 1928). These initial observations have been confirmed in subsequent studies. Adenomas have the same anatomical location as cancer, predominating distal to the splenic flexure (Clark, Collan et al. 1985) and their prevalence peaks five to ten years before that of cancer (Muto, Bussey et al. 1975). Foci of cancer are found in up to 8% of polyps and adenomatous tissue is found in continuity with around 20% of cancers (Leslie, Carey et al. 2002).

That cancers arise from adenomas is widely accepted, but it is also clear that not all adenomas turn into cancer. Around 40% of the population will have an adenoma by the age of sixty (Levine and Ahnen 2006), which compares with the lifetime risk of colorectal cancer of around 6%. The risk of developing cancer is greater for larger adenomas particularly in those >1cm. One study has demonstrated that polyps >1cm or more progress to cancer at a rate of about 1% per year (Stryker, Wolff et al. 1987). The risk is less for smaller adenomas, but even small adenomas can have foci of cancer. Thus, removal of all adenomas detected endoscopically is recommended. In addition to progression of adenomas to cancer, they may remain stable or regress (Loeve, Boer et al. 2004).

Of patients who have an adenoma removed, 30-35% will have a recurrent adenoma within 3-4 years (Neugut, Johnsen et al. 1985). Colonoscopic

surveillance of these patients is therefore essential; guidelines based on the features of the baseline adenoma have been developed (Atkin and Saunders 2002). Consequently, polyp surveillance results in a large number of the colonoscopies performed in the NHS. The ability to identify those at greatest risk of developing further adenomas would be of clinical utility.

1.2.7 Molecular pathology of colorectal cancer

The molecular mechanisms underlying the development of colorectal cancer via the adenoma-carcinoma sequence were first proposed by Vogelstein and Fearon (Fearon and Vogelstein 1990). In this model, colorectal cancer develops by a multi-step genetic process, with mutational inactivation of tumour suppressor genes and mutational activation of oncogenes.

1.2.8 Genetic alterations occurring in colorectal cancer

Screening of colorectal cancer for somatic mutations has identified up to ninety mutated genes, of these fourteen are considered to occur at high enough frequency to indicate functional importance. Of these the three commonestly mutated genes are APC, K-ras and P53 (Sjoblom, Jones et al. 2006).

1.2.8.1 Adenomatous polyposis coli (APC)

Inactivating mutations of the adenomatous polyposis coli (APC) gene are found in up to 80% of sporadic colorectal cancers (Powell, Zilz et al. 1992). Mutational inactivation of APC results in loss of its ability to bind β-catenin, this results in increased accumulation of β-catenin. One important consequence of β-catenin accumulation is increased T-cell factor (TCF) mediated transcription. Increased β-catenin/TCF mediated transcription and gene expression has been demonstrated in colorectal cancers (Kinzler and

Vogelstein 1996). Inactivating mutations of β-catenin have been found in the majority of tumours with wild type APC (Morin, Sparks et al. 1997), highlighting the importance of this pathway in colorectal tumorigenesis.

1.2.8.2 K-ras

K-ras (Kirsten ras) is an oncogene, which undergoes activating mutations, which are found in over a third of colorectal cancers (Vogelstein, Fearon et al. 1988). K-ras is a (GTP) guanosine- triphosphate binding protein, mutations result in decreased GTPase activity and constitutive activation, influencing diverse signalling pathways (Bos 1989).

1.2.8.2 P53

P53 has been identified as “the guardian of the genome”, as it blocks cell proliferation in the presence of DNA damage and to result in apoptosis if the damage is irreparable (Lane 1992). As well as being frequently mutationally inactivated in colorectal cancer, P53 is the most commonly mutated gene in all cancers (Caron de Fromentel and Soussi 1992).

In addition to genetic mutation, other somatic changes have been recognised in cancers, these include: chromosomal losses and gains, aneuploidy and promoter methylation (Leslie, Carey et al. 2002).

1.2.9 Staging

The pathological staging of colorectal cancer is of clinical significance in predicting the outcome of treatment. The first staging system for colorectal cancer was that devised by Dukes (Dukes 1932). Tumours were classified into stage A, B, or C, where stage A tumours were confined to the rectal wall, stage B tumours breached extra-rectal tissues, and stage C tumours had metastasised to lymph nodes. The significance of Dukes findings was his

demonstration of poorer outcomes with advancing stage of the disease. Due to its clinical utility and ease of use the Dukes staging system remains in common usage. And though not described by Dukes, stage D, representing the presence of distant metastases has been added to the staging bearing his name (Turnbull, Kyle et al. 1967). The site of distant metastases for colorectal cancer is most commonly the liver, with pulmonary metastases also found.

The other staging system commonly used for colorectal cancer, often in conjunction with Dukes staging, is the TNM system (Sobin and Wittikind 2010). This describes features of the primary tumour (T stage), the presence and features of nodal metastases (N stage) and the presence of distant metastases (M stage). In the past, the only staging of colorectal cancer occurred following surgery with the pathological examination of the surgical specimen. Pre-operative staging using various imaging modalities: Computed tomography (CT) and Magnetic resonance imaging (MRI), are now used to provide pre-operative staging. These are used to guide the use of pre-operative therapy. The pathological staging however remains the final arbiter on prognosis following surgical treatment.

1.3: Diagnosis of colorectal cancer

There are two main paradigms in which diagnosis of colorectal cancer occurs: symptomatic presentation and detection during screening of asymptomatic individuals

1.3.1 Symptomatic presentation

Patients presenting with symptoms, such as: rectal bleeding, change in bowel habit, or abdominal pain is the commonest mode of diagnosis and historically was the only method. It is important to note, that of patients presenting with symptoms, half have advanced disease (Dukes stage C or D) including 20% with metastases (Hardcastle, Chamberlain et al. 1996). As outcome following treatment for colorectal cancer is related to stage of the disease, the outcome for this group is poor. Also, up to 30% of patients with colorectal cancer will present as an emergency (Scott, Jeacock et al. 1995). These patients have bowel obstruction or perforation, requiring urgent treatment. The outcome for these emergency patients is particularly poor (McArdle and Hole 2002).

1.3.2 Screen detected

The advanced stage of presentation at symptomatic presentation led to the development of tools for screening asymptomatic individuals. The aim was to identify the disease at an earlier stage thus improving the outcome from treatment. In the United Kingdom two approaches have been shown to be effective. The use of faecal occult blood testing (FOBT) followed by colonoscopy for those who test positive has been shown in a randomised controlled trial to reduce colorectal cancer mortality by 15% after eight years follow up. This is principally based on the diagnosis of a greater proportion of early stage tumours in the screened group (Hardcastle, Chamberlain et al.

1996). This study, as well as similar findings from others around the world (Kronborg, Fenger et al. 1996; Mandel, Church et al. 1999), has led to the introduction of a national bowel cancer screening programme in the UK. In this, individuals aged from 60 to 69 are invited for biennial FOBT. Initial results indicate that the cancers detected by screening are of earlier stage compared to those presenting symptomatically (Steele 2004).

Direct endoscopic screening has also been studied. A trial of screening for colorectal cancer using a one off flexible sigmoidoscopy between the ages of 55 and 64 has been carried out. This also showed high rates of early stage cancer (Atkin, Edwards et al. 2010). After eleven years follow up, there was a 31% reduction in colorectal cancer mortality in the intention to treat analysis and in those who actually underwent sigmoidoscopy (per protocol analysis) there was a 43% reduction (Atkin, Edwards et al. 2010).

1.3.2.1 Effect of screening on the incidence of colorectal cancer

Another consequence of screening, by either of these two methods, is a reduction in the incidence of colorectal cancer in the screened compared to the control group. In the flexible sigmoidoscopy study there was a 23% reduction in the incidence of colorectal cancer after a mean of eleven years follow up(Atkin, Edwards et al. 2010). This decrease in incidence was 33% in those who actually underwent screening, with a 50% reduction in distal colorectal cancer. A 20% reduction in incidence of colorectal cancer has also been shown in those undergoing annual FOBT screening after eighteen years of follow up (Mandel, Church et al. 2000). The reduction in incidence of colorectal cancer in subjects who have undergone screening is due to the

detection and removal of polyps. The effect of sigmoidoscopy is of greater magnitude and apparent earlier. This is likely to be because of the greater rate of polyp detection with sigmoidoscopy, which is 12% (Atkin, Cook et al. 2002) compared to 0.6% with FOBT (Steele 2004).

The effectiveness of screening in reducing colorectal cancer incidence and mortality has been demonstrated. There are, however, a number of issues with screening that limit its potential effectiveness. Uptake of screening is poor whichever method is employed, with only just over half of those invited taking up the offer of screening (Atkin, Cook et al. 2002; Steele 2004). FOBT testing has a sensitivity of around 50%, with subsequent interval cancer rate. Similarly it has a positive predictive value of only 50%, such that around half of patients undergoing colonoscopy have no abnormality (Steele 2004). There is also the question of managing the patients identified with polyps, who are at increased risk of developing colorectal cancer. Currently the only option is to offer them continued colonoscopic surveillance, which is very demanding of resources.

1.3.3 Treatment of colorectal cancer

The curative treatment of colorectal cancer is predominantly by surgery. The principles of surgery are: resection of the primary tumour and draining lymph nodes, dissection in anatomical planes and adequate resection margins. Additionally radiotherapy and chemotherapy have a role in reducing local recurrence and improving the survival from colorectal cancer in selected patients (Cunningham, Atkin et al. 2010).

1.3.4 Prognosis

The main determinant of outcome of colorectal cancer is the tumour stage. Cancer registry data has shown that 5year survival rates for Dukes stage A is 85%, 67% for stage B and 37% for stage C (Cunningham, Atkin et al. 2010). The long term survival of patients with metastases is very low. Due to the stage distribution at presentation, overall survival for colorectal cancer is around fifty percent. This confirms the principle that, stage of disease at presentation is a major determining factor in the outcome of colorectal cancer.

1.4: Prevention of colorectal cancer

Prevention of colorectal cancer would appear to be self evidently a good thing. Additionally, the evidence above that current treatment strategies are not able to cure a significant proportion of patients makes this more desirable. There are three mechanisms by which the incidence of colorectal cancer can be reduced, these are: Surgery, colonoscopic polypectomy and chemoprevention. Surgery and polypectomy form part of current clinical practice. Chemoprevention remains a research intervention and is not yet part of routine clinical practice.

1.4.1 Surgery

Surgical removal of the colon and rectum, removes all of the epithelium at risk of neoplastic transformation. Prophylactic removal of the colon and rectum is the recommended treatment for patients with FAP, to prevent the inevitable development of colorectal cancer (Dunlop 2002). It is also the treatment for patients with long standing colitis who develop dysplastic change in the colonic mucosa (Eaden, Abrams et al. 2001). For the majority of patients who

are not at significantly increased risk of colorectal cancer, surgical prophylaxis is clearly not an acceptable approach to preventing colorectal cancer.

1.4.2 Polypectomy

The adenoma-carcinoma sequence model is now widely accepted as the mode by which colorectal cancer develops. It is predicted from the adenoma-carcinoma model that removal of adenomatous polyps would reduce colorectal cancer incidence. There is substantial evidence that this is the case in a number of clinical scenarios. Colonoscopic surveillance in patients with HNPCC has been shown to reduce mortality from colorectal cancer (Jarvinen, Aarnio et al. 2000). This is attributed to earlier stage disease at diagnosis. However, surveillance is also associated with a reduction in incidence of colorectal cancer which is secondary to the removal of polyps by polypectomy (Jarvinen, Aarnio et al. 2000). Colonoscopic removal of polyps in patients with sporadic adenomas at standard population risk has been shown to reduce colorectal cancer incidence in two cohort studies by 70-90% (Winawer, Zauber et al. 1993; Citarda, Tomaselli et al. 2001). Further evidence that removal of polyps reduces the incidence of colorectal cancer comes from screening studies. As was described in section 1.5.2.1, both FOBT and endoscopic screening reduce the incidence of colorectal cancer.

The efficacy of endoscopic polypectomy in reducing colorectal cancer incidence is reasonably clear. There are however, a number of difficulties in the clinical application of colonoscopy and polypectomy as a preventive strategy. These include: identifying groups of individuals in whom the benefits of colonoscopy outweigh the risks, improving the uptake of population

screening, defining the role of endoscopy as a screening tool and manpower issues relating to increased number of colonoscopies performed.

1.4.3 Chemoprevention

Chemoprevention is the use of oral agents, nutrient or pharmacological, to prevent the development of colorectal cancer (Martinez, Marshall et al. 2008). That such an approach may be feasible is based on the observed wide variation in incidence and mortality between geographically distinct populations and the changes in incidence in migrant populations. These differences have been attributed to variation in environmental and dietary factors. Indeed, it has been suggested that around a third of cancer deaths might be due to dietary factors (Doll and Peto 1981). The recognition that the development of symptomatic colorectal cancer from normal mucosa occurs over ten to fifteen years provides a window of opportunity to intervene.

1.4.3.1 Chemo-preventive agents

Epidemiological studies have provided evidence for an association between a number of dietary factors and the incidence of colorectal cancer. These include: fibre, folate and calcium, as well as pharmacological agents: aspirin and other non-steroidal anti-inflammatory drugs (NSAID's).(Martinez, Marshall et al. 2008). Subsequently, randomised controlled adenoma prevention trials of these agents have been carried out. Adenoma prevention trials are secondary prevention trials in which the subjects have had a previous adenoma removed and are randomised to intervention or placebo and undergo follow up colonoscopy after 3-4 years, with adenoma recurrence as the primary endpoint. Despite the epidemiological evidence for a preventive effect of fibre and folate, randomised controlled trials of both of these agents

have shown no effect on adenoma recurrence (Asano and McLeod 2002; Cole, Baron et al. 2007; Logan, Grainge et al. 2008). However, there are agents with proven efficacy, the agent for which there is the greatest evidence is Aspirin.

1.4.3.1.1 Aspirin

There is evidence from both epidemiological and intervention studies that aspirin reduces the incidence of colorectal cancer. Epidemiological studies have shown that those taking aspirin had a lower incidence of colorectal cancer after 10 years (Flossmann and Rothwell 2007). There have also been four adenoma prevention trials of aspirin. In a meta-analsyis of these four trials there was a statistically significant 17% relative reduction in the risk of any adenoma and a 28% relative risk reduction in the risk of an advanced neoplasm (Cole, Logan et al. 2009). As polypectomy has been shown to reduce colorectal cancer incidence, it would be hypothesised that this reduction in adenoma incidence would result in a reduction of colorectal cancer incidence. None of these trials yet have long enough follow up to demonstrate this. Nor are they likely to as participants will be under colonoscopic surveillance which will substantially reduce the subsequent rate of colorectal cancer. The question of whether a reduction in adenoma recurrence rate will translate into reduction in colorectal cancer incidence has been recently addressed. A meta-analysis of other studies, using aspirin for thrombosis prevention, was carried out to look at the effect of aspirin on the risk of colorectal cancer over 20 years. This demonstrated that aspirin reduced the incidence of colorectal cancer by 30%, with no effect seen until after ten years follow up (Flossmann and Rothwell 2007). This latency of

effect of aspirin is consistent with our understanding of the development of colorectal cancer and suggests a mechanism of action in the early stages of colorectal tumorigenesis.

These combined data, demonstrating that: aspirin prevents adenomas after three years and also reduces colorectal cancer incidence after ten years demonstrates the utility of adenoma prevention trials. The mechanism by which aspirin acts is not fully known, though it is thought that this is via inhibition of cyclo-oxygenase-2 (COX2). COX2 promotes proliferation and is commonly up-regulated in colorectal tumours (Eberhart, Coffey et al. 1994). Further support for this mechanism of action was provided by a large cohort study comparing COX2 expression in 636 incident cancers with self-reported intake of aspirin. In this a reduction in the incidence of tumours with moderate or high expression of COX2 in those taking aspirin was demonstrated (Chan, Ogino et al. 2007).

This finding provided a rationale for testing the effect of selective COX2 inhibitors; these have an improved gastrointestinal side effect profile compared to Aspirin. The Adenomatous Polyp Prevention on Vioxx (APPROVe) trial of the selective COX2 inhibitor Rofecoxib (Vioxx) demonstrated a significant 34% relative reduction in adenoma incidence compared to placebo after three years (Baron, Sandler et al. 2006). This was however associated with a significantly increased risk of thrombotic cardiovascular events (Bresalier, Sandler et al. 2005), resulting in withdrawal of Rofecoxib. The use of aspirin is also associated with a number of side effects particularly gastrointestinal bleeding. In view of this, and despite evidence of efficacy, it is felt that more research is required before Aspirin can

be recommended for colorectal cancer chemoprevention (Cuzick, Otto et al. 2009).

The data relating to NSAID's and Aspirin in particular indicate that chemoprevention is possible and that adenoma prevention trials are capable of identifying appropriate agents. However, in view of the uncertain risk-benefit profile, alternative agents with acceptable safety and tolerability are of interest in the further study of colorectal cancer chemoprevention.

1.5: Dietary calcium and colorectal cancer

Calcium is the only other agent that has been shown to reduce adenoma formation in randomised placebo controlled trials. The aim of this thesis is to investigate the mechanism of action of calcium. The evidence relating to calcium and colorectal chemoprevention will be presented below.

1.5.1 Epidemiology

Evidence that diets high in calcium may reduce the incidence of colorectal cancer came initially from epidemiological studies. A meta-analysis of ten studies of calcium intake, which included 534,536 individuals, was carried out. This demonstrated a reduced incidence of colorectal cancer in those with the highest compared to the lowest intake of calcium (Cho, Smith-Warner et al. 2004).

1.5.2 Animal studies

There is also evidence from studies of rodents that calcium reduces dietary or experimentally induced colonic tumours either alone (Appleton, Davies et al. 1987; Pence and Buddingh 1988) or in conjunction with vitamin D(Newmark, Yang et al. 2009).

1.5.3 Randomised controlled trials

These epidemiological and animal data supported the testing of calcium in adenoma prevention trials. Of which there have been two, one carried out in the USA (Baron, Beach et al. 1999) and one in Europe (Bonithon-Kopp, Kronborg et al. 2000). These two trials were the subject of a meta-analysis (Weingarten, Zalmanovici et al. 2008). In these two studies a total of 1346 patients with a history of adenomas were enrolled and the recurrence of adenoma assessed by colonoscopy. The two studies differed in the duration (4 vs 3 years) and daily dose of calcium (1.2g vs 2g). The outcome of the meta-analysis was a moderate but statistically significant reduction in adenoma recurrence in those patients receiving calcium compared to placebo, with an Odds Ratio of 0.74 (95% confidence interval 0.58-0.95). Importantly, there was no increase in serious adverse events in the calcium treated patients.

Further analysis of the larger American study demonstrated a number of other important outcomes. Firstly the effect of calcium was most pronounced on the recurrence of advanced neoplasms (Tubulovillous or villous adenomas or carcinoma-in-situ) with a RR of 0.65 (95%CI 0.46-0.93) compared to tubular adenoma RR 0.89 (0.77-1.03) (Wallace, Baron et al. 2004). Subsequently the protective effect of calcium was demonstrated to be prolonged after the end of randomised treatment. There was a statistically significant reduction in the incidence of adenomas (RR 0.63 (0.46-0.87)) in the first five years after the end of treatment (Grau, Baron et al. 2007). Unfortunately, neither of these trials will be able to demonstrate a reduction of colorectal cancer incidence in the long term because the patients are under

colonoscopic surveillance, which in itself reduces the incidence of colorectal cancer.

The authors of the meta-analysis concluded that; there is evidence that calcium supplementation may reduce adenoma recurrence, but the evidence is not strong enough to recommend the use of calcium supplements in the primary or secondary prevention of adenomas. They recommend that "further work is needed on the factors involved in individual susceptibility to colorectal cancer in order to identify any subgroups that might benefit from calcium supplementation" (Weingarten, Zalmanovici et al. 2008). Understanding the mechanism by which calcium reduce the incidence of colorectal neoplasia will facilitate its rational use in strategies to reduce an individual's risk of developing colorectal cancer.

1.5.4 Mechanism of action of calcium

The mechanism by which calcium reduces the development of colorectal neoplasia has not been elucidated. However, a number of mechanisms of action have been proposed. Firstly, it has been hypothesised that calcium acts in the intestine binding bile acids and reduce their carcinogenic effect (Lupton, Steinbach et al. 1996). Alternatively it has been hypothesised that calcium acts directly on the colonic mucosa via the calcium sensing receptor and changes in c-myc and e-cadherin expression have been demonstrated in-vitro (Chakrabarty, Radjendirane et al. 2003). An alternative hypothesis is proposed below along with the evidence supporting it; this hypothesis will be addressed in part by the experiments described in this thesis.

1.5.5 Hypothesis to explain mechanism of action of calcium in preventing colorectal neoplasia

The hypothesis has three parts:

1. Raised serum PTH increases the risk of developing colorectal neoplasia.
2. Parathyroid hormone acts directly on colonic epithelial cells to alter cell signalling and gene expression. The changes induced by PTH will be consistent with a pro-neoplastic effect.
3. Dietary calcium acts to reduce colorectal neoplasia by reducing serum PTH levels and thus results in changes in colonic epithelial cells opposite to those seen in response to PTH.

There is both direct and indirect evidence that raised serum PTH increases the risk of colorectal cancer. This will be presented here in the introduction. The second and third parts of the hypothesis will be addressed by the experimental studies described in this thesis.

1.5.6 Parathyroid hormone physiology

The physiological role of Parathyroid hormone is of relevance to understanding how it relates to dietary calcium intake and therefore potentially to the incidence of colorectal cancer.

Parathyroid hormone (PTH) is a classical endocrine hormone produced by the parathyroid glands in the anterior region of the neck. PTH is an 84 amino acid polypeptide, derived from a 115 amino acid precursor molecule and is the product of a single gene on chromosome 11. The major function of PTH is the maintenance of normal levels of calcium in extra-cellular fluid, including serum. The physiological actions of PTH are mediated by activation

of the PTH receptor (PTHR1) on kidney and bone and also indirect actions on the intestine. In response to a fall in ionised calcium levels in the extra-cellular fluid, decreased stimulation of the Calcium sensing receptor (CaSR) on the parathyroid glands results in increased PTH secretion which acts to increase calcium levels in three ways:

1. Acting on the kidney to increase tubular calcium re-absorption thus decreasing urinary calcium loss.
2. Stimulation of osteoclast resorption on bone to mobilise skeletal calcium.
3. Via action on the kidney PTH promotes the conversion of 25OH-vitamin D to 1,25OH-vitamin D which increases calcium absorption from the gastrointestinal tract.

These actions increase extra-cellular fluid calcium and via a classic endocrine feedback loop increased calcium acts via the CaSR to decrease PTH secretion (Wass and Shalett 2002). It is therefore no surprise that dietary intake of calcium is a significant, but not the only determinant of serum PTH level (Saleh, Jorde et al. 2006).

1.5.7 Causes of raised serum PTH (hyperparathyroidism)

1.5.7.1 Primary hyperparathyroidism

Primary hyperparathyroidism (pHPT) is due to excess secretion of parathyroid hormone by an autonomously acting parathyroid adenoma and affects around 1% of the adult population. Patients with primary hyperparathyroidism have high serum calcium secondary to elevated levels of serum PTH.

1.5.7.2 Secondary hyperparathyroidism

Secondary hyperparathyroidism (SHPT) is a raised serum PTH which occurs as a compensatory response to a reduction in serum calcium. It is characterised by a raised serum PTH and a normal or low/normal serum calcium level. There are several factors which have been positively associated with SHPT, these include: increasing age (Jorde, Bonaa et al. 1999), high body mass index (BMI) (Kamycheva, Sundsfjord et al. 2004), serum creatinine (Stein, Flicker et al. 2001), vitamin D deficiency (Saleh, Jorde et al. 2006), low dietary calcium intake (Saleh, Jorde et al. 2006) and dosage of loop diuretic (Stein, Flicker et al. 2001).

1.6: Raised serum Parathyroid hormone as a risk factor for the development of colorectal neoplasia

There is both direct and indirect evidence, that raised serum PTH is associated with an increased risk of developing colorectal cancer. This evidence comes from several sources. The principal purpose of this discussion is to understand the mechanism of action of calcium. Therefore, the evidence relating calcium, PTH and colorectal neoplasia will be presented first.

1.6.1 Calcium acts together with vitamin D on colorectal neoplasia

There is evidence that calcium acts together with vitamin D to reduce the incidence of colorectal neoplasia. Perhaps the most significant is from the American adenoma prevention trial (Baron, Beach et al. 1999). In this study serum 25OH-vitamin D and 1, 25OH-vitamin D levels were measured at the start of the study. The results of the study were analysed with the participants divided into two groups, those with serum levels of both vitamin D metabolites above and below the median level. For participants with 25OH-vitamin D

levels below the median no effect of calcium was seen on adenoma recurrence. For participants with serum 25OH-Vitamin D levels above the median there was a significant reduction in risk of adenoma recurrence (Grau, Baron et al. 2003). There was no difference between the two groups when stratified by 1,25OH-Vitamin D measurements. There was also no relationship between serum 25OH-Vitamin D levels and the risk of colorectal neoplasia in the placebo treated group but increasing serum 25OH-Vitamin D levels were associated with a reduction in risk in those taking calcium supplements (Grau, Baron et al. 2003). These findings led the authors to conclude that; "Vitamin D and calcium appear to act jointly, not separately on colorectal carcinogenesis" and "Overall, the associations we describe provide a strong indication that vitamin D and calcium have a joint anti-neoplastic effect in the large bowel" (Grau, Baron et al. 2003).

There is similar evidence from a case control study; this examined the relationship between serum levels of 25OH-Vitamin D, dietary calcium intake and colorectal adenoma occurrence (Peters, McGlynn et al. 2001). In this study, there was an inverse association between serum 25OH-Vitamin D level and colorectal adenoma occurrence. There was also evidence of an interaction between 25OH-Vitamin D and calcium intake, the inverse association between 25OH-Vitamin D and adenoma occurrence was stronger in those with higher dietary calcium intake (Peters, McGlynn et al. 2001). A similar interaction between vitamin D and calcium and colorectal neoplasia has also been observed in two cohort studies (Zheng, Anderson et al. 1998; Wu, Willett et al. 2002). A synergistic effect of calcium and vitamin D on colorectal carcinogenesis has also been observed in a rodent study, in this

the protective effect of calcium was absent in the presence of vitamin D deficiency (Sitrin, Halline et al. 1991).

Calcium and vitamin D are inter-related particularly in relation to skeletal physiology. That calcium and vitamin D appear to act jointlym, is therefore perhaps not surprising and has implications for the mechanism of action of calcium. It suggests that the effect is not mediated in the intestinal lumen as most vitamin D is synthesised in the skin and is not found in the intestinal lumen. Indeed, it seems likely that the action is due to calcium absorption that is increased with higher levels of 25OH-Vitamin D (Heaney, Dowell et al. 2003; Heaney 2004). Consistent with the stated hypothesis, serum PTH is lowest in individuals with the highest levels of both dietary calcium and serum 25OH-Vitamin D (Adami, Viapiana et al. 2008).

The evidence that calcium and vitamin D act together, has led to the running of adenoma prevention trials testing the combined effect of calcium and vitamin D on adenoma occurrence (www.clinicaltrials.gov ID number, NCT00153816). There have also been randomised trials of the effect of calcium and vitamin D on the incidence of cancer. In the women's health initiative study, the effect of calcium (1000mg) and vitamin D (400 IU) supplementation on colorectal cancer incidence showed no effect on colorectal cancer incidence after seven years follow up (Wactawski-Wende, Kotchen et al. 2006). This study has been criticised for a number of reasons, including the high calcium intake of participants at baseline(Martinez, Marshall et al. 2008) and the low dose of vitamin D used (Schwartz and Blot 2006). Comparison with the studies of aspirin also suggests that the length of follow up was too short, as the effect of aspirin did not become apparent until after

ten years of treatment (Flossmann and Rothwell 2007). In contrast, a different study of daily supplementation with 1500mg of calcium and 1100 IU of vitamin D resulted in a significant reduction in incidence of any cancer (including colorectal) after four years follow up (Lappe, Travers-Gustafson et al. 2007). This study can also be criticised for its short follow up time and the use of any cancer as an end point.

1.7: Vitamin D

As calcium and vitamin D act by the same mechanism, to understand the mechanism of action of calcium, it is important to study the data relating vitamin D to colorectal cancer. As well as an association with colorectal cancer, there is an increasingly large body of evidence demonstrating an inverse association between vitamin D and the risk of a number of other cancers (Grant 2010).

1.7.1 Physiology of Vitamin D

Vitamin D is a fat soluble vitamin with an essential role in calcium homeostasis. By strict definitions, it is not a vitamin as all requirements can be met from the none-dietary source of ultraviolet radiation. It is well recognised that here is a relationship between sunlight and UVB exposure and serum levels of 25OH vitamin D. Lower levels of vitamin D are found at higher latitudes where exposure to UVB radiation is lower (Mithal, Wahl et al. 2009), even within the United Kingdom, serum 25OH-Vitamin D is lower at more northerly latitudes (Macdonald, Mavroeidi et al.). Serum 25OH-Vitamin D also varies by season being higher in the summer and lower in the winter (Pasco, Henry et al. 2004; Mithal, Wahl et al. 2009), when exposure to UVB

radiation is low (Kimlin 2004). Important to this discussion is the fact that: serum PTH level is negatively correlated with serum 25OH-vitamin D level (Lips 2001) and supplementation with vitamin D suppresses serum PTH level (Malabanan, Veronikis et al. 1998).

1.7.2 Vitamin D and colorectal cancer

The evidence linking vitamin D and colorectal cancer is predominantly observational. The starting point for all of these studies is the hypothesis proposed by Garland and Garland linking sunlight, exposure to ultraviolet B (UVB) radiation and vitamin D to the risk of dying from colorectal cancer (Garland and Garland 1980).

1.7.3 Sunlight exposure and colorectal cancer

This hypothesis arose from the observation of increased death rates from colorectal cancer in areas of low sunlight exposure (Garland and Garland 1980). Ground level radiation values, from the United States weather service were used to create a map of solar radiation exposure for the whole of the United States. Comparison of this, with published rates of colorectal cancer deaths, demonstrated an inverse association between solar radiation exposure and deaths from colorectal cancer. It was also observed that urban areas had higher death rates from colorectal cancer compared to rural areas. In these urban areas the relationship between solar radiation exposure and death rates from colorectal cancer were even stronger. This paper was not the first to report a relationship between higher latitude and death from cancer. In 1941 a link between all cancer mortality and reduced exposure to solar radiation in the United States was demonstrated (Apperly 1941). In this

study, the observed lower mortality from cancer was attributed to a relative immunity to cancer induced directly by exposure to sunlight.

What was new in the 1980 paper (Garland and Garland 1980) was the linking of the relationship between reduced solar radiation and colorectal cancer death to vitamin D levels. The authors drew a parallel with rickets, the childhood bone disease. Rickets is associated with urban living and lack of exposure to solar radiation. It was subsequently found that rickets was caused by low levels of vitamin D and could be treated with vitamin D supplements or sunlight exposure (Rajakumar 2003). The authors proposed a similar explanation for the inverse association between solar radiation exposure and the risk of colorectal cancer death. It was hypothesised that low levels of vitamin D were associated with an increased risk of death from colorectal cancer and that this effect is mediated by a direct effect of vitamin D on the colorectal epithelium.

1.7.4 Serum vitamin D levels

Following on from this ecological evidence, it was hypothesised that if the effect of sunlight was mediated by vitamin D that individuals with high levels of serum vitamin D would have a lower incidence of colorectal neoplasia compared to those with lower levels. This has been studied in a number of observational studies of colorectal cancer and adenoma.

1.7.4.1 Colorectal cancer incidence and serum 25OH-vitamin D

The relationship between serum 25OH-vitamin D and a diagnosis of colorectal cancer was the subject of a meta-analysis (Gorham, Garland et al. 2007). Five studies were included with a total of 535 cases and 913 controls. The five studies were all nested case control studies of healthy volunteers, with

pre-diagnostic blood samples who were followed up for 2-25 years after blood sample collection. Four of these studies were carried out in the United States and one in Finland. Individually, three of these studies showed statistically significant odds ratio in individuals with higher 25OH-vitamin D levels (Tangrea, Helzlsouer et al. 1997; Feskanich, Ma et al. 2004; Wactawski-Wende, Kotchen et al. 2006) and two showed none significant trends in the same direction (Garland, Comstock et al. 1989; Braun, Helzlsouer et al. 1995). In the meta-analysis, the odds ratio of the highest versus lowest quintile was 0.49 (0.32-0.64), which was highly significant ($p<0.0001$). Two of these studies investigated the association between both serum 25OH-vitamin D and 1,25OH-vitamin D, in both of these there was a significant association between colorectal cancer and 25OH-vitamin D but no association was seen for 1,25OH-vitamin D (Tangrea, Helzlsouer et al. 1997; Feskanich, Ma et al. 2004).

The meta-analysis demonstrates with greater certainty than the five individual studies alone that there is an inverse relationship between serum 25OH-vitamin D and colorectal cancer incidence. Importantly, the relationship observed is between 25OH and not 1,25OH-Vitamin D. 25OH-Vitamin D is the storage form of vitamin D and represents the body's reserves; it also has an inverse relationship with PTH.

1.7.4.2 Colorectal adenoma incidence and serum 25OH-vitamin D

Colorectal adenomas are the precursor to the majority of colorectal cancers. As it seems likely that vitamin D acts early in colorectal tumorigenesis; studying the relationship between serum 25OH-vitamin D levels and the presence of adenomas may provide support for the hypothesis. A number of

studies measuring serum 25OH-vitamin D and determining the risk of colorectal adenoma have been carried out. Recently, a meta-analysis of these has been performed (Wei, Garland et al. 2008). Seven studies were identified for inclusion, all of which were carried out in the United States between 1989 and 2000. There were: three case control studies (Levine, Harper et al. 2001; Peters, McGlynn et al. 2001; Peters, Hayes et al. 2004), one nested case control study (Platz, Hankinson et al. 2000), two cohort studies (Grau, Baron et al. 2003; Jacobs, Alberts et al. 2007) and one cross-sectional study (Miller, Keku et al. 2007). A total of 2628 cases and 3787 controls were included. In the meta-analysis, the odds ratio of colorectal adenoma occurrence was 0.7 (95% CI 0.56-0.87), for the highest versus the lowest levels of circulating 25OH-vitamin D. The finding of a risk reduction was also observed in six of the studies alone, with the seventh showing no effect (Platz, Hankinson et al. 2000).

1.7.5 Mechanism of action of vitamin D

Having observed an apparent protective effect of 25OH-Vitamin D on colorectal cancer incidence, a direct effect of vitamin D on the colon was hypothesised. In this 25OH-Vitamin D is converted to the active form: 1,25OH-Vitamin D by 25OH-Vitamin D-1α-hydroxylase, which activates the vitamin D receptor (VDR). Expression of both, 25OH-Vitamin D-1α-hydroxylase and the VDR has been shown in benign and malignant colonic tissues (Matusiak, Murillo et al. 2005).

1.7.6 Vitamin D and causation of colorectal neoplasia

These data relating vitamin D to colorectal cancer provides evidence for it having a role in the development of cancer. However, there is only limited

randomised controlled trial data. Therefore, it could be argued that there is insufficient evidence that the relationship between vitamin D and reduced colorectal cancer incidence is directly causal. This brings into question whether the proposed mechanism of action of vitamin D is direct as described above. It has been argued, using the criteria of Hill (Hill 1965), that the most likely explanation for the vitamin D-colorectal cancer observations is causation (Grant 2009). It will be argued here, using the same criteria that though there is significant evidence in support of a protective effect of vitamin D, that there are weaknesses. And, that this suggests an alternative mechanism of action of vitamin D.

In his seminal lecture; „On the environment and disease: association or causation' (Hill 1965) Sir Austin Bradford Hill highlighted the difficulties of inferring causation from epidemiological evidence. In acknowledging the difficulties of differentiating between association or causation, he observed that the vital question was whether the presence of a disease B (e.g. colorectal cancer) would be reduced by changing an environmental feature A (e.g. vitamin D levels). To aid in the differentiation between causation and association, he proposed nine features that could be considered to make causation the most likely interpretation. It was clearly stated that none of these criteria can be used to confirm causation, only to aid in the decision. These nine criteria are listed below.

1. Strength
2. Consistency
3. Specificity
4. Temporality.

5. Biological gradient
6. Plausibility
7. Coherence
8. Experiment
9. Analogy

As has been argued (Grant 2009), for most of the criteria the evidence for vitamin D being directly protective is reasonably compelling. However it will be argued, that two of these criteria (specificity and biological gradient) are not met and that the data suggest an alternative explanation.

1.7.6.1 Specificity

As discussed above, there is a body of evidence indicating that calcium and vitamin D act to inhibit colorectal neoplasia together, rather than separately. It is of note that, it is not just the effect of calcium that depends on the level of vitamin D, but that the effect of vitamin D depends on calcium intake (Peters, McGlynn et al. 2001; Grau, Baron et al. 2003). This implies that both agents act jointly through the same mechanism, via a factor modulated by both of them. This argues against Vitamin D having a directly protective effect against colorectal neoplasia.

1.7.6.2 Biological gradient

The presence of a linear biological gradient, or dose response curve, has not been demonstrated for vitamin D and colorectal neoplasia. Indeed, there are studies demonstrating a none-linear relationship between serum levels of 25OH-Vitamin D and colorectal neoplasia. The most clear evidence of a non linear relationship between 25OH-Vitamin D and colorectal neoplasia is from an adenoma case-control study; in this an inverse association between 25OH-

Vitamin D and colorectal adenoma was demonstrated (Peters, McGlynn et al. 2001). This inverse relationship was greatest in the deficiency range, which was defined by a node at 12.6ng/ml, identified using piecewise linear regression modelling. Above 12.6ng/ml, the risk of adenoma decreased by only 2% for each 1ng/ml increase in 25OH-Vitamin D, in contrast below 12.6ng/ml the risk of adenoma decreased by 45% for each 1ng/ml increase in 25OH-Vitamin D. In most studies of colorectal cancer, categorical comparisons have been performed. These do not precisely show the nature of the relationship with 25OH-Vitamin D. In a meta-analysis of these studies a non linear relationship was demonstrated between the risk of colorectal cancer and serum 25OH-Vitamin D (Grant 2010). The risk of colorectal cancer was greatest in the vitamin D deficiency range with the benefit per unit of nano-mole per litre decreasing at higher serum 25(OH)-Vitamin D levels (Grant 2010). The absence of a linear relationship between serum 25OH-Vitamin D and colorectal neoplasia argues against a direct protective effect of vitamin D on colorectal neoplasia and suggests an alternative explanation.

There is a striking similarity between the relationship of 25OH-Vitamin D and the risk of colorectal neoplasia (Peters, McGlynn et al. 2001; Grant 2010) and the relationship between serum 25OH-Vitamin D and PTH (Need, O'Loughlin et al. 2004). The relationship between 25OH-Vitamin D and PTH is similarly non linear, with little relationship between the two variables above a 25OH-Vitamin D level of around 45nmol/l and a significant inverse relationship below this level. These observations provide indirect evidence that the risk of colorectal neoplasia is greatest when serum PTH is highest and suggest an

indirect action of 25OH-Vitamin D in reducing the risk of colorectal neoplasia by modulating serum PTH levels.

1.8: Serum PTH levels and colorectal neoplasia

In addition to the indirect evidence described above there is direct evidence that raised serum PTH is associated with an increased risk of colorectal cancer. This comes from two studies published ten years apart (Niv, Sperber et al. 1999; Charalampopoulos, Charalabopoulos et al. 2010). In these two small case control studies, both vitamin D and PTH levels were measured and both showed the same results. Higher levels of PTH and lower levels of vitamin D were found in cases with colorectal cancer compared to controls. Strikingly both studies demonstrated that PTH levels were higher in subjects with more advanced disease, suggesting a possible effect on the progression as well as the development of cancer. In both of these studies the primary hypothesis of the study relate to the levels of vitamin D and only in the second study was a possible role for PTH in colorectal cancer development suggested (Charalampopoulos, Charalabopoulos et al. 2010). These studies could be criticised as the serum levels were measured after diagnosis and may have been influenced by the disease process rather than causing it. However, a report (only published in abstract form) of a nested case-control study of 1248 cases from within the EPIC (European investigation into cancer and nutrition) cohort study examined the relationship between pre-diagnostic levels of serum PTH and the subsequent risk of colorectal neoplasia (Fedirko, Riboli et al. 2010). This showed an increased risk of colorectal cancer in patients with serum PTH levels >65 compared to those below this with a

relative risk of 1.41 (1.03-1.93). This increased risk was independent of 25OH-Vitamin D levels.

1.8.1 Hyperparathyroidism

There are a number of clinical situations in which a raised serum PTH is found. Epidemiological evidence that these conditions are associated with an increased risk of colorectal neoplasia is presented here and provides further indirect evidence that raised serum PTH is a risk factor for the development of colorectal cancer.

1.8.1.1 Primary hyperparathyroidism

An increased risk of developing colorectal cancer has been demonstrated in patients with primary hyperparathyroidism (Palmer, Adami et al. 1988; Nilsson, Zedenius et al. 2007), an effect that persists for up to 15 years after parathyroidectomy.

1.8.1.2 Secondary hyperparathyroidism

The risk factors for secondary hyperparathyroidism have been described in section 1.5.7. It is predicted that these factors may also be associated with an increased risk of colorectal cancer. The evidence that increasing age, low dietary calcium and vitamin D deficiency are risk factors for developing colorectal cancer have been presented above. High Body Mass Index (Lin, Zhang et al. 2004; Samanic, Chow et al. 2006) and loop diuretic intake (Tenenbaum, Grossman et al. 2001) have also been associated with an increased risk of colorectal cancer. The evidence for raised serum creatinine is less clear but patients with end stage renal failure have an increased risk of colorectal cancer (Iseki, Osawa et al. 1993). These observations are intriguing and consistent with the hypothesis, but of only limited support for it.

1.8.2 Raised serum PTH and the risk of other cancers

There is also similar evidence for other cancers that serum PTH may be a risk factor for the development of cancer or involved in its progression.

1.8.2.1 Breast cancer

Vitamin D

Similar to the findings with colorectal cancer, there is an inverse association between the risk of breast cancer and sunlight exposure (Grant 2002). In a meta-analysis, low levels of serum vitamin D are also associated with an increased risk of breast cancer (Grant 2010). As with colorectal cancer, there is evidence of a non linear relationship between 25OH-Vitamin D and breast cancer incidence. There is an individual study (Abbas, Linseisen et al. 2008), as well as a meta-analysis (Grant 2010) that show a non linear relationship. These show minimal variation in risk at higher 25OH-Vitamin D levels, with a marked inverse association in the vitamin D deficiency range, when PTH is highest.

Serum PTH level

There is also direct evidence that serum PTH levels are higher in patients with breast cancer compared to controls (Fierabracci, Pinchera et al. 2001). Serum PTH levels are also significantly higher in patients with advanced compared to early breast cancer (Palmieri, MacGregor et al. 2006).

Primary hyperparathyroidism

Patients with primary hyperparathyroidism also have an increased risk of breast cancer (Palmer, Adami et al. 1988; Michels, Xue et al. 2004; Nilsson, Zedenius et al. 2007).

1.8.2.2 Prostate cancer

The evidence relating PTH to prostate cancer, relates more to advanced stage of disease and influence on prognosis. Raised serum PTH is commonly found in patients with prostate cancer, particularly those with bony metastases (Murray, Grill et al. 2001). In another study, higher levels of serum PTH were associated with poorer survival (Schwartz 2008). These effects are hypothesised to be mediated in part via the action of PTH on bony metastases. An effect on the early development of prostate cancer has not been reported. However a recent study, demonstrated that in patients without prostate cancer serum prostate specific antigen (PSA) is positively correlated with serum PTH levels (Skinner and Schwartz 2009). This is consistent with PTH having a growth promoting effect on normal prostate.

The observation that raised serum PTH is found in three different tumour types suggests a similar pathological mechanism is in play for each of them.

1.9: Normal colorectal mucosa

Based on the findings above, raised serum PTH appears to be a potential risk factor for the development of colorectal neoplasia. It is hypothesised that PTH acts directly on colorectal epithelium. This is based on the evidence that, calcium works early in colorectal tumorigenesis to reduce the occurrence of adenomas. There are several studies which have demonstrated an effect of dietary calcium supplementation on the normal epithelium and these will be reviewed below. In this thesis, an alternative approach to that of others using oligonucleotide micro-arrays will be used to investigate the influence of calcium and PTH on gene expression in the normal colorectal mucosa. The following section will briefly review: current understanding of colonic crypt organisation, the signalling pathways that regulate this, the effect of calcium

on the colonic crypt and normal epithelial markers of the risk of developing colorectal cancer.

1.9.1 Organisation of the colonic crypt

The colorectal epithelium is a rapidly renewing tissue in which cell homeostasis is a balance between proliferation, growth arrest, differentiation and apoptosis. It is made up of a single sheet of columnar epithelial cells lying on top of the underlying connective tissue into which this sheet invaginates forming millions of crypts, which are the basic unit of the colon. At the base of each crypt are stem cells, these form part of the stem cell niche along with the surrounding peri-cryptal myofibroblasts (Humphries and Wright 2008). These myofibroblasts secrete a number of factors involved in the regulation of stem cell behaviour (Fevr, Robine et al. 2007). There are four types of differentiated epithelial cells in the colon: colonocytes or absorptive cells, mucous secreting goblet cells, peptide hormone secreting entero-endocrine cells and paneth cells which are uncommon and usually only found in the ascending colon. These different cell types all arise from the multi-potent stem cells at the crypt base. At a simplistic level, the proliferating cells are found at the crypt base and as cells migrate up the crypt they undergo growth arrest, differentiate and subsequently undergo apoptosis.

It is clear from this description that stem cells are important in maintaining the colonic crypt, as the rest of the cells within it will inevitably die. This fact has led to the hypothesis that stem cells are the cells of origin of colorectal cancer, as it is only in these long lived cells that mutations leading to colorectal cancer will have the time to develop. Consistent with this hypothesis, is the finding that deletion of the APC gene in stem cells results in

progressive neoplasia, which does not occur if APC is deleted in transit amplifying cells (Barker, Ridgway et al. 2009). Signalling pathways that regulate colonic stem cells are therefore of interest. These stem cell regulating pathways along with those that regulate proliferation and differentiation in colonic crypts are discussed below.

1.9.1.1 Wnt signalling pathway

The Wnt signalling pathway has important functions in the normal colonic epithelium and deregulation of the Wnt signalling pathway is a defining feature of colorectal neoplasia. The Wnt signalling pathway is named after the wingless Drosophila that was found to be due to mutations in the gene which became recongised as components of the Wnt signalling pathway. Subsequently the Wnt signalling pathway has been found to have an important role in human embryological development and the regulation of proliferation and differentiation in several adult tissues as well as the colon.

Description of Wnt signalling pathway

The components of the Wnt signalling pathway and their interactions are described below and are shown in Figure 1.1. There are a number of components with overlapping functions and tissue specific expression of different components is recognised and has been described for the colon (Gregorieff, Pinto et al. 2005). The focus of this introduction will be a general overview of the pathway and a specific focus on those components of functional relevance in the colon and those altered in colorectal neoplasia. There are classically two wnt signalling pathways activated by wnt ligands; the canonical and non-canonical pathways. The canonical pathway acts via β-

catenin mediated transcription and as this is the pathway deregulated in colorectal cancer it will be the focus of this introduction.

To simplify the pathway, the components can be described in three cellular domains; extra-cellular signals and the plasma membrane receptors they interact with, the cytoplasmic machinery that controls β-catenin stability and the contents of the nucleus that control β-catenin mediated transcription.

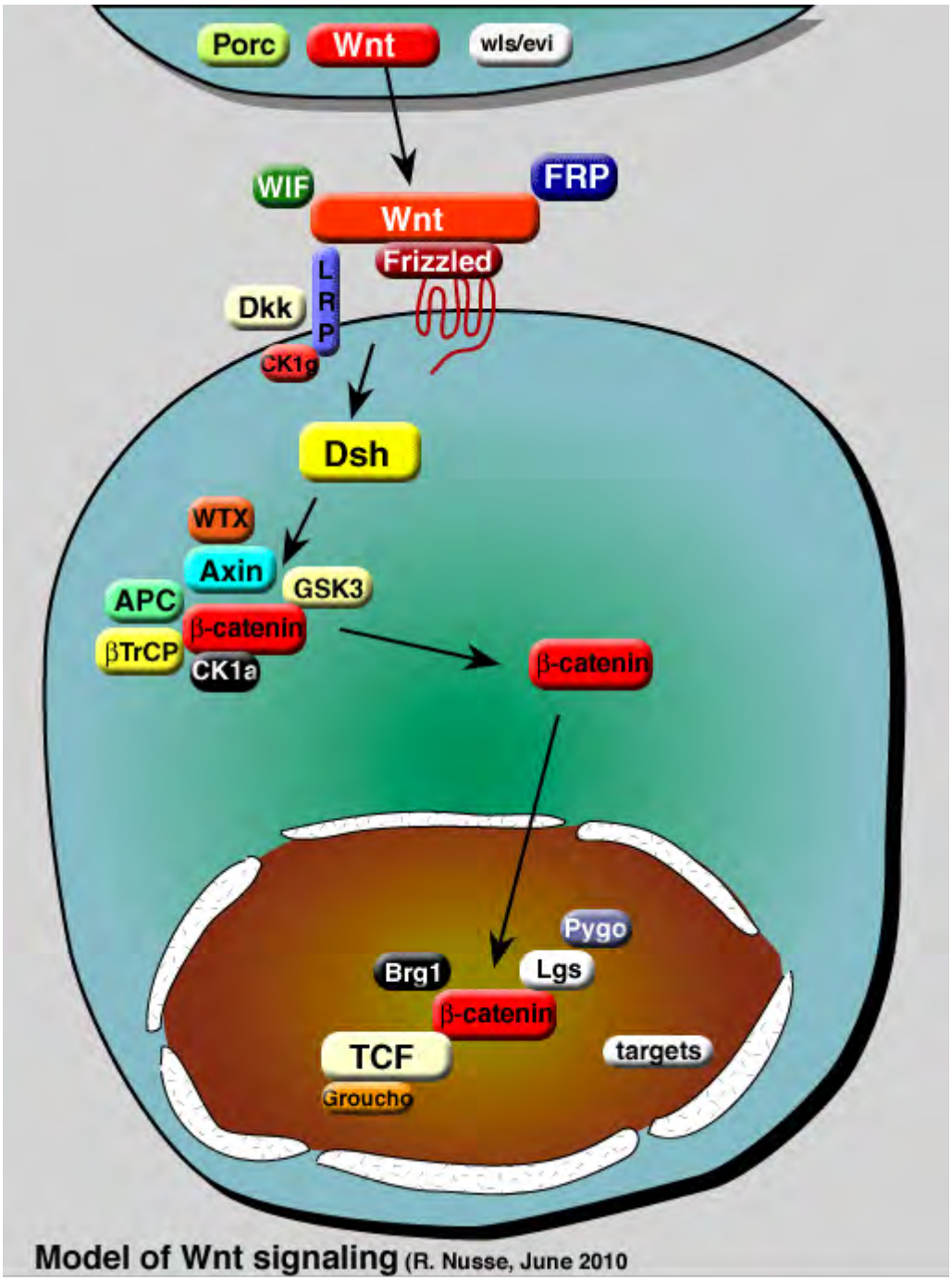


Figure 1.1 The Wnt signalling pathway.
http://www.stanford.edu/group/nusselab/cgi-bin/wnt/

Extra-cellular/plasma membrane

There are a number of (19) secreted Wnt proteins that bind to Frizzled receptors in the plasma membrane and their LRP5/6 (low density lipoprotein receptor-related protein) co-receptors. The presence of Wnt ligand results in the activation of a signalling cascade that controls the stability of β-catenin. There are several inhibitors of Wnt signalling that act to prevent wnt-Frizzled interactions. These include; the SFRP's (Secreted Frizzled-related proteins), the Dikkopf's (DKK's) and Wnt inhibitory factor 1 (WIF1). The expression of these inhibitors adds another level of control of the signalling cascade in addition to the presence or absence of the wnt ligands.

Cytoplasmic machinery

In the absence of an extra-cellular wnt signal β–catenin in the cytoplasm is recruited to a destruction complex, this consists of APC (Adenomaous polyposis coli) and Axin. In this complex β–catenin is phosphorylated by GSK3β (Glycogen syynthase kinase 3β) and CK1α (Casein kinase 1α). This phosphorylation results in β–catenin being targeted for proteasome dependent degradation, involving interaction with β-TRC, a component of the ubiquitin ligase complex. This process results in low levels of cytoplasmic β–catenin.

In contrast, the presence of wnt ligand activates a signalling cascade that results in the stabilisation of cytoplasmic β-catenin. The results of Wnt ligand signalling are phosporylation of LRP5/6 by GSK3β and CK1α. Recruitment of Dishevelled to the plasma membrane leads to polymerisation of multiple dishevelled moieties and interaction with Frizzled receptors. These processes lead to the inactivation of the destruction complex via translocation

of axin to the plasma membrane. Inactivation of the destruction complex leads to the cytoplasmic stablisation of β-catenin, where it is free to translocate into the nucleus.

Nucleus

Following translocation to the nucleus β-catenin forms a complex with the transcription factors LEF (Lymphoid enhancing factor) and TCF (T-cell factor). This results in the expression of downstream target genes, which have been widely studied. These genes have been characterised (van de Wetering, Sancho et al. 2002) and include several genes with key roles in cell proliferation and colorectal tumorigenesis, particularly c-Myc (He, Sparks et al. 1998).

Deregulation of the Wnt signalling pathway in colorectal cancer

The most relevant observation implicating deregulation of the Wnt signalling pathway in colorectal tumorigenesis is the finding of mutation of the APC gene in 85% of sporadic colorectal cancers(Kinzler and Vogelstein 1996). Germline mutations in APC also underlie FAP(Bulow 1989). Mutations in other components of the Wnt signalling pathway are found less frequently. These include CTNNB1 (the gene for β-catenin) and Axin (Satoh, Daigo et al. 2000). These mutations lead to the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus. This results in the increased expression of Wnt (TCF/LEF) target genes. Consistent with this is the finding that colorectal cancer cells show constitutive activation of β-catenin/TCF complex (Korinek, Barker et al. 1997) and high expression of downstream Wnt/β-catenin target genes are found in colorectal cancer (Yan, Wiesmann et al. 2001).

In additional to mutational activation of the Wnt signalling pathway, promoter methylation resulting in decreased expression of several wnt antagonists (sFRP1 and DKK1) has been found in colorectal cancers (Caldwell, Jones et al. 2004; Gonzalez-Sancho, Aguilera et al. 2005).

Normal intestine

The Wnt signalling pathway is the main regulator of the colonic crypt and is implicated in maintenance of intestinal stem cells (Crosnier, Stamataki et al. 2006). Over activation of the Wnt signalling pathway results in crypt hyper-proliferation (Sansom, Reed et al. 2004) and blockage of the pathway results in loss of proliferating crypt cells (Korinek, Barker et al. 1998). Similarly, loss of Wnt signalling results in differentiation of crypt cells (Mariadason, Bordonaro et al. 2001). Consistent with these observation are the finding that Wnt/TCF target genes are strongly expressed in proliferating cells at the crypt base and conversely genes negatively regulated by Wnt/TCF are expressed in differentiated cells found at the top of the colonic crypt (Kosinski, Li et al. 2007).

PTH activation of the Wnt signalling pathway

Of relevance to this study is the observation that PTH has been shown to activate the Wnt sginallng pathway in bone. PTH treatment of osteosarcoma cell lines results in increased β-catenin expression and increased activity of a Wnt reporter gene (Kulkarni, Halladay et al. 2005) with a similar finding in mouse osteoblastic cells(Tobimatsu, Kaji et al. 2006). More recently it has been shown that PTHR1 interacts directly with dishevelled and that PTH activates β-catenin independent of Wnt (Romero, Sneddon et al. 2010).

Whether PTH activates the Wnt signalling pathway in the colon is therefore of interest in this study.

1.9.1.2 Hedgehog pathway

The Indian hedgehog (IHH) signalling pathway has been shown to regulate the intestinal stem cell niche. Knockout of IHH results in: increased proliferation, decreased differentiation, increase Wnt signalling and increased numbers of stem cells (Kosinski, Stange et al. 2010). Increased activity of the IHH pathway showed the opposite effects (van Dop, Uhmann et al. 2009). There is evidence that some of the effects of hedgehog on the colonic crypt are mediated by BMP (Bone morphogenetic protein) signalling, with BMP signalling limiting the expansion of the proliferative crypts (Crosnier, Stamataki et al. 2006).

1.9.2 Features that predict the development of colorectal cancer

There are two features of normal colorectal epithelium that have been shown to predict the risk of developing colorectal cancer. These are of relevance to studying the effect of chemo-preventive agents on normal colorectal mucosa.

1.9.2.1 Apoptosis

Reduced apoptosis has been shown to predict current (Anti, Armuzzi et al. 2001) and future neoplasia (Keku, Amin et al. 2008).

1.9.2.2 Proliferation and Differentiation

The presence of proliferating cells in the upper part of the colonic crypt has been demonstrated to predict the risk of current (Ponz de Leon, Roncucci et al. 1988; Risio, Lipkin et al. 1991) or future neoplasia (Scalmati, Roncucci et al. 1990; Anti, Marra et al. 1993).

1.9.3 Effect of calcium on normal colorectal mucosa

1.9.3.1 Proliferation and Differentiation

Several studies have examined the effect of calcium on proliferation markers. Some studies have shown no effect of calcium (Gregoire, Stern et al. 1989) or only in some individuals (Rozen, Fireman et al. 1989). In several studies calcium reduced the proliferation index, sometimes with a reduction in the total number of proliferating cells (Wargovich, Isbell et al. 1992). The commonest finding was a reduction in the number of proliferating cells in the upper parts of the crypt, with a redistribution of the proliferation zone to the lower portion of the crypt (Nobre-Leitao, Chaves et al. 1995; Holt, Atillasoy et al. 1998; Holt, Wolper et al. 2001). In the only randomised double blind placebo controlled trial, with 170 patients, no change in overall proliferative index was seen. However, again there was a decrease in the number of proliferating cells in the upper portion of the crypt, resulting in what the authors describe as a normalisation of the distribution of proliferating cells (Bostick, Fosdick et al. 1995). A study of ninety two patients demonstrated that calcium increased the expression of p21 (CDKN1A), a cyclin-dependent kinase inhibitor only expressed in fully differentiated cells but had no effect on the expression of the proliferation marker MIB-1 (Fedirko, Bostick et al. 2009). These findings suggest that calcium induces the earlier differentiation of crypt cells as they migrate up the crypt, restricting proliferation to the crypt base.

1.9.3.1 Apoptosis

Other studies have investigated whether calcium results in increased apoptosis in colorectal epithelium, some of these have shown no effect on apoptosis (Holt, Wolper et al. 2001; Holt, Bresalier et al. 2006), while others

have suggested that calcium may enhance apoptosis (Miller, Keku et al. 2005; Fedirko, Bostick et al. 2009).

1.9.4 Effect of PTH on normal colorectal mucosa

There are no studies of the effect of PTH on the colorectal mucosa; this will be addressed in this study**.**

Chapter 2
Methods

2.1: Cell culture

Caco-2 cells (passages 36) were maintained in culture at 37°C in a humidified atmosphere of 5% CO_2 in Dulbecco's Modified Eagle Medium supplemented with 10% heat inactivated foetal calf serum. The methods used to prepare Caco2 for the experiments are described in chapter three.

2.2: RNA extraction

Frozen colonic tissues were thawed on ice and homogenised in 1ml Trizol (Invitrogen) using a tissue homogeniser whilst being kept chilled. The samples were incubated at room temperature for five minutes to enable complete dissociation of nucleoprotein complexes. To each sample 0.2ml of chloroform was added and the tube vortexed for 15 seconds, then incubated at room temperature for three minutes. Samples were then centrifuged at 12,000g for fifteen minutes at 4°C and the upper aqueous phase harvested. The RNA was precipitated by addition of 0.5ml isopropanol and incubation for ten minutes at room temperature. The RNA was spun to a pellet by centrifugation at 12,000g at 4°C for ten minutes and the supernatant removed. The pellet was washed with 75% ethanol, spun at 7,500g at 4°C for five minutes. The supernatant was removed and the pellet allowed to air dry at room temperature for ten minutes, the RNA was then re-suspended in an appropriate volume of nuclease free water (30-70µl). For cells-lines, $1x10^6$ cells were washed with PBS x2 and harvested in 1 ml of TRIzol Reagent (Invitrogen) and RNA extracted as described above. RNA was stored at -80°C.

2.2.1 Quantification of RNA

Total RNA was quantified by measuring the optical density at 260nm of 1µl of the RNA suspension using the NanoDrop spectrophotometer (Labtech International). The quality of the RNA was measured using the 260/280nm ratio, with values >1.8 and <2.1 indicative of high nucleic acid content comparet to non-nucleic acid content.

2.3: cDNA synthesis

The Promega reverse transcription system A3500 was used. For each sample a mastermix of 18µl was made with the following reagents:

1.0µl (500ng) random primers

0.75µl (15 units) of Avian Myeloblastosis Virus (AMV) reverse transcriptase

2.0µl (1/10 volume) of 10X reverse transcriptase buffer

0.5µl (20 units) of RNase inhibitor

4.0µl (5mM $MgCl_2$)

2.0µl (1mM) dNTPs

7.75µl dH_2O

2µl (approximately 200-300ng) of total RNA was added to this mastermix giving a final volume of 20µl. An equivalent volume of dH_2O was added to an additional tube (instead of RNA) which acted as a negative control. The mixture was then reverse transcribed at 42°C for 30 min, followed by 94°C for 2 min to inactivate the reverse transcriptase. The cDNA was then stored at -20°C for future use.

2.4: SYBR-Green semi-quantitative real-time polymerase chain reaction

Semi-quantitative real-time polymerase chain reaction (SQRT-PCR) was carried out using SYBR-Green. Analsyses were carried out in triplicate 25µl reactions. Each of which contained 1x Sensimix (Quantace), 20-40ng cDNA, 9pmoles each primer, 1x SYBRGreen solution (Quantace), 4mM MgCl2 and 0.5 units UNG (Quantace). SQRT-PCR was carried out in a 96 well optical plate sealed with a plastic cover. This was carried out using an ABI Prism 7000 (Applied Biosystems) following the manufacturer instructions. The PCR cycle included incubation at 37°C for 10 minutes followed by incubation at 95°C for 10 minutes. Then 40 cycles of 95°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds were performed.

2.4.1 SQRT-PCR data analysis

SQRT-PCR data was first analysed using ABI Prism 7000 software (Applied Biosystems) following the manufacturer guidelines. Cycle threshold (Ct) values were determined for both KRT8 internal control and gene of interest in each sample by placing a threshold line through the exponential phase of the PCR cycle profiles. This data was then exported to Microsoft Office Excel where the average Ct values were calculated from the triplicates.

2.4.2 Primers for semi-quantative real time PCR

For each gene of interest, the cDNA sequence was downloaded from Ensembl and primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3). The primer pairs used in the SQRT-PCR ananlyses amplified an amplicon which crosses an exon-exon boundary. To confirm the presence of a single amplicon of the expected size and the

absence of amplified genomic DNA the PCR product was visualised on an agarose gel.

2.4.3 Primer sequences

PTHR1	Left:	GACACACGGCAGCAGTACC
	Right:	GTGTGGCCATGAAGACAATG
AREG	Left	TGGTGCTGTCGCTCTTGATA
	Right	ACTGTGGTCCCCAGAAAATG
CYP1A1	Left	GACAGATCCCATCTGCCCTA
	Right	GGTTGATCTGCCACTGGTTT
GSN	Left	TGGGTTGGAAAGGATTCTCA
	Right	ACAAAGGAGGGAGGCTCAAA
CTBP1	Left	CATCGAGATGCGAGAGGAG
	Right	GGCTGTCAGATGGTCCTTGT
STT3A	Left	GTGTGGACCGTGAAGGTTCT
	Right	CAATCTCAGCATTTCGGACA
GJA1	Left	CAATCACTTGGCGTGACTTC
	Right	CCTCCAGCAGTTGAGTAGGC
PDCD4	Left	GCTGGAGCGGTTTGTAGAAG
	Right	CCTCCATCTCCTTCGCTTAC

2.5: Micro-array expression analysis

Micro-array expression analysis allows the simultaneous analysis of the expression levels of thousands of genes. Affymetrix expression gene chips are single colour oligo-nucleotide micro-arrays. Two different chips were used in the experiments described in this thesis. The human genome focus array was used in chapter four and has around 8,700 probes representing 8,400 genes. The human genome U133A 2.0 array was used in chapters six and seven and has around 22,000 probes representing 14,500 genes; this includes all those on the focus array. The oligo-nucleotide probes consist of 25mers and the hybridisation of biotin labelled target sequences in the experimental sample to these probes is detected by staining with a streptavidin-phycoerythrin and measuring the light emitted at 570 nm. The signal produced is proportional to the target gene bound at each location on the array, which reflects the transcript levels present in the original target population. A number of control genes are used to normalise and scale between each array to enable differences in signal intensities to be compared.

2.5.1 Sample preparation for micro-array analysis

Sample RNA needs to undergo a number of processes prior to micro-array analysis.

2.5.1.1 RNA clean up

Firstly sample RNA was purified using the Qiagen® RNeasy mini kit as per the manufacturer's instructions. Briefly, the RNA samples were adjusted to 100 µl volume with RNAse free water. To this 350 µl of buffer RLT is added and mixed, this inactivates RNAses. To this mixture is added 250 µl of 100%

ethanol. The mixture is added to the silica-gel membrane within the column and centrifuged for 15 seconds at 8000 x g and the flow-through is discarded. Subsequently, 500 µl of RPE buffer is added to the column which is again centrifuged and the flow-through discarded. This is repeated with a prolonged period of centrifugation, to ensure the column is dry and to avoid carry over of ethanol. Finally, 30 µl of RNAse free water is passed through the column to elute the RNA.

2.5.1.2 First-strand cDNA synthesis

10 µg RNA was reverse transcribed to first-strand cDNA by adding 100 pmol $T7(dT)_{24}$ primer, 2 µl poly-A controls and made up to 11 µl with DEPC-treated water. This reaction was incubated at 70ºC for 10 minutes, then a master mix consisting of: 4 µl 5X First Strand buffer, 2 µl 0.1M DTT, and 1 µl 10 mM dNTPs was added. The samples were then incubated at 42ºC for 2 minutes. Then 2 µl Superscript II Reverse Transcriptase was added and the sample incubated at 42ºC for 1 hour followed by 2 minutes incubation on ice.

2.5.1.3 Second-strand cDNA synthesis

For the second-strand cDNA synthesis a master mix containing: 30 µl 5 X Second strand buffer, 3 µl 10 mM dNTPs, 1 µl 10U/µl *E.Coli* DNA ligase, 4 µl 10U/µl *E. Coli* DNA Polymerase I, 1 µl 2U/µl *E. Coli* RNAse H and 91 µl DEPC treated water was made up. The second-strand cDNA synthesis reaction was performed using 130 µl of the master mix added to each of the first strand reaction tubes and incubated at 16ºC for 2 hours. Next 2 µl T4 DNA Polymerase was added and incubated at 16ºC for a further 2 minutes, this was followed by the addition of 10 µl 0.5M EDTA.

2.5.1.4 Clean-up of double stranded cDNA

To clean up the cDNA, 600 µl of cDNA binding buffer was added to the cDNA sample. The sample was place on a cDNA cleanup spin column in a collection tube and centrifuged for 1 minute at full speed. The flow-through was discarded. The column was washed with 750 µl wash buffer applied to the column and the sample was centrifuged again for 1 minute at full speed. The spin column was transferred to a new collection tube and centrifuged for 5 minutes at maximum speed. The purified cDNA was eluted by adding 14 µl of elution buffer and spinning for 1 minute at 10,000 rpm.

2.5.1.4 In vitro transcription reaction

This converts the cDNA to cRNA and labels the molecules with biotinylated ribonucleotides. *In vitro* transcription (IVT) was carried out in the presence of T7 RNA polymerase, a biotinylated nucleotide analog/ribonucleotide mix for complementary RNA (cRNA) amplification and biotin labelling. To 6 µl of the cDNA sample was added 34 µl of a master mix comprising of: 4 µl 10 X IVT labelling buffer¸ 12 µl IVT labelling NTP mix, 4 µl IVT labelling enzyme mix and 14 µl DEPC treated water. The sample mixture was incubated over night at 37ºC.

2.5.1.5 cRNA Clean-up

To purify the cRNA 60 µl RNAse free water was added to the IVT reaction. Then 350 µl cRNA binding buffer was added and the mixture vortexed, next 250 µl of 100% ethanol was mixed with this. The sample was passé through a cRNA cleanup column, centrifuged at full speed for 15 seconds and he flow-through was discarded. The column was washed with 500 µl wash Buffer, centrifuged and the flow through discarded. The column was washed again this time with 500 µl of 80% ethanol. The sample was centrifuged for

15 seconds at full speed and transferred to a new collection tube and centrifuged for a further 5 minutes. The cRNA eluted from the column with RNase free water, this was incubated at room temperature for one minute and then centrifuged into an Eppendorf tube. The RNA concentration and A260/A280 ratio was noted using the NanoDrop spectrophotometer (Labtech International).

2.5.1.6 cRNA fragmentation

The final step prior to hybridisation is fragmentation of the cRNA. 25 µg cRNA was fragmented in 10 µl 10X fragmentation buffer which was made up to 50 µl with DEPC treated water. The reaction was incubated at 94ºC for 35 minutes and then placed on ice. The fragmented cRNA was stored at -80ºC until required.

2.5.1.7 Hybridization to gene chip and scanning

Hybridization, washing and staining was performed according to the manufacturer's instructions. This was carried out by Sim Sahota, research technician in the department of cancer studies. A hybridization cocktail was prepared containing control oligonucleotide B2 (3nM) 5 µl, 20 x eukaryotic hybridisation controls 15 µl, hybridisation mix 150 µl, 30 µl of DMSO and nuclease free water made up to 300 µl . The sample of fragmented cRNA was added to this. This mixture was hybridized to the array for 16 hours. Following hybridization the array was processed through an automated washing and staining protocol on the fluidics station. The arrays were scanned using a gene-chip Scanner (Affymetrix GeneChip Scanner 3000; Affymetrix). The cell intensity data for each probe was calculated from the scanned image using the GCOS software.

2.6: Identification of differentially expressed genes

2.6.1 Rank product

The Rank products method (Breitling, Armengaud et al. 2004) was used to identify differentially expressed genes (chapters 4 and 6), following RMA (Robust Multiarray Analysis) (Irizarry, Bolstad et al. 2003). Briefly, this method involves the ranking of genes based on fold change for each replicate experiment; and then calculation of the Rank Product. This identifies those genes that appear near the top (or bottom) of the list in all of the replicate experiments. The significance of the results is determined by permutation testing a number of random experiments and calculating the percentage of false positives (PFP) for each gene. This allows the determination of the false positive rate, if all genes with a similar estimate of the percentage of false positives were considered differentially expressed; giving a conservative estimate of the chance that a gene is significantly regulated.

2.6.2 GCOS Change Algorithm

Differentially expressed genes in rectal mucosa before and after calcium treatment were identified using the GCOS change algorithm, which was applied with default settings. For each subject paired comparison was made between the arrays before and after calcium supplementation. The Wilcoxon's Signed Rank test for paired data was used in a comparison analysis; each probe set on the calcium treated array was compared to its counterpart on the untreated array. A change *p*-value is calculated indicating an increase (I), decrease (D), or no change (NC) in gene expression.

The number of increase and decrease calls of each probe set was calculated using MS Excel and probe sets with the highest number of changes (either increased or decreased) among the six subjects were identified. A threshold of a similar change in half the subjects (3/6) was used to identify the group of genes considered differentially expressed.

Chapter 3
INVESTIGATION OF THE EXPRESSION OF PTHR1 IN NORMAL AND NEOPLASTIC COLON USING SEMI-QUANTITATIVE REAL TIME PCR AND IMMUNOHISTOCHEMISTRY

3.1: Aims

The primary aims of this study were to:

1. Using semi-quantitative real-time PCR: investigate the relative expression of PTHR1 mRNA in colorectal cancer and adenoma cell lines to establish suitability for future experiments.

2. Using semi-quantitative real-time PCR: investigate the relative expression of the PTHR1 mRNA transcript in colorectal cancer and adenomas compared to normal mucosa.

3. Using Immuno-histochemistry: investigate the expression of PTHR1 protein in colorectal cancers.

3.2: Introduction

The hypothesis of this thesis is that PTH acts directly on colonic epithelial cells, whether normal or neoplastic. It is therefore a requirement that colonic epithelium is sensitive to PTH. The physiological action of PTH is mediated in an endocrine manner via activation of the PTH receptor (PTHR1), which is expressed on its two classical target organs kidney and bone (Gensure, Gardella et al. 2005). PTHR1 is a class B G-protein coupled receptor, containing the characteristic seven trans-membrane domains (Juppner, Abou-Samra et al. 1991). It mediates the action of both parathyroid hormone (PTH) and parathyroid hormone related peptide (PTHrP) on bone (Lanske, Divieti et al. 1998). The downstream signalling of PTHR1 is mediated by activation of adenylate cyclase and phospholipase C (Karaplis and Goltzman 2000).

The colorectal epithelium is not involved in calcium homeostasis and is not a classical PTH target organ, so may not be expected to express PTHR1. However, as well as being expressed in bone and kidney, PTHR1 has been demonstrated in a wide range of other normal rat tissues including: brain, lung, heart, breast, muscle, skin and various parts of the gastrointestinal tract (Urena, Kong et al. 1993). Expression of PTHR1 has also been demonstrated in several different human cancers including: breast (Linforth, Anderson et al. 2002), renal cell (Massfelder, Lang et al. 2004), prostate (Bryden, Hoyland et al. 2002), glioma (Evliyaoglu, Carroll et al. 2000) and osteosarcoma (Yang, Hoang et al. 2007).

Expression of PTHR1 in colonic tissues has not been widely studied. In the only human study that has investigated colorectal cancers, 2/5 tumours demonstrated expression of PTHR1 when assessed by RT-PCR (reverse transcriptase polymerase chain reaction) (Carron, Fraser et al. 1997), but expression of PTHR1 was not investigated in normal colon or adenomatous polyps.

In this study the relative expression of PTHR1 mRNA in neoplastic compared to normal colon has been investigated. This was done to determine whether PTHR1 is expressed in colorectal cell lines and normal and neoplastic colon and whether there is any association between relative PTHR1 expression and tumour characteristics. Similarly expression of PTHR1 protein was investigated in a series of colorectal cancers.

3.3: Methods and Subjects

3.3.1 Calculation of Fold changes in PTHR1 expression

Semi-quantitative real time PCR for PTHR1 was carried out on ten cancers, six adenomas and nine samples of normal mucosa. For each sample Ct values were measured for PTHR1 and KRT8. Mean Ct values were calculated from the triplicate analyses. KRT8 (Cytokeratin 8) is an epithelial specific marker and was used to normalise PTHR1 expression. Fold change was calculated in the following standard manner. For each tumour, polyp and normal mucosa sample Dct was calculated. Fold change was calculated by comparison was made with matched normal mucosa as shown below as an example for Tumour 1.

1. DCt for T1= Ct (T1 PTHR1) – Ct (T1 KRT8)
2. DdCt = DCt (T1) – DCt (N1)
3. Fold change= 2^{-DdCt}

Where matched normal mucosa was not available the mean DCt value for all the normal samples was used. Relative expression of PTHR1 in the cell lines was compared to the mean DCt value for all the normal mucosa samples.

3.3.2 Patient characteristics, Cancers

The characteristics of the patients and their tumour samples used in the real time PCR studies of PTHR1 expression in cancers are described below and shown in Table 3.1. There were 5 males and 5 females. Mean age was 69.8 (range 43-83). In six patients the cancer was proximal to the splenic flexure and in four it was distal. Four of the samples were stage 2 (Dukes B) and six were stage 3 (Dukes C) cancers, the TNM stage is shown in Table 3.1.

Seven tumours showed moderate and three showed poor differentiation. One tumour (T7) was from a patient with a mutation in MSH2 and showed evidence of micro-satellite instability, the other tumours were sporadic with no significant family history. Three out of ten patients were alive and free of recurrence at three years; these three patients had stage 2 tumours. All four patients with stage 3 tumours had recurrence.

Table3.1 Patient and tumour characteristics of samples studied
The patient and tumour features of the ten samples (T1-T10) studied in the real time PCR studies of PTHR1 expression are shown. 3YRFS= three year recurrence free survival.

	Site of tumour	TNM	Differentiation	Sex	Age	3 YRFS
T1	Rectum	300	Moderate	M	74	No
T2	Transverse	410	Poor	F	76	No
T3	Sigmoid	300	Moderate	M	75	Yes
T4	Sigmoid	410	Moderate	M	66	No
T5	Ascending	300	Moderate	F	83	Yes
T6	Caecum	400	Poor	F	79	No
T7	Caecum	410	Moderate	M	45	No
T8	Rectum	300	Moderate	M	78	No
T9	Ascending	310	Moderate	F	43	No
T10	Caecum	400	Poor	F	79	Yes

3.3.3 Patient characteristics, Adenomas

The characteristics of the six patient samples used in the real time PCR studies of PTHR1 expression in adenomas are described below and shown in Table 3.2. One polyp P3 was from a patient with FAP, the remainder were from patients with sporadic polyps. Three were tubulo-villous adenomas (TVA) and three were tubular adenomas. They ranged in size from 7-10mm in diameter, four showed moderate and two mild dysplasia and all six adenomas were from distal to the splenic flexure. Four of the patients were male and two female and the mean age was 54, (range 21-64).

Table 3.2 Patient and pathological features of the six adenomas
The patient and pathology features of the six polyps (P1-P6) studied in the real time PCR studies of PTHR1 expression.

	Site of Adenoma	Size (mm)	Dysplasia	Pathology	Sex	Age
P1	Sigmoid	10	Moderate	TVA	M	64
P2	Sigmoid	8	Moderate	TVA	F	59
P3	Descending	7	Mild	Tubular	M	21
P4	Rectum	8	Moderate	Tubular	M	57
P5	Sigmoid	7	Mild	Tubular	F	63
P6	Sigmoid	10	Moderate	TVA	M	62

3.3.4 Normal colonic mucosa

Nine samples of normal mucosa were analysed. Eight of these were matched normal samples from the patients with cancer and one from a patient with a polyp, P3. These samples are taken from macroscopically normal colon away from the tumour or polyp. No matched normal samples were available from tumours T4 and T10 and the other patients with polyps. Five of the normal mucosa samples were from proximal to the splenic flexure and four were distal.

3.3.5 Expression of PTHR1 protein using immuno-histochemistry

Expression of PTHR1 was studied in fourty-four cancers. Apart from two cases these were a different cohort of patients from those studied using PCR. Immunohistochemistry was performed using the streptavidin–biotin indirect immunoperoxidase method. Slide mounted MCF7 breast cancer cells were used as a positive and negative control with the primary antibody omitted in the latter. The primary antibody ab3271 (Abcam) is a mouse monoclonal antibody that detects amino acids 146-169 of human PTHR1 (Ferguson, Seaner et al. 1998) and was used at a concentration of 1:1000.

Slide mounted sections were de-waxed by immersion in xylene twice followed by 100% ethanol twice, each for five minutes, with a further wash in 70% ethanol for five minutes. The slides were then placed in a hydrogen peroxide/methanol (1:10) mixture for 5 minutes in order to remove endogenous peroxidase activity. Non-specific binding was blocked by incubation with 20% normal goat serum for 30 min. Sections were then incubated for with the primary antibody diluted in 20% normal goat serum (see above) at 4°C overnight. Following two washes with PBS (phosphate buffered saline) sections were then incubated with 1: 500 dilution of biotinylated goat anti-mouse (Dako) for 30 min. Serial PBS washes were undertaken and the slides then incubated with streptavidin-peroxidase conjugate (Dako) prior to visualisation of immunoreactivity by incubation with diaminobenzidine tetrahydrochloride (Sigma). Sections were counterstained with haematoxylin. Sections were then washed in tap water, dehydrated by taking through ethanol (70% then 100%) for 10 minutes each, xylene for 10 minutes and then mounted in Depex. Mounted sections were analysed by light microscopy.

The sections were scored as positive or negative, depending on whether immunoreactivity was seen in the sections with reference to the positive and negative control sections. These were scored independently by me and Dr Philippe Taniere, Consultant Pathologist.

3.3.6 Patient and tumour characteristics

Of the fourty-four patients studied using immunohistochemistry, twenty-five were male and nineteen female. The location of the tumour was proximal to the splenic flexure in fifteen (34%) and distal in twenty nine (66%). The Dukes stage of the tumours was as follows: Dukes A; 5 (11%), Dukes B; 14 (32%), Dukes C; 16 (36%) and Dukes D; 9 (21%).

3.4: Results

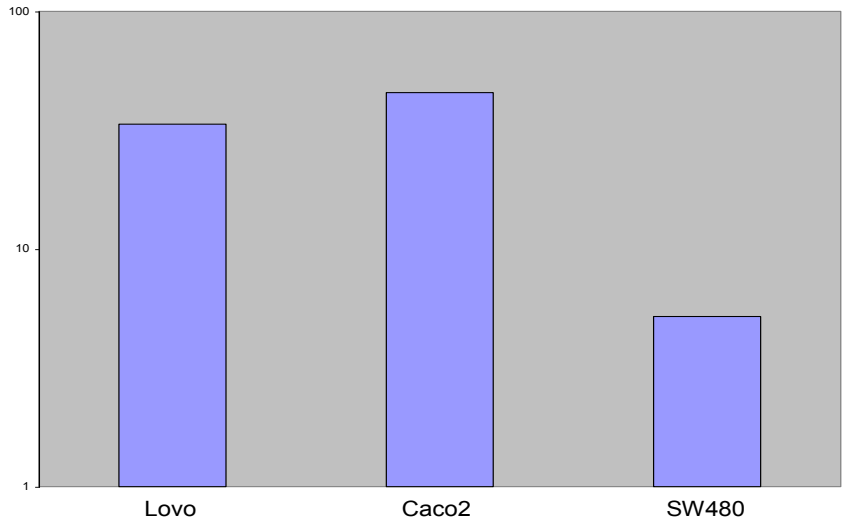
3.4.1 Expression of PTHR1 mRNA in normal and neoplastic colon

Semi-quantitative real time PCR for PTHR1 was carried out on five cell lines, nine normal colonic samples, ten cancers and six adenomas. Mean Ct values were calculated from the triplicate analyses and expression compared to normal mucosa. Expression of PTHR1 was seen in all normal, adenoma and tumour samples. Positive controls also demonstrated expression (Mean Ct for PTHR1 of 25.34 and 25.67) and the mean Ct values for the negative controls were >39.

3.4.2 Expression of PTHR1 mRNA in Cell Lines

The three colorectal cancer cell lines tested (CACO2, SW480, LOVO) expressed PTHR1, in contrast the two adenoma cell lines (RGC2, AAC1) did not express PTHR1 with Ct values similar to the negative controls. Compared to the mean expression of PTHR1 in all of the nine normal mucosa samples all three cancer cell lines showed increased expression with fold changes of 33.5 in CACO2, 45.4 in LOVO and 5.2 in SW480. The relative expression of PTHR1 in RGC2 and AAC1 compared to normal mucosa was decreased by a magnitude greater than 10,000 fold and are therefore not shown in Figure 3.1.

Figure 3.1 Relative expression of PTHR1 mRNA in colorectal cancer cell lines compared to normal mucosa
The Y-axis is fold change (log_{10} scale)



3.4.3 Relative expression of PTHR1 mRNA in Cancers

For the ten cancers the mean (range) Ct value for PTHR1 was 31.8 (28.1-34.3) and for KRT8 23.5 (20.7-25.5). Comparison was made to matched normal mucosa for eight of the samples. For the two tumours with no matched normal sample (T4 and T10) comparison was made to the mean PTHR1 expression for all of the normal samples. Four out of ten tumours showed increased (>2 fold) expression of PTHR1, range +5 to +33.4 fold. Expression levels of PTHR1 similar to normal mucosa (<2 and >-2 fold change) were seen in 2/10 tumours (+1.03 and +1.69 fold difference). The remaining four samples showed decreased expression of PTHR1 (<-2 fold change) compared to normal mucosa, range -4.6 to -7.6. The fold changes in tumours showing increased expression were greater than those showing decreased expression. Mean fold change for these ten cancers was +6.3 fold. These results are shown graphically in figure 3.2 and table 3.3.

Figure 3.2 Relative expression of PTHR1 mRNA in colorectal cancers compared to normal mucosa.
The Y-axis is fold change (log_{10} scale)

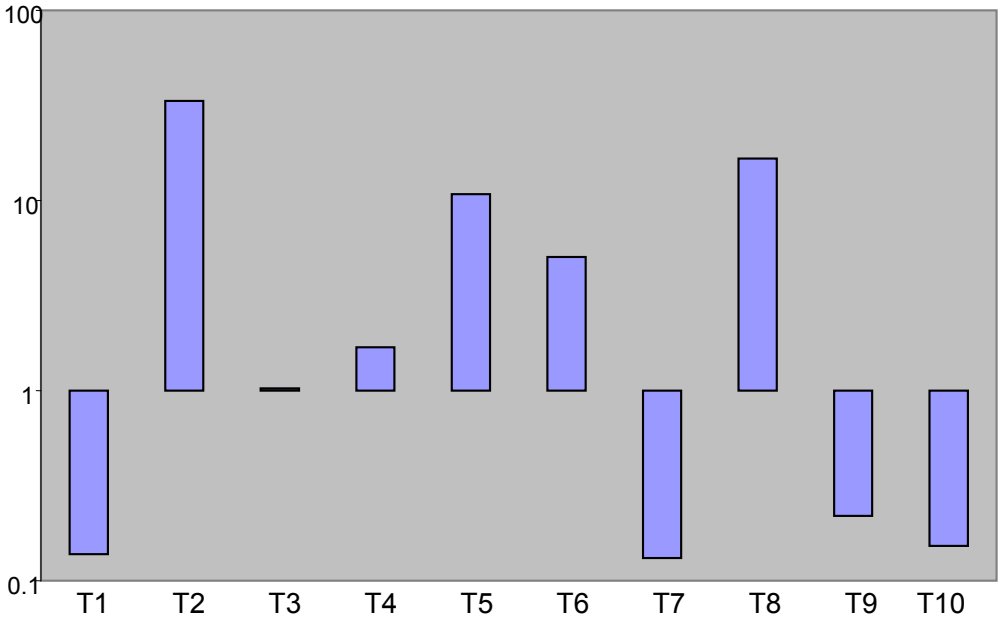


Table 3.3 Relative expression of PTHR1 mRNA in the ten cancers compared to normal mucosa.
Relative expression of PTHR1 in the ten cancers is shown along with the patient and tumour characteristics. The samples have been put into three groups showing increased, similar or decreased expression compared to normal mucosa. For those tumours showing decreased expression of PTHR1 two figures are given to aid clarity. The first is fold change and the second in brackets is fold reduction, calculated by -1/fold change.

	Site of tumour	TNM	Differentiation	3 YRFS	Relative expression of PTHR1 (T vs N)
Tumours showing increased expression of PTHR1 compared to normal mucosa					
T2	Transverse	410	Poor	No	+33.4
T5	Ascending	300	Moderate	Yes	+10.8
T6	Caecum	400	Poor	No	+5.1
T8	Rectum	300	Moderate	No	+16.7
Tumours showing similar expression of PTHR1 compared to normal mucosa					
T3	Sigmoid	300	Moderate	Yes	+1.03
T4	Sigmoid	410	Moderate	No	+1.69
Tumours showing decreased expression of PTHR1 compared to normal mucosa					
T1	Rectum	300	Moderate	No	0.14 (-7.25)
T7	Caecum	410	Moderate	No	0.13 (-7.6)
T9	Ascending	310	Moderate	No	0.22 (-4.56)
T10	Caecum	400	Poor	Yes	0.15 (-6.55)

3.4.4 Comparison of PTHR1 mRNA expression with patient and tumour characteristics

PTHR1 expression levels were compared to the tumour features shown in Table 3.3. In this small group no clear trends or significant association of any of these features with PTHR1 expression was seen. These results are described below and summarised in Table 3.4.

Tumour site

Of the six cancers proximal to the splenic flexure three showed increased and three showed decreased expression compared to normal. Of the four distal

cancers one showed increased and one showed decreased expression and the other two showed expression levels similar to normal mucosa.

Tumour stage

Of the six stage 2 (Dukes B) cancers, three showed increased expression, two showed decreased relative expression and one showed expression similar to normal mucosa. Of the four stage 3 (Dukes C) cancers, one showed increased expression, two showed decreased expression and one expression levels similar to normal mucosa.

Differentiation

Two out of three poorly differentiated tumours showed increased relative expression of PTHR1, the third showed decreased relative expression. Of the seven moderately differentiated cancers, two showed increased and three decreased relative expression and two showed expression similar to normal mucosa.

Recurrence free survival

Three year recurrence free survival was not associated with either decreased or increased expression of PTHR1. One out of four patients with increased expression was alive and free of recurrence after three years and similarly one out of four patients with decreased expression was alive and free of recurrence after three years. One out of two patients with expression levels similar to normal mucosa was alive and recurrence free at three years.

Table3.4 Relationship between relative expression of PTHR1 mRNA and tumour features.
Comparison is made between cancers showing increased, decreased or equivalent expression of PTHR1 and pathological and clinical features.

Relative expression of PTHR1 (T vs N)		Increased	Equivalent	Decreased
Tumour Site	Proximal	3	0	3
	Distal	1	1	2
Tumour stage	Two	3	1	2
	Three	1	1	2
Differentiation	Poor	2	0	1
	Moderate	2	2	3
3 Year RFS	Yes	1	1	1
	No	3	1	3

3.4.5 Relative expression of PTHR1 mRNA in adenomas

For the six adenomas the mean (range) Ct values for PTHR1 was 32.8 (31.6-33.8) and for KRT8, 23.7 (22.3-25.2). Comparison to matched normal mucosa was made for P3, for the rest comparison was made to the mean PTHR1 expression for all of the normal samples. Of the six adenomas two showed increased and four showed decreased relative expression of PTHR1 compared to normal mucosa. These results are shown in Table 3.5 and figure 3.3. The magnitude of the expression changes ranged from +3.2 fold to -3.3 fold change and Mean fold change was +1.3. The size of the change in expression was smaller than those seen in the cancers and the variability in relative expression was less compared to that seen in the cancers. No association between relative PTHR1 expression and any pathological features were seen. Increased expression of PTHR1 was seen in one out of three tubular adenomas and one out of three TVA's. One out of two mildly dysplastic polyps and one of four moderately dysplastic polyps showed increased expression of PTHR1.

Figure 3.3 Relative expression of PTHR1 mRNA in colorectal adenomas compared to normal mucosa.
The Y-axis is fold change (log_{10} scale)

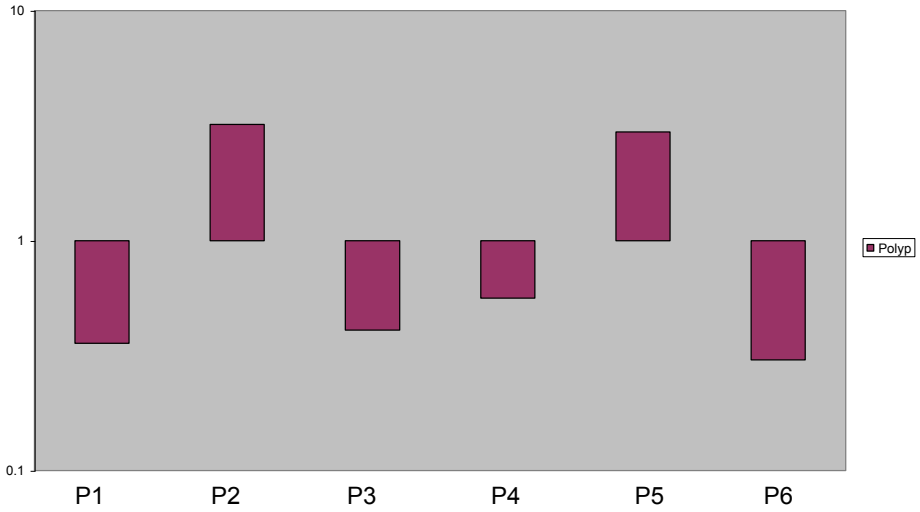


Table 3.5 Relative expression of PTHR1 mRNA in adenomas compared to normal mucosa and adenoma characteristics.
For those adenomas showing decreased expression of PTHR1 two figures are given to aid clarity. The first is fold change and the second in brackets is fold reduction, calculated by -1/fold change.

	Site of Adenoma	Size (mm)	Dysplasia	Pathology	Relative expression of PTHR1
P1	Sigmoid	10	Moderate	TVA	0.3 (-2.8)
P2	Sigmoid	8	Moderate	TVA	+3.1
P3	Descending	7	Mild	Tubular	0.4 (-2.5)
P4	Rectum	8	Moderate	Tubular	0.6 (-1.8)
P5	Sigmoid	7	Mild	Tubular	+3
P6	Sigmoid	10	Moderate	TVA	0.3 (-3.3)

3.4.6 Immuno-histochemical expression of PTHR1 protein in colorectal cancers

Of the forty-four cancers tested, forty (91%) were positive and four (9%) tumours were negative for PTHR1 expression. Of the forty positive tumours the majority (36) showed homogenous expression, whilst four showed patchy positivity. The PTHR1 protein was localised in the epithelial tumour cells, with

no significant expression in the tumour stroma apart from the occasional neutrophil. PTHR1 was expressed in the cytoplasm and plasma membrane of the epithelial cells with expression in the apical brush border, indicating expression at the cell membrane. There was no nuclear staining. Expression of PTHR1 by Dukes stage was; Dukes A 5/5 (100%), Dukes B 14/15 (93%), Dukes C 15/16 (94%) and Dukes D 7/9 (78%). There was no relationship between Dukes stage or tumour localisation and PTHR1 expression.

Figure 3.4. Immuno-histocehmistry for PTHR1 in colorectal cancer samples.
Slide mounted MCF7 cells were used as a positive control. Figure A shows MCF7 positive for PTHR1 and B is MCF7 with no primary antibody which shows no immunoreactivity for PTHR1 (both x400magnification).

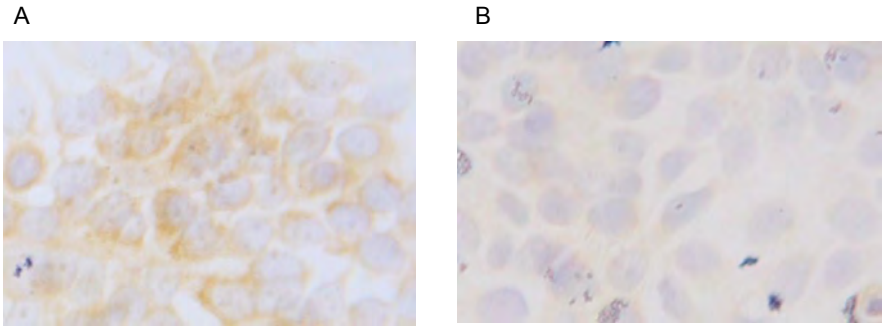
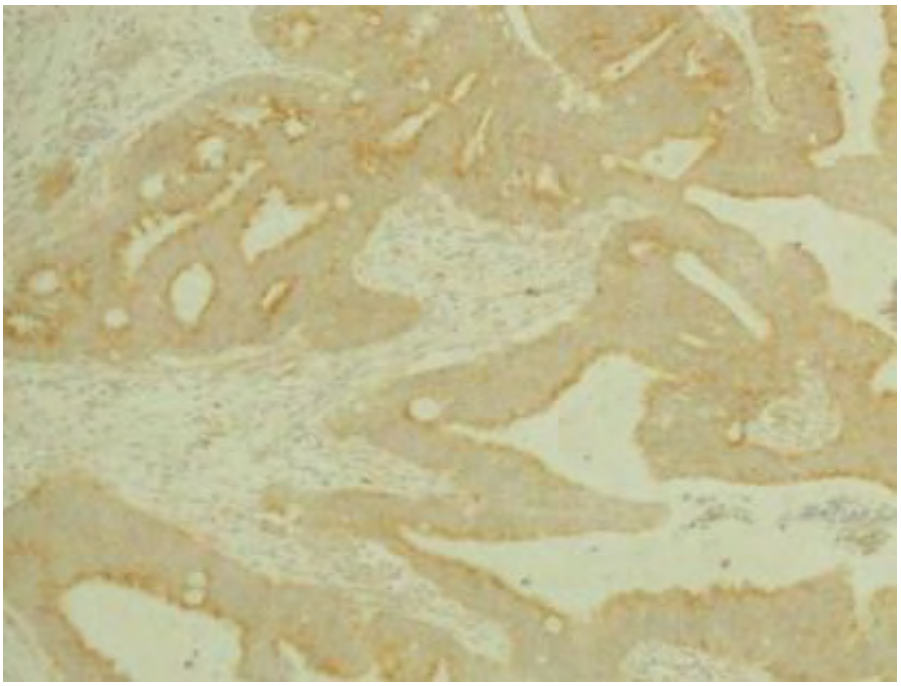


Figure 3.4 Continued. Figs. C and D are of a moderately differentiated rectal cancer (C x100, D x400 magnification) shows expression of PTHR1 in the glandular epithelium with no nuclear expression or staining of stromal tissue. The mos positive staining is seen at the apical border of the tumour glands.

C



D

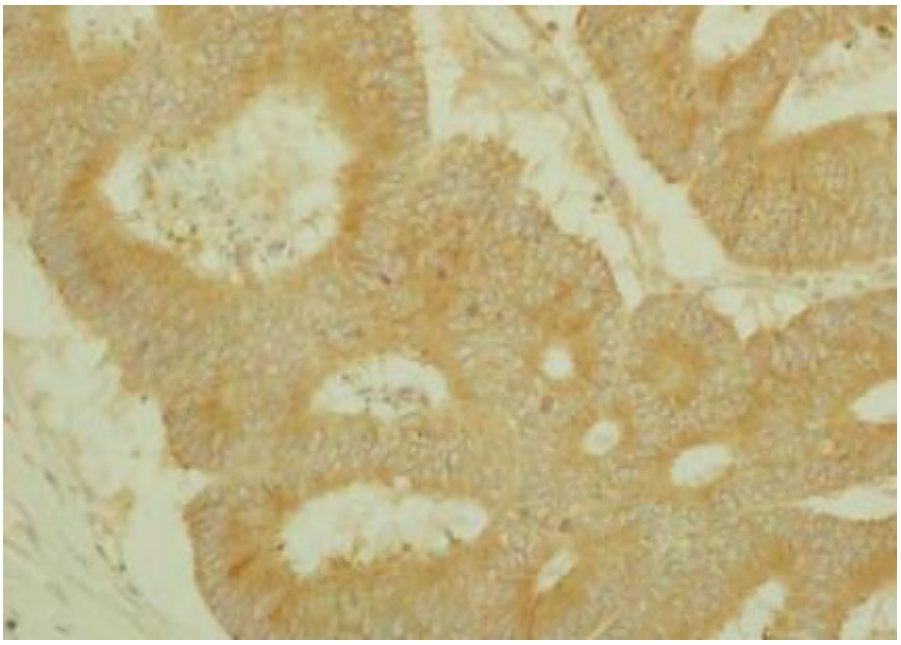
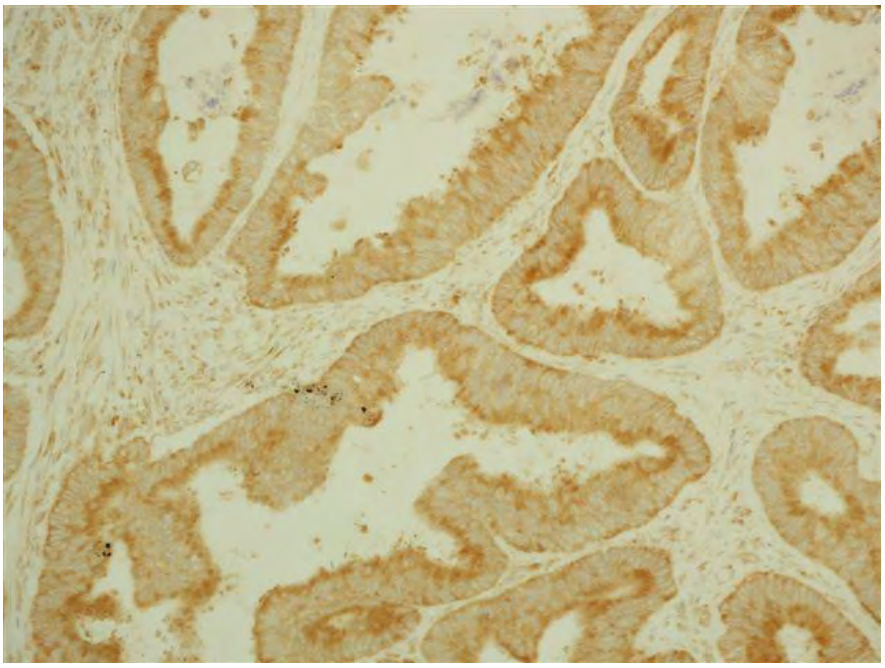
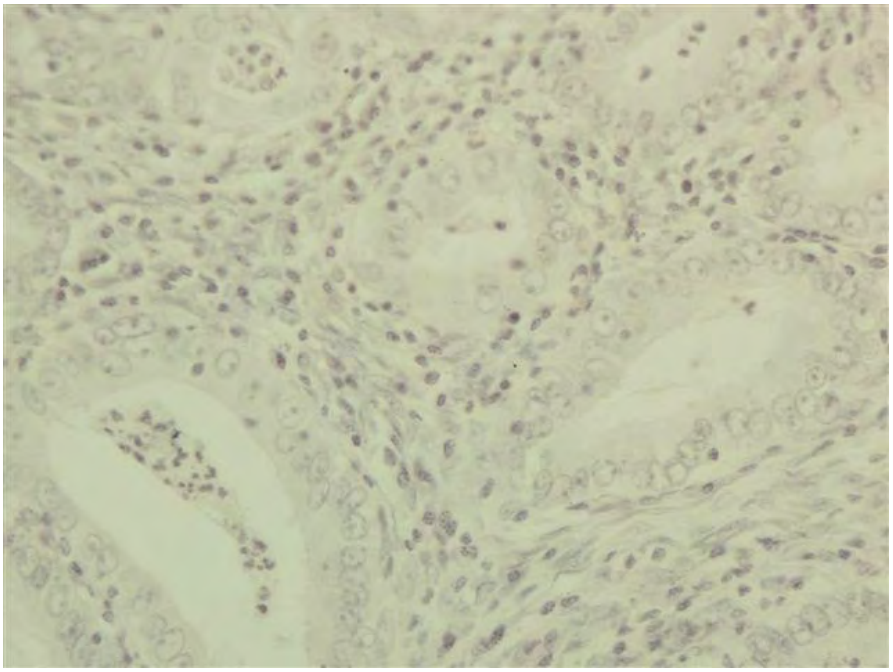


Figure 3.4 Continued. Figure E (x200) shows a well differentiated caecal cancer, similarly showing staining of the glandular epithelium with staining seen throughout the cytoplasm but most marked at the apical membrane. Figure F (x400) is of a moderately differentiated sigmoid cancer showing no positivity for PTHR1.

E



F



3.5: Discussion

This study has demonstrated that normal and neoplastic colon express PTHR1 mRNA and protein. The colonic epithelium and neoplasms developing from it are therefore likely to be sensitive to the level of circulating serum PTH. PTHR1 mRNA was more frequently expressed in cancers than in the study of Carron who showed expression of mRNA in only 2/5 (Carron, Fraser et al. 1997). In contrast in this study 10/10 tumours demonstrated PTHR1 mRNA expression. This may be explained by the use in this study of semi-quantitative real time PCR which is more sensitive than RT-PCR. In other tumour types similar frequencies of PTHR1 expression have been reported: 99/107 breast tumours (Downey, Hoyland et al. 1997), 12/12 prostate tumours (Bryden, Hoyland et al. 2002) and 55/55 osteosarcomas (Yang, Hoang et al. 2007) demonstrated PTHR1 expression.

This study has demonstrated expression of PTHR1 mRNA and protein. Expression of PTHR1 protein was also recently demonstrated in a study published since this work was carried out; using immuno-histochemistry, PTHR1 protein expression was shown in normal colorectal mucosa, with expression localised to the basolateral membrane of epithelial cells(Lupp, Klenk et al. 2010). Interestingly, expression of PTHR1 was seen throughout the colonic crypt in most of the colon but in the ascending colon expression was limited to cells found at the base of the crypt, a pattern that was also seen in the small bowel(Lupp, Klenk et al. 2010). However in the current study there was no relationship between relative expression level and tumour site. No adenomas were studied but all eleven colorectal cancers showed expression of PTHR1 protein (Lupp, Klenk et al. 2010). This contrasts with

this larger study in which expression was not universal but only found in 90% of tumours. The reason for this is not clear, it may be that expression is heterogenous and is present in other parts of the tumour. Alternatively the level of PTHR1 expression may be below the level of detection or not found in a small proportion of tumours.

The presence of PTHR1 in normal colon as well as adenomas and cancers indicate that it is not an oncofoetal protein but has a function in normal as well as neoplastic colon. The presence of PTHR1 in the normal as well as neoplastic tissue is a also feature of a number of other tumours, including the breast (Linforth, Anderson et al. 2002), kidney (Massfelder, Lang et al. 2004), prostate (Bryden, Hoyland et al. 2002), brain (Evliyaoglu, Carroll et al. 2000) and bone (Yang, Hoang et al. 2007).

The samples for the mRNA study were selected to provide initial information on PTHR1 expression in colorectal cancers. There were tumours from throughout the colon, with varying degrees of differentiation. Dukes stage B and C cancers were studied to investigate whether there was any influence of PTHR1 expression on recurrence, because of this, no early (Dukes A) or tumours with metastases were studied. In contrast in the immunohistochemistry study, all tumour stages were studied with no clear difference in expression. One of the polyps P3 was from a patient with FAP and one tumour T7 from a patient with a mutation in MSH2. Both T7 and P3 showed decreased expression of PTHR1 compared to normal, but were not significantly different from the other samples. The cell line Lovo also carries mutation in MSH2 (Umar, Boyer et al. 1994) as does T7 and Lovo showed high level expression of, suggesting that decreased expression of PTHR1

need not be a feature of mismatch repair deficient tumours. The three cancer cell lines strongly expressed PTHR1 indicating they would be suitable to study the effect of PTH in-vitro. The reason for the absence of PTHR1 expression from the two adenoma cell lines is not clear as PTHR1 is expressed in adenomas in-vivo at a similar level to normal mucosa. It demonstrates the differences between the in-vitro and in-vivo situation and means that these cell lines are not suitable for studying the effect of PTH in-vitro.

The high relative expression of PTHR1 in the colorectal cancer cell lines and its absence from the adenoma cell lines suggests that PTHR1 may be over-expressed in the cancer samples. In fact, a similar number of tumours showed increased and decreased relative expression of PTHR1, suggesting the cell line data is slightly misleading. For, though there were a similar number of tumours showing increased or decreased relative expression of PTHR1: the magnitude of the relative expression change was greater in the tumours, showing increased expression with a mean fold change for all ten tumours of +6.3. Mean expression of PTHR1 increased marginally from normal mucosa to adenoma (+1.3 fold) and again to cancer (+6.3 fold). This suggests a possible role in the progression of colorectal neoplasia. Increasing expression of PTHR1has been shown with advancing stage of breast cancer. Increased expression of PTHR1 compared to normal was reported in half of breast tumours (Downey, Hoyland et al. 1997) and bony metastases showed expression levels greater than primary breast tumours (Hoey, Sanderson et al. 2003). In this small study of colorectal cancers, no clear association with pathological features was demonstrated. A larger study would be required to determine whether over-expression of

PTHR1 was of clinical significance in colorectal cancer. This is a possibility, as a functional role for PTHR1 in tumorigenesis has been suggested in a number of other cancers. Early breast cancers expressing PTHR1 had poor clinical outcomes, particularly disease free survival and overall survival (Linforth, Anderson et al. 2002). Consistent with this clinical observation, over-expression of PTHR1 in the breast cancer cell line MCF-7 resulted in increased proliferation (Hoey, Sanderson et al. 2003). Similarly, osteosarcomas which relapsed or had metastases had higher levels of PTHR1 mRNA than those without (Yang, Hoang et al. 2007). Over-expression of PTHR1 in HOS; an osteosarcoma cell line, resulted in: increased proliferation, motility, invasion and delayed differentiation (Yang, Hoang et al. 2007).

Having demonstrated expression of PTHR1 in the colon, the question remains, what is its function? It would seem likely as has been proposed by others that in non-classical PTH target organs, the „natural' ligand for PTHR1 is PTHrP acting in a paracrine or autocrine fashion. PTHrP was first recognised as the mediator of humoral hypercalcaemia of malignancy (Suva, Winslow et al. 1987) due to its over expression by tumours and entry into the circulation where it activates PTHR1 on bone and kidney raising serum calcium. Over expression of PTHrP is a common finding in several cancers including colorectal. PTHrP is not expressed in adenomas but is found in cancers with higher levels of expression associated with advanced stage of disease (Nishihara, Ito et al. 1999). These findings of expression of both PTHrP (Nishihara, Ito et al. 1999) and PTHR1 (current study) (Lupp, Klenk et al. 2010) in colorectal cancers suggest a role for PTHrP signalling via PTHR1

in colorectal neoplasia. Indeed, over-expression of PTHrP in a colorectal cancer cell line results in increased proliferation, invasion and migration (Shen, Mula et al. 2007).

In addition to a pathological role in the development of cancer, PTHrP and PHTR1 interactions have a number of physiological functions in embryological development and normal tissue homeostasis. The skeleton is the most obvious organ system in which PTHrP/PTHR1 interactions have a role in development (Lanske, Karaplis et al. 1996). There is also evidence that PTHrP and PTHR1 are involved in the embryological development of a large number of other tissues including, heart, lung, breast, skin and blood vessels (Clemens, Cormier et al. 2001). Based on highly regulated expression level of PTHR1, a role in intestinal development has also been suggested (Lepourcelet, Tou et al. 2005). Regulation of normal adult tissues by PTHrP/PTHR1 signalling has been demonstrated for the skin, where PTHrP acting via PTHR1 has been shown to delay keratinocyte differentiation and apoptosis and promote proliferation (Foley, Longely et al. 1998). Based on co-expression of PTHrP and PTHR1 in several normal adult tissues, similar roles are proposed in other tissues. These include the rat intestine where PTHrP was expressed only in the upper part of the crypts and a role in controlling proliferation and differentiation proposed (Li, Seitz et al. 1995). Any such role in the intestine has not been defined, but it can be seen how circulating PTH would be able to interfere with any regulation of the colonic crypt mediated by PTHrP interacting with PTHR1.

3.5.1 Conclusion

This study has demonstrated expression of PTHR1, the receptor for parathyroid hormone, in normal and neoplastic colorectal tissue. This indicates that they are likely to be sensitive to circulating PTH levels. The mean relative expression of PTHR1 increased with the progression of neoplasia but with variability between samples. In this small series there was no association of PTHR1 expression with any adverse pathological features.

Chapter 4A

INVESTIGATION OF THE EFFECT OF PARATHYROID HORMONE ON GENE EXPRESSION IN AN IN-VITRO MODEL OF THE COLORECTAL EPITHELIUM

4A.1: Aim

1. Identification of genes differentially expressed in response to parathyroid hormone treatment of an in-vitro model of normal colonic epithelium using oligo-nucleotide micro-arrays.
    a. Identify genes regulated by PTH and also known to be altered in colorectal tumorigenesis
    b. Identify genes regulated by PTH in this study and known to be regulated by PTH in other systems.

4A.2: Introduction

In the previous chapter, expression of PTHR1 was demonstrated in normal and neoplastic colon, indicating these tissues are likely to be sensitive to PTH. The hypothesis of this study is that PTH acts directly on colorectal epithelium resulting in changes in gene expression consistent with a pro-tumorigenic effect. The aim of the work described in this chapter is to identify genes differentially expressed in response to parathyroid hormone treatment in an in-vitro model of normal colorectal epithelium; the cell line Caco2. In the previous chapter, the colorectal cancer cell line Caco2 was shown to strongly express PTHR1. Caco2 forms a polarised monolayer and differentiates with time in culture forming crypt like architecture. Upon differentiation it has been

shown to have a gene expression profile similar to normal colonic epithelium (Saaf, Halbleib et al. 2007). And this feature will enable closer comparison with experiments on normal colonic epithelium described in chapter three.

Gene expression profiling using micro-arrays allows the investigation of the expression of multiple genes simultaneously. The gene expression level can be compared in diseased versus normal tissue: or, in response to chemical or genetic manipulation. This technique has been used to identify genes differentially expressed in colonic adenomas and carcinomas compared to normal mucosa in several different studies (Cardoso, Boer et al. 2007). Gene expression profiling has also been used to identify PTH regulated genes in rat osteoblasts in-vitro(Qin, Qiu et al. 2003) and in particular to differentiate between the in-vivo effects of intermittent and continuous PTH on bone, which are known to have different effects (Li, Liu et al. 2007). The functional effect of PTH on colonic epithelium is unknown, although a role in colorectal tumorigenesis is hypothesised. Gene expression profiling is therefore well suited to use in this study in which there are no known targets of parathyroid hormone in colonic mucosa and only a general a priori hypothesis.

4A.3: Methods

4A.3.1 Cell culture

In this study, the Caco-2 human colorectal cancer cell line (Passage 36) was used. For the micro-array studies, cells were plated in twelve T25 flasks. The media was changed every 48 hours and the cells were grown until confluence was reached, assessed by light microscopy as >90% of cells in contact with surrounding cells. The cells were grown for a further seven days (confluence +7), after this time point no further changes in gene expression have been shown to occur (Kosinski, Li et al. 2007). At this point the cells were washed with warm PBS and were transferred from Dulbecco's Modified Eagle Medium (DMEM) + 10% foetal calf serum (FCS) to DMEM + 1% FCS and incubated overnight (Approximately 16 hours), as was carried out in another similar study (Yu, Seitz et al. 1992) . The medium was then changed and replaced with DMEM + 1% FCS containing 10^{-7} Parathyroid hormone (Bachem) in the test or a similar volume of vehicle in the control samples. The cells were treated with N-terminal PTH 1-34, which has the same activity as full length PTH (1-84)(Habener, Rosenblatt et al. 1984). The effect of PTH was studied at two time points (4 and 24 hours) to identify early and late targets of PTH. The samples were harvested at 4 hours (3 test and 3 controls) and 24 hours (2 test and 2 controls). RNA extraction was carried out and the concentration and purity of the RNA was measured as described in the methods. These values are shown in table 4A.1.

Table 4A.1 Concentration and purity of RNA extracted from cell lines used in the studies described in chapter four. PTH=PTH treated sample; CONT=Control samples and 4 and 24 refer to the number of hours of treatment.

Sample	RNA concentration (ng/µl)	RNA purity (260/280nm ratio)
PTH4A	1790	2.05
PTH4B	1581	2.08
PTH4C	1436	2.03
CONT4A	1768	2.05
CONT4B	1840	2.07
CONT4C	1524	2.07
PTH24A	1627	2.06
PTH24B	1425	2.07
CONT24A	1067	1.99
CONT24B	2158	2.05

4A.3.2 Identification of differentially expressed genes

Affymetrix Focus micro-arrrays were used in this study; these are single colour, oligo-nucleotide arrays with over 8,000 probe sets representing over 8000 individual genes. The robust multi-array average (RMA) (Irizarry, Bolstad et al. 2003) approach was used to normalise the array data and calculate gene expression values.

Several methods have been used to identify differentially expressed genes in micro-array studies, in this study the Rank products method (Breitling, Armengaud et al. 2004) was used. Briefly, this method involves the ranking of genes based on fold change for each replicate experiment; and then calculation of the Rank Product. This identifies those genes that appear near the top (or bottom) of the list in all of the replicate experiments. The significance of the results is determined by permutation testing a number of random experiments and calculating the percentage of false positives (PFP) for each gene. This allows the determination of the false positive rate, if all genes with a similar estimate of the percentage of false positives were

considered differentially expressed; giving a conservative estimate of the chance that a gene is significantly regulated. The Rank Products method has been shown to perform well compared to t-test based methods such as SAM (Significance analysis of micro-arrays) (Breitling, Armengaud et al. 2004) and performs well in the presence of a small number of replicates or noisy data (Jeffery, Higgins et al. 2006). In this study genes were defined as differentially expressed using Rank Product based on a PFP (percentage false positive) cut off below 10%.

4A.3.3 Fold change

Fold change was calculated in the standard fashion. The mean expression level of each gene for all post-treatment replicates was divided by the mean expression level of each gene for all pre-treatment replicates.

4A.3.4 Analysis of the results from Micro-array experiments

The first part of the analysis addresses specific hypotheses by identifying differentially expressed genes that are:

1. Known targets of parathyroid hormone.
2. Known to be differentially expressed in colorectal neoplasia.

This was done by reviewing the literature using online search tools Pubmed and google scholar.

4A.4: Results

4A.4.1 Differentially expressed genes in Caco2 following PTH treatment

Differentially expressed Genes were identified in the PTH treated samples compared to control at 4 and 24 hours. This was done using Rank Product with a PFP cut off of 10% as described in the methods.

4A.4.1.1 Four hours

After four hours treatment with PTH eighty two genes were differentially expressed. Increased expression was seen in five genes and decreased expression in seventy seven genes. The fold changes in expression in the five genes showing increased expression ranged from +1.4 to +3.2 fold. The fold changes in gene expression in the genes showing decreased expression ranged from -1.3 to -1.9 fold. The genes changed at four hours are shown in table 4A.2 (Up-regulated) and table 4A.3 (Down-regulated), along with the fold change and the PFP (percentage false positive) for each gene.

Table 4A.2 The five differentially expressed genes showing increased expression in caco2 after four hours of PTH treatment.
PFP=percentage false positive.

Gene Symbol	Gene description	Fold change	PFP
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	+3.2	0
MAFF	v-maf musculoaponeurotic fibro-sarcoma oncogene homolog F (avian)	+1.5	0.04
AREG	amphiregulin	+1.5	0.01
APOBEC3B	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	+1.5	0.02
ARF6	ADP-ribosylation factor 6	+1.4	0. 04

Table 4A.3 The seventy seven differentially expressed genes with decreased expression in Caco2 after four hours of PTH treatment.

Gene Symbol	Gene description	Fold change	PFP
STT3A	STT3, subunit of the oligosaccharyl-transferase complex, homolog A	-1.9	0
EIF2S3	eukaryotic translation initiation factor 2, subunit 3 gamma	-1.9	0
TMED2	transmembrane emp24 domain trafficking protein 2	-1.9	0
PDCD4	programmed cell death 4	-1.8	0
RTN3	reticulon 3	-1.8	0
RDX	radixin	-1.7	0
TXM1	thioredoxin-related transmembrane protein 1	-1.7	0
RBBP4	retinoblastoma binding protein 4	-1.6	0.005
SKP2	S-phase kinase-associated protein 2 (p45)	-1.6	0.002
PSPH	phosphoserine phosphatase	-1.6	0
CANX	calnexin	-1.6	0.007
SLC16A1	solute carrier family 16, member 1	-1.5	0.01
MBNL2	muscleblind-like 2 (Drosophila)	-1.5	0.003
TOMM22	translocase of outer mitochondrial membrane 22 homolog (yeast)	-1.5	0.02
COPG	coatomer protein complex, subunit gamma	-1.5	0.01
GSN	gelsolin (amyloidosis, Finnish type)	-1.5	0.01
NUTF2	nuclear transport factor 2	-1.5	0.002
RAB8A	RAB8A, member RAS oncogene family	-1.5	0.02
LIN7C	lin-7 homolog C (C. elegans)	-1.5	0.02
HSPA1B	heat shock 70kDa protein 1B	-1.5	0.01
RHOB	ras homolog gene family, member B	-1.5	0.02
NUP50	nucleoporin 50kDa	-1.5	0.03
ATF2	activating transcription factor 2	-1.5	0.03
CALM1	calmodulin 1 (phosphorylase kinase, delta)	-1. 5	0.03
ACTB	actin, beta	-1.5	0.03
SRPK1	SFRS protein kinase 1	-1.4	0.03
TMOD3	tropomodulin 3 (ubiquitous)	-1.4	0.02
SELT	selenoprotein T	-1.4	0.04
TTR	transthyretin (prealbumin, amyloidosis type I)	-1.4	0.02
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	-1.4	0.01
CROP	cisplatin resistance-associated overexpressed protein	-1.4	0.03

OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	-1.4	0.02
EML4	echinoderm microtubule associated protein like 4	-1.4	0.02
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	-1.4	0.03
CD9	CD9 molecule	-1.4	0.04
ELL2	elongation factor, RNA polymerase II, 2	-1.4	0.04
ARFIP1	ADP-ribosylation factor interacting protein 1	-1.4	0.05
AGPS	alkylglycerone phosphate synthase	-1.4	0.05
SCARB2	scavenger receptor class B, member 2	-1.4	0.05
APLP2	amyloid beta (A4) precursor-like protein 2	-1.4	0.05
KLF3	Kruppel-like factor 3 (basic)	-1.4	0.05
NLK	nemo-like kinase	-1.4	0.05
DUSP5	dual specificity phosphatase 5	-1.4	0.05
FBLN1	fibulin 1	-1.4	0.05
UTP20	UTP20, small subunit (SSU) processome component, homolog	-1.4	0.05
RAB5B	RAB5B, member RAS oncogene family	-1.4	0.05
JMJD1B	jumonji domain containing 1B	-1.4	0.06
ICAM1	intercellular adhesion molecule 1	-1.4	0.03
HSPA9B	heat shock 70kDa protein 9B	-1.4	0.05
B3GNT2	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2	-1.4	0.06
CNN3	calponin 3, acidic	-1.4	0.04
PTPN11	protein tyrosine phosphatase, non-receptor type 11	-1.4	0.06
GPC3	glypican 3	-1.4	0.05
SPOP	speckle-type POZ protein	-1.4	0.05
SERPINH1	serpin peptidase inhibitor, clade H member 1	-1.4	0.07
HNRPU	heterogeneous nuclear ribonucleoprotein U	-1.3	0.08
PVR	poliovirus receptor	-1.3	0.08
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	-1.3	0.08
MOCS3	molybdenum cofactor synthesis 3	-1.3	0.04
GPD2	glycerol-3-phosphate dehydrogenase 2	-1.3	0.06
CTBP1	C-terminal binding protein 1	-1.3	0.08
SRRM2	serine/arginine repetitive matrix 2	-1.3	0.05
GSR	glutathione reductase	-1.3	0.07
NCBP1	nuclear cap binding protein subunit 1, 80kDa	-1.3	0.06
GATM	glycine amidinotransferase	-1.3	0.05

ARHGAP5	Rho GTPase activating protein 5	-1.3	0.06
BTRC	beta-transducin repeat containing	-1.3	0.07
STAT2	signal transducer and activator of transcription 2,	-1.3	0.09
SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	-1.3	0.04
NDRG3	NDRG family member 3	-1.3	0.05
PPAP2B	phosphatidic acid phosphatase type 2B	-1.3	0.07
ANKRD11	ankyrin repeat domain 11	-1.3	0.06
SFRS1	splicing factor, arginine/serine-rich 1	-1.3	0.08
ROD1	ROD1 regulator of differentiation 1	-1.3	0.09
CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	-1.3	0.03
HOXA7	homeobox A7	-1.3	0.05
SNRNP40	Small nuclear ribonuclear protein	-1.3	0.09

4A.4.1.2 Twenty four hours

After twenty four hours treatment with PTH sixty three genes were differentially expressed. Increased expression was observed for two genes and sixty one genes showed decreased expression. The two up regulated genes showed increased expression of +1.4 and +1.5 fold. The genes showing decreased expression had fold changes ranging from -1.3 to -5.2 fold. The genes changed at twenty four hours are shown in table 4A.4 (Up-regulated) and table 4A.4 (Down-regulated), along with the fold change and the PFP (percentage false positive) for each gene.

Table 4A.4 The two differentially expressed genes showing increased expression after twenty four hours of PTH treatment

Gene Symbol	Gene description	Fold change	PFP
ACF	apobec-1 complementation factor	+1.5	0.09
GLULD1	glutamate-ammonia ligase (glutamine synthetase) domain containing 1	+1.4	0.09

Table 4A.5 The sixty one genes showing significantly decreased expression after twenty four hours PTH treatment of Caco2

Gene Symbol	Gene description	Fold change	PFP
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	-5.2	0
MMP1	matrix metallopeptidase 1	-1.8	0
SLC26A2	solute carrier family 26 member 2	-1.8	0.002
PRKCI	protein kinase C, iota	-1.7	0.005
EREG	epiregulin	-1.7	0.004
MYC	v-myc myelocytomatosis viral oncogene homolog	-1.6	0.004
RBM3	RNA binding motif protein 3	-1.6	0.004
AREG	amphiregulin	-1.6	0.003
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	-1.6	0.007
ID1	inhibitor of DNA binding 1,	-1.5	0.004
NRIP1	nuclear receptor interacting protein 1	-1.5	0.01
PAFAH1B1	Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit	-1.5	0.006
SLC25A32	solute carrier family 25, member 32	-1.5	0.01
PHLDA1	pleckstrin homology-like domain, family A, member 1	-1.5	0.007
DUSP6	dual specificity phosphatase 6	-1.4	0.02
ZNF148	zinc finger protein 148	-1.4	0.03
HSPA1B	heat shock 70kDa protein 1B	-1.4	0.02
FUS	fusion (involved in t(12;16) in malignant liposarcoma)	-1.4	0.02
HOXA7	homeobox A7	-1.4	0.03
DYNC1LI2	dynein, cytoplasmic 1, light intermediate chain 2	-1.4	0.02
LRRFIP1	leucine rich repeat (in FLII) interacting protein 1	-1.4	0.03
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	-1.4	0.01
ASNS	asparagine synthetase	-1.4	0.01
NUP98	nucleoporin 98kDa	-1.4	0.02
SFRS7	splicing factor, arginine/serine-rich 7, 35kDa	-1.4	0.02
DKK1	dickkopf homolog 1	-1.4	0.02
SFRS3	splicing factor, arginine/serine-rich 3	-1.4	0.02
PDCD2	programmed cell death 2	-1.4	0.03
SGK1	serum/glucocorticoid regulated kinase 1	-1.4	0.04
GJA1	gap junction protein, alpha 1 (connexin 43)	-1.4	0.01
WNK1	WNK lysine deficient protein kinase 1	-1.4	0.02

PHLDA2	pleckstrin homology-like domain, family A, member 2	-1.4	0.06
SLC2A1	solute carrier family 2 member 1	-1.4	0.05
SS18	synovial sarcoma translocation, chromosome 18	-1.4	0.06
RFC3	replication factor C (activator 1) 3, 38kDa	-1.4	0.03
ABCG2	ATP-binding cassette, sub-family G, member 2	-1.4	0.04
PRNP	prion protein	-1.4	0.05
TAF15	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa	-1.3	0.04
MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4	-1.3	0.08
NOX1	NADPH oxidase 1	-1.3	0.06
DARS	aspartyl-tRNA synthetase	-1.3	0.04
RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	-1.3	0.06
STT3A	STT3, subunit of the oligosaccharyltransferase complex, homolog A (S. cerevisiae)	-1.3	0.07
SOX9	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	-1.3	0.06
UTS2	urotensin 2	-1.3	0.05
NOL5A	nucleolar protein 5A	-1.3	0.06
SLC12A2	solute carrier family 12 member 2	-1.3	0.07
GNL2	guanine nucleotide binding protein-like 2 (nucleolar)	-1.3	0.08
UBIAD1	UbiA prenyltransferase domain containing 1	-1.3	0.03
YTHDF2	YTH domain family, member 2	-1.3	0.07
TGIF	TGFB-induced factor (TALE family homeobox)	-1.3	0.07
ANKRD11	ankyrin repeat domain 11	-1.3	0.09
PSPH	phosphoserine phosphatase	-1.3	0.05
EFNB2	ephrin-B2	-1.3	0.08
CKAP4	cytoskeleton-associated protein 4	-1.3	0.08
FZD7	frizzled homolog 7 (Drosophila)	-1.3	0.06
ARL4C	ADP-ribosylation factor-like 4C	-1.3	0.08
MSH2	mutS homolog 2, colon cancer, nonpolyposis type	-1.3	0.09
BRD2	bromodomain containing 2	-1.3	0.09
BRD4	bromodomain containing 4	-1.3	0.09
PPAP2B	phosphatidic acid phosphatase type 2B	-1.3	0.09

4A.4.1.3 Genes differentially expressed at both 4 and 24 hours

There were eight genes differentially expressed at both four and twenty four hours. For six of these genes (STT3A, HOXA7, HSPA1B, ANKRD11, PPAP2B and PSPH) expression was decreased at both four and twenty four hours. In contrast, for two genes (CYP1A1, AREG) expression was increased at four hours but decreased at twenty four hours.

4A.4.2 Identification of differentially expressed genes known to be targets of PTH/PTHrP signalling

Comparison of the differentially expressed genes with genes already identified as targets of PTH signalling was carried out to identify common themes of PTH signalling in different tissue types.

4A.4.2.1 Genes differentially expressed at four hours

Of the eighty two genes differentially expressed after four hours of treatment with PTH, thirty one have been identified as PTH/PTHrP modulated genes in different organ systems. These PTH regulated genes are listed in Table 4A.6 along with the studies in which they were identified. Twenty one genes were differentially expressed in the same direction in both studies. Twenty genes (HOXA7, ROD1, SFRS1, GPD2, GSR, SERPINH1, FBLN1, DNAJB6, NUP50, SLC16A1, CANX, RBBP4, SKP2, PDCD4, TMED2, EIF2S3, HSPA9, DUSP5, BRD4, and CALM1) were down-regulated in both studies and AREG was up-regulated. Nine genes showed different responses to PTH in this study. Eight genes (PVR, SCARB2, RHOB, PSPH, APLP2, CD9, PPAP2B and HSPA1B) were down-regulated by PTH in this study and up-regulated in the other studies and CYP1A1 showed the converse. One gene, CEBPA is a

transcription factor thats expression correlates with known PTH regulated genes.

Table 4A.6 The genes changed in expression in Caco2 after four hours of treatment with PTH that have also been previously demonstrated to be targets of PTH in other studies.
Each study is briefly described and the direction of change of expression for each gene is given. One gene (AREG) was changed in more than one study.

Brief description of Study	Genes in which expression changed in response to PTH/PTHrP and the direction of change
Expression profiling of Parathyroid hormone treated rat osteoblastic cell line UMR106-01 (Qin, Qiu et al. 2003)	Increased: AREG
PCR study of the influence of Parathyroid hormone on PTH treated hepatocytes in-vitro (Michaud, Naud et al. 2006)	Decreased: CYP1A1
Gene expression profiling study of bone biopsies from patients with primary hyperparathyroidism comparing before and after parathyroidectomy (Reppe, Stilgren et al. 2006)	Decreased: HOXA7, ROD1, SFRS1, GPD2, GSR, SERPINH1, FBLN1, DNAJB6, NUP50, SLC16A1, CANX, RBBP4, SKP2, PDCD4, TMED2, EIF2S3
Gene expression profiling study of bone from rats treated with parathyroid hormone (Li, Liu et al. 2007)	Increased: PVR, SCARB2, RHOB, PSPH, AREG
Gene expression profiling study of bone from rats treated with parathyroid hormone (Onyia, Helvering et al. 2005)	Decreased: HSPA9 Increased: APLP2, CD9
Gene expression profiling study of MCF7 breast cancer cell lines treated with siRNA to PTHrP (Dittmer, Vetter et al. 2006)	Increased: PPAP2B, HSPA1B Down-regulated: DUSP5, AREG
Genes altered in expression following in-vitro treatment of chondrocytes with PTHrP (Hoogendam, Farih-Sips et al. 2007)	Decreased: BRD4, CALM1
Promoter analysis study identifying transcription factors associated with PTH regulated genes (Qiu, Qin et al. 2003)	Correlated with PTH regulated genes: CEBPA

4A.4.2.2 Genes differentially expressed at twenty four hours

Of the sixty three genes changed after twenty four hours, twenty five have been identified as PTH/PTHrP modulated genes in different studies and three of them (AREG, GJA1 and SFRS3) were identified in more than one study. These PTH regulated genes are listed in Table 4A.7 along with the studies in which they were identified. Fifteen genes (CYP1A1, MSH2, ARL4C, FZD7, PSPH, NOL5A, RFC3, ASNS, SFRS3, LRRFIP1, PHLDA1, AREG, GJA1, DKK1 and ID1) out of the twenty five were differentially expressed in the same direction, all of them being down-regulated in both studies. Neither of the two genes showing increased expression after twenty four hours has been demonstrated to be a target of PTH signalling. The other ten genes that showed decreased expression in this study (SOX9, GJA1, PHLDA2, HOXA7, ATP1B1, CKAP4 SGK1, DUSP6, MYC, PPAP2B and EFNB2) were up-regulated in the other studies. Two genes AREG and GJA1 have been demonstrated to be both up and down regulated in response to PTH.

Table 4A.7 The twenty five genes changed in expression in Caco2 after twenty-four hours of treatment with PTH that have also been previously demonstrated to be targets of PTH/PTHrP signalling in other studies. Each study is briefly described and the direction of change of expression for each gene is given. Three genes (AREG, GJA1 and SFRS3) were changed in more than one study.

Brief description of Study	Genes in which expression changed and the direction of change
Expression profiling of Parathyroid hormone treated rat osteoblastic cell line UMR106-01 (Qin, Qiu et al. 2003)	Increased: AREG
PCR study of the influence of Parathyroid hormone on PTH treated hepatocytes in-vitro (Michaud, Naud et al. 2006)	Decreased: CYP1A1
Gene expression profiling study of bone biopsies from patients with primary hyperparathyroidism comparing before and after parathyroidectomy (Reppe, Stilgren et al. 2006)	Decreased: MSH2, ARL4C, FZD7, SFRS3 Increased: SOX9, GJA1, PHLDA2, HOXA7, ATP1B1
Gene expression profiling study of bone from rats treated with parathyroid hormone (Li, Liu et al. 2007)	Decreased: PSPH, NOL5A, RFC3, ASNS Increased: CKAP4 SGK1, DUSP6, MYC
Gene expression profiling study of MCF7 breast cancer cell lines treated with siRNA to PTHrP (Dittmer, Vetter et al. 2006)	Up-regulated: PPAP2B, Down-regulated: SFRS3, LRRFIP1, PHLDA1, AREG
Gene expression changes during endodermal differentiation induced by PTHrP treatment (van der Heyden, Veltmaat et al. 2000)	Decreased: GJA1
Influence of PTH on bone (Allan, Hausler et al. 2008)	Increased: EFNB2
Influence of parathyroid hormone treatment on the Wnt pathway components in bone (Kulkarni, Halladay et al. 2005)	Decreased: DKK1
Gene expression profiling of the rat osteosarcoma cell line UMR106-01 treated with PTH (Qin, Qiu et al. 2003)	Decreased:ID1

4A.4.3 Differentially expressed genes known to be changed in colorectal tumorigenesis

To investigate the hypothesis that PTH influences signalling pathways involved in colorectal tumorigenesis, a review of the literature was performed. This identified any previously reported changes in expression of these genes in colorectal neoplasia.

4A.4.3.1 Genes showing increased expression after four hours of PTH treatment

Increased expression in colorectal neoplasia

Of the five genes showing increased expression after four hours, two of them (AREG and MAFF) have been demonstrated to show increased expression in colorectal neoplasia. Increased expression of AREG has been demonstrated in colorectal cancer compared to normal colonic mucosa (Croner, Foertsch et al. 2005) and MAFF shows increasing expression with advancing stage of colorectal cancer (Friederichs, Rosenberg et al. 2005).

4A.4.3.2 Genes showing decreased expression after four hours of PTH treatment

Decreased expression in colorectal neoplasia

Of the seventy seven genes showing decreased expression after four hours of PTH treatment, decreased expression of fifteen of them has been implicated in colorectal tumorigenesis. Decreased expression in adenomatous polyps compared to normal mucosa has been demonstrated for six genes ICAM1, PPAP2B, FBLN1, RDX (Segditsas, Sieber et al. 2008), PDCD4 (SEUNGKOO LEE 2006), and DUSP5 (Nikola A. Bowden 2007). Decreased expression in

colorectal cancer compared to normal mucosa was found in five genes, ARHGAP5 (Lin YM 2002), GPD2 (Bianchini, Levy et al. 2006), HSPA9 (Bertucci, Salas et al. 2004), GSN (Gay, Estornes et al. 2008) and BTRC (Grade, Hormann et al. 2007).

For two genes (OGT and CD9), decreased expression was found in advanced stage disease compared to early stage disease. Decreased expression of OGT and CD9 is more common in cancers with nodal involvement (Hashida, Takabayashi et al. 2003; Grade, Hormann et al. 2007). Decreased expression of OGT (Fritzmann, Morkel et al. 2009) and CNN3 (Bertucci, Salas et al. 2004) is found in tumours with distant metastases. Inactivating mutations of OGT are also commonly found in tumours with micro-satellite instability (Woerner, Gebert et al. 2001).

Increased expression in colorectal neoplasia

Of the seventy seven genes showing decreased expression after four hours of PTH treatment, increased expression of six of them has been implicated in colorectal tumorigenesis. Increased expression in adenomatous polyps compared to normal mucosa was found in two genes, SFRS1 and HNRPU (Gaspar, Cardoso et al. 2008) and increased expression in colorectal cancer compared to normal mucosa was found in four genes, MOCS3 (Wiese, Auer et al. 2007), SERPINH1 (Croner, Foertsch et al. 2005) SKP2 and SRPK1 (N Ancona and S Liuni 2006).

Overall after four hours treatment with PTH, twenty three of the differentially expressed genes have been shown to be altered in colorectal neoplasia. Of

these, seventeen are changed in the same direction and six in the opposite direction by PTH. These are shown in tables 4A.8 and 4A.9 respectively.

Table 4A.8 The seventeen genes changed after four hours of PTH treatment, which are also changed in a similar manner in colorectal neoplasia. The studies identifying these changes are included

	Gene symbol
Increased in cancer	**MAFF** (Friederichs, Rosenberg et al. 2005), **AREG** (Croner, Foertsch et al. 2005)
Decreased in cancer	**ARHGAP5** (Lin YM 2002), **BTRC** (Grade, Hormann et al. 2007), **GPD2** (Bianchini, Levy et al. 2006), **GSN** (Gay, Estornes et al. 2008)
Decreased in polyps	**PPAP2B**, **FBLN1**, **ICAM1**, **RDX** (Segditsas, Sieber et al. 2008), **PVR** (Gaspar, Cardoso et al. 2008), **DUSP5** (Bowden, Croft et al. 2007), **PDCD4** (Lee, Bang et al. 2006)
Decreased in advanced tumours	**HSPA9**, **CNN3** (Bertucci, Salas et al. 2004), **OGT (**Fritzmann, Morkel et al. 2009**)**,
Poor prognostic marker	**CD9**, (Hashida, Takabayashi et al. 2003)

Table 4A.9 The six genes changed after four hours of PTH treatment, which are changed in the opposite direction to that seen in colorectal neoplasia. The studies identifying these changes are shown

	Gene symbol
Increased in cancer	**SRPK1**, **SKP2** (N Ancona and S Liuni 2006)
Increased in polyps	**SFRS1**, **HNRPU**, (Gaspar, Cardoso et al. 2008), **MOCS3** (Wiese, Auer et al. 2007), **SERPINH1** (Croner, Foertsch et al. 2005)

4A.4.3.3 Genes with increased expression after twenty four hours of PTH treatment

Neither of the two genes showing increased expression after twenty four hours has been demonstrated to show altered expression in colorectal neoplasia.

4A.4.3.4 Genes showing decreased expression after twenty four hours of PTH treatment

Decreased expression in colorectal neoplasia

Of the sixty three genes showing decreased expression after twenty four hours of PTH treatment, decreased expression of ten of them has been implicated in colorectal tumorigenesis. Decreased expression in adenomatous polyps compared to normal mucosa has been demonstrated in six genes, PPAP2B, ABCG2, SGK, GJA1 (Segditsas, Sieber et al. 2008), PRNP (Nikola A. Bowden 2007) and PRKCI (Kim, Park et al. 2008). Decreased expression in colorectal cancer compared to normal mucosa has been demonstrated for four genes, DKK1 (Gonzalez-Sancho, Aguilera et al. 2005), ABCB1 (Birkenkamp-Demtroder, Olesen et al. 2005), ATP1B1 (Chen, Zhang et al. 2006) and SLC26A2 (Cardoso, Boer et al. 2007).

Increased expression in colorectal neoplasia

Of the sixty three genes showing decreased expression after twenty four hours of PTH treatment, increased expression of ten of them has been implicated in colorectal neoplasia. Increased expression in adenomatous polyps compared to normal mucosa has been demonstrated for seven genes, NUP98 (Salahshor, Goncalves et al. 2005), NOL5A, SLC12A2, RFC3, PHLDA1, SOX9 and MYC (Gaspar, Cardoso et al. 2008). Increased expression in colorectal cancer compared to normal mucosa has been demonstrated for three genes, TGIF (Cardoso, Boer et al. 2007), AREG and MMP1 (Croner, Foertsch et al. 2005).

Overall at twenty-four hours, twenty differentially expressed genes have been shown to be altered in colorectal neoplasia. Of these ten are changed in the same direction and ten in the opposite direction by PTH in this study. These are shown in tables 4A.10 and 4A.11.

Table 4A.10 The ten genes changed after twenty-four hours of PTH treatment, which are also changed in a similar manner in colorectal neoplasia. The studies identifying these changes are shown

	Gene symbol
Decreased in cancer	**DKK1**(Gonzalez-Sancho, Aguilera et al. 2005), **ABCB1**(Birkenkamp-Demtroder, Olesen et al. 2005) **ATP1B1**(Chen, Zhang et al. 2006), **SLC26A2** (Cardoso, Boer et al. 2007)
Decreased in polyps	**PPAP2B**, **ABCG2**, **GJA1**, **SGK1** (Segditsas, Sieber et al. 2008) **PRNP** (Bowden, Croft et al. 2007) **PRKCI** (Kim, Park et al. 2008)

Table 4A.11 The ten genes changed after twenty-four hours of PTH treatment, which are changed in the opposite direction to that seen in colorectal neoplasia. The findings of the studies identifying these changes are included

	Gene symbol
Increased in cancer	**TGIF**, **SOX9** (Cardoso, Boer et al. 2007), **NUP98** (Salahshor, Goncalves et al. 2005), **AREG**, **MMP1** (Croner, Foertsch et al. 2005)
Increased in polyps	**NOL5A**, **SLC12A2**, **RFC3**, **PHLDA1** (Gaspar, Cardoso et al. 2008), **MYC** (Segditsas, Sieber et al. 2008)

4A.5 Discussion

In these experiments, parathyroid hormone treatment of the colorectal cell line Caco2 resulted in the differential expression of a number of genes, demonstrating that parathyroid hormone alters gene expression in colonic cells: a non-classical PTH target. At both time points, the majority of genes showed decreased expression: this suggests that in the colon PTH acts as a transcriptional repressor. This differs from other in-vitro micro-array studies of PTH action in bone that show similar number of increased and decreased genes (Qin, Qiu et al. 2003).

4A.5.1Parathyroid hormone responsive genes

In this study a number (56/145) of the differentially expressed genes are recognised PTH/PTHrP targets, identified in other tissues, predominantly bone. Inclusion of genes modulated by PTHrP is based on the observation that both PTH and PTHrP activate the PTH receptor, PTHR1, with similar efficacy. This finding indicates that the same signalling mechanisms activated by PTH in bone are likely to be active in the colon. The classical signalling mechanism for PTH is via stimulation of adenlyl-cyclase, production of cAMP (cyclic adenosine monophosphate) and activation of protein kinase A (PKA) (Swarthout, D'Alonzo et al. 2002). PTH stimulates the production of cAMP in the colonic cell line Lovo (Yu, Seitz et al. 1992), indicating that the cAMP/PKA pathway is activated by the PTH signalling pathway in the colon. This is consistent with the findings of differential expression of some of the genes in this study known to be regulated by the PTH/cAMP/PKA pathway. For example, the most highly up-regulated gene after four hours is Amphiregulin (AREG). Amphiregulin has been demonstrated to be up-regulated by PTH in

rat osteoblasts in-vitro (Qin, Tamasi et al. 2005) and rat bone in-vivo (Li, Liu et al. 2007) and is regulated by the cAMP/PKA pathway(Qin and Partridge 2005). Similarly, down-regulation of Dikkopf1 (DKK1) in bone, in response to PTH is mediated by cAMP (Kulkarni, Halladay et al. 2005). Decreased expression of GJA1 is also mediated similarly by PTHrP and production of cAMP (van der Heyden, Veltmaat et al. 2000). The downstream signalling mechanisms of PTH signalling in the colon have not been fully investigated. It seems likely, based on the above observations, that the cAMP/PKA pathway is active in colonic cells. Parathyroid hormone has also been shown to activate a number of other signalling pathways in the small intestine, including: phospholipase C (PLC) (Gentili, Boland et al. 2001), calcium influx (Picotto, Massheimer et al. 1997) and MAP kinases (Gentili, Morelli et al. 2001). The contribution of specific signalling pathways to these PTH mediated changes has not been investigated in this study.

Some of the differentially expressed genes are regulated in a different direction to that seen in other tissues. Differential regulation of other genes by PTH from different tissues (van der Heyden, Veltmaat et al. 2000; Qin, Qiu et al. 2003; Dittmer, Vetter et al. 2006; Reppe, Stilgren et al. 2006; Li, Liu et al. 2007), or within the same tissue but different contexts(Li, Liu et al. 2007), has been observed. These findings suggest that the changes induced by PTH may be tissue or context specific. Having observed overlap with known PTH regulated genes it is important to note that the majority of the genes differentially expressed in this study have not been previously shown to be PTH target genes. These genes therefore represent novel PTH target genes

in the colon, which it is predicted would be differentially expressed in response to PTH in-vivo.

4A5.2 Colorectal neoplasia associated genes

Identification of a number of genes differentially expressed in response to PTH, that are similarly differentially expressed in neoplastic colon compared to normal colon, indicates that PTH may have a role in colorectal tumorigenesis. After four hours, seventeen genes were changed in a direction consistent with a role in tumorigenesis and six genes were changed in the opposite direction. At twenty four hours a differential effect was not apparent with a similar number of genes (ten) showing changes both consistent with or opposite to those seen in neoplastic colon. One possible explanation for this is that the pro-neoplastic effects of PTH are seen early after treatment and as the effect of the single PTH treatment diminishes, these pro-neoplastic changes are less apparent. Another explanation is that the predominant effect of PTH is not to just to change the expression of genes known to be altered in colorectal tumorigenesis. This is apparent from the observation that the majority of the differentially expressed genes are not differentially expressed in colorectal neoplasia. Identification of the function of the differentially expressed genes is therefore of interest.

Of the differentially expressed genes also altered in colorectal neoplasia, the most interesting is Amphiregulin (AREG), which was up-regulated by PTH at four hours. Amphiregulin is a member of the epidermal growth factor family and acts via the EGFR (epidermal growth factor receptor). Amphiregulin is a well recognised PTH target gene in bone and is involved in the growth of osteoblasts (Qin, Tamasi et al. 2005). In addition to

being a recognised PTH target, Amphiregulin is strongly implicated in colorectal neoplasia. Amphiregulin expression is higher in tumours compared to normal mucosa (Croner, Foertsch et al. 2005) and high expression level also predicts the development of liver metastases (Yamada, Ichikawa et al. 2008). As well as altered expression in neoplastic colon Amphiregulin also has growth promoting effects on colorectal cells in-vitro (Damstrup, Kuwada et al. 1999). Thus, PTH induced up-regulation of Amphiregulin provides evidence for a growth promoting effect of PTH on colorectal cells. However, increased expression of AREG at four hours was followed by decreased expression at twenty-four hours. Whether this is a homeostatic response, a downstream result of PTH or due to the diminishing effect of PTH with time from the treatment, is not clear. It again demonstrates that the effect of PTH is not simply to alter the expression of genes known to be altered in colorectal neoplasia.

4A.5.3 Comparison with other studies

This is the first study identifying global changes in gene expression in a colorectal cell line in response to PTH. Only one other study has investigated the effect of PTH on gene expression in a colorectal cell line. In that study, PTH treatment of the colorectal cell line Lovo resulted in increased expression of ornithine decarboxylase (ODC) mRNA and increased ODC activity after two hours (Yu, Seitz et al. 1992). Differential expression of ODC was not observed in this study, probably due to investigation of different time points. This suggests that increased ODC expression is an early response to PTH treatment, which returns promptly to normal in the absence of persistent PTH stimulation.

Studies investigating the effect of PTHrP in colorectal cell lines have been more commonly carried out. Over expression of full length PTHrP in Lovo has been shown to increase the expression of integrins α6 and β4 (Shen, Rychahou et al. 2007). These changes were not seen in this study. This could be for many reasons, including the use of a different cell line, Lovo. But there is also evidence that PTHrP mediates its effects via an intracrine pathway (Bhatia, Saini et al. 2009) as well as by activation of PTHR1.

4A.5.4 Caveats and limitations

The approach used in this study of identifying early (4hour) and late (24 hour) transcriptional targets, was the same taken in studies of parathyroid hormone treatment of bone (Qin, Qiu et al. 2003) and in studying the effect of other agents on colorectal cell lines (Palmer, Sanchez-Carbayo et al. 2003). The nature of these in-vitro studies, using administration of a single treatment of PTH with a limited half life, differs from the in-vivo situation where a steady state of PTH stimulation is likely to be found. The half-life of naturally occurring PTH in-vivo is around 4 minutes (Irvin and Carneiro 2000). Of more relevance to this study, is that the half life of recombinant PTH used as treatment for osteoporosis has a half life of one hour (Deal 2004). The in-vitro half life of PTH used in this study is not known (Personal communication) but it is likely to be similar to these two values. It is therefore recognised that the nature of the response to the treatment schedule employed here represents a change in PTH level, rather than that seen in response to a steady state difference in serum PTH. The two time points employed can be seen to represent the immediate transcriptional effects of PTH treatment (4 hours)

and the downstream consequences of this treatment (24 hours) and possibly homeostatic consequences.

It could be argued that the ideal cell line for investigating events early in tumorigenesis, would be an adenoma cell line, but those studied in chapter one (RGC2 and AAC1) did not express PTHR1. Though it has some features of normal colonic epithelium, Caco2 is derived from a malignant colorectal tumour and has gene expression profiles similar in part to normal colonic mucosa and in part to tumours (Saaf, Halbleib et al. 2007). Of note is that the caco2 gene expression profile becomes more like normal colonic gene expression profile with prolonged time in culture (Saaf, Halbleib et al. 2007). It is therefore predicted that the response of caco2 to PTH shows some features of normal colon and some of a colorectal tumour.

At twenty-four hours, the array studies were only carried out in duplicate compared to at four hours when the studies were performed in triplicate which is considered standard procedure, this was for logistical reasons. This was taken into account by using Rank product to identify differentially expressed genes, as this method is well suited when few replicates are used (Jeffery, Higgins et al. 2006).

The fold changes seen in the array study are relatively small, suggesting that the overall effect of PTH is small; this may not be the case and could be due to a number of reasons. As discussed above caco2 has altered gene expression profiles consistent with its malignant origin and may already highly express PTH target genes, limiting the ability of PTH to further stimulate expression. Expression of PTHrP by Caco2 acting at the same

PTHR1 receptor will also limit the ability of PTH to further stimulate changes in gene expression.

4A.5.5 Alternative techniques and future studies

In completing this study it became clear how things could have been improved on and what further studies should be carried out. Repeated treatments with PTH over a longer period (eg. 72-96 hours) would be expected to result in differential expression of genes regulated by PTH in a manner found in-vivo which is exposed to a continuous level of PTH. Alternative strategies that could be employed to identify PTH regulated genes include interfering RNA inhibition of PTHR1 expression, or transfection with a constitutively active PTHR1 (Calvi, Sims et al. 2001).

4A5.6 Conclusion

In this study it has been demonstrated that PTH influences gene expression in an in-vitro model of the colonic epithelium. A novel group of PTH target genes in the colon has been identified. Some of these have a role in colorectal tumorigenesis, the majority do not. The functions of these genes are of interest in determining the effect of PTH on the colon.

Chapter 4B

FUNCTIONAL ANNOTATION OF THE GENES DIFFERENTIALLY EXPRESSED IN RESPONSE TO PARATHYROID HORMONE AND VALIDATION USING SEMI-QUANTITATIVE REAL-TIME PCR

4B.1: Aims

1. Functional analysis of differentially expressed genes.
2. Validation of selected findings of array studies using real time PCR

4B.2: Introduction

In the previous part of this chapter, genes differentially expressed in response to PTH treatment of Caco2 were identified. It was demonstrated that a number of these genes are similarly altered during colorectal neoplasia. However, the majority of genes differentially expressed in response to PTH have no recognised role in colorectal tumorigenesis. Determining the biological activity of these genes is therefore of interest in identifying the function of PTH in the colon. Analysis of the list of differentially expressed genes produced by micro-array studies is an important task in understanding the biological process being studied. A commonly utilised approach is to map the list of genes to biological annotation terms and then to identify the statistically enriched or overrepresented biological annotation terms. This approach has been used in a number of studies. These include identifying the enriched biological processes and molecular functions of the genes differentially expressed in response to PTH treatment of bone (Onyia, Helvering et al. 2005). There are also a number of studies that have identified

the annotation terms enriched in the genes differentially expressed in colorectal cancer or adenoma compared to normal mucosa (Sabates-Bellver, Van der Flier et al. 2007; Chan, Griffith et al. 2008); as well as those in proliferating or differentiated colonic epithelium (Kosinski, Li et al. 2007). As enrichment analysis of large gene lists is more of an exploratory process than a strictly statistical solution, comparison of the results with those from other studies of colorectal cancer and PTH results will enable an additional layer of analysis. Identification of annotation terms or signalling pathways enriched in the genes differentially expressed in response to PTH will provide clues to the function of PTH in the colon.

Validation of the micro array data was carried out for selected genes using semi-quantitative real time PCR. Eight (AREG, CYP1A1, STT3A, PDCD4, CTBP1, GSN, DKK1 and GJA1) of the genes identified in the array studies were investigated. These genes were chosen for a number of reasons. Some of them are known targets of PTH signalling: AREG (Qin, Tamasi et al. 2005), GJA1(van der Heyden, Veltmaat et al. 2000), DKK1(Kulkarni, Halladay et al. 2005), PDCD4(Reppe, Stilgren et al. 2006) and CYP1A1 (Michaud, Naud et al. 2006). Some are altered in colorectal tumorigenesis, GSN (Gay, Estornes et al. 2008): involved in wnt signalling CTBP1 (Cuilliere-Dartigues, El-Bchiri et al. 2006) or changed at both four and twenty four hours (AREG, CYP1A1, STT3A).

4B.3: Methods

4B.3.1 Functional annotation of the genes differentially expressed in response to PTH

Two lists of genes were created from those identified using Rank Product. As there were only a small number of up-regulated genes, the up and down regulated genes were combined for each time point. These two gene lists were uploaded to DAVID (Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/)) (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009). This online resource enables the analysis of lists of gene resulting from micro-array expression studies. The purpose of this analysis was to identify the biological function and meaning from a list of genes. DAVID maps the genes from the list to associated biological annotation terms and employs enrichment analysis to statistically identify the most overrepresented annotation terms. This is done by identifying which annotation terms are enriched in the list of genes compared to the background genes. The significance of gene term enrichment is examined by the calculation of an EASE score; this is derived from the name of the original software used for DAVID, Expression Analysis Systematic Explorer. The EASE score is a modified Fishers exact test giving a p-value. The default cut-off is 0.1, the smaller the score the more significant the enrichment. Multiple testing is corrected for by the calculation of a false detection rate (FDR). The FDR is known to be a conservative approach and a value of up to 0.5 maybe considered significant. It is recommended that the enrichment scores are used as a scoring system suggesting roles, rather than decision making roles (Huang da, Sherman et al. 2009). The enriched

annotation terms were identified using two different utilities available on DAVID.

1. Functional annotation chart

   This identifies enriched terms within the gene list and performs a "term centric singular enrichment analysis". The default settings were employed, apart from using an EASE score of 0.05 (instead of 0.1), to identify enriched annotation terms.

2. Functional annotation clustering

   This clusters functionally similar enriched terms into groups, performing a "term centric modular enrichment analysis". Default settings were employed, apart from stringency being increased to high. Clusters of terms with enrichment scores of >1.3, which is equivalent to non-log scale value of 0.05, were identified. Clusters with enrichment scores less than 1.3 were also identified as enriched, if they included individual terms with EASE scores <0.05.

For each analysis the background was set as Affymetrix HG-Focus. This process was carried out separately for the genes differentially expressed at four and twenty four hours. Additionally, clusters of annotation terms that did not meet the above criteria, but were enriched to any degree at both time points were identified.

4B.3.2Annotation methods

DAVID allows the use of several annotation methods, in this study two were use; the gene ontology (GO) annotation (Ashburner, Ball et al. 2000)(http://www.geneontology.org/)) and Kegg pathways (Kanehisa, Goto et al. 2009) http://www.genome.jp/kegg. The GO ontology provides a systematic method for the description of gene products in three biological domains; cellular component (CC), biological process (BP) and molecular function (MF). A cellular component (CC) is as described the component of a cell but specifically a defined anatomical component of a cell such as the nucleus. A biological process (BP) is a series of events carried out by various molecular functions. Biological processes include broad terms such as signal transduction and more specific terms such as alpha-glucoside transport. Molecular functions (MF) describe activities that occur at the molecular level and these terms can be broad (catalytic activity) or narrow (adenylate cyclase activity).

Kegg (Kyoto Encyclopaedia of Genes and Genomes) pathways are an online resource of pathway maps representing our knowledge on the molecular reaction and interactions in the areas of: metabolism, genetic information processing, environmental information processing, cellular processes and Human diseases.

4B.4: Results

4B.4.1 Functional annotation of the genes differentially expressed in response to PTH treatment

Functional annotation of the differentially expressed genes was performed using the online utility DAVID. The two approaches used identified:

1. Enriched single GO annotation terms and Kegg pathways
2. Enriched clusters of functionally related GO annotation term

4B4.2 Enriched single GO annotation terms and Kegg pathways

Identification of enriched single GO terms was carried out separately for the genes differentially expressed at four and twenty-four hours.

4B4.2.1 Enriched single GO annotation terms after four hours

Enriched single annotation terms for each of the three biological domains of the GO annotation (BP, MF and CC), were identified in the genes differentially expressed at four hours. Twenty one biological process terms, ten cellular component terms and five molecular function annotation terms were enriched in the genes differentially expressed at four hours. These are shown in Table 4B.1.

4B4.2.2 Enriched Kegg pathways after four hours

One Kegg pathway, the Wnt signalling pathway was enriched in the genes differentially expressed at four hours. The four genes from the wnt signalling pathway (CTBP1, NFAT5, NLK and BTRC) are all down regulated in response to PTH.

Table 4B.1: List of individual GO annotation terms from each of the three biological domains (BP, CC and MF) enriched in the genes differentially expressed after four hours of treatment with PTH. The p-value and FDR (false detection rate) are included for each term.

Biological process (BP)	*P* value	FDR
macromolecule localisation	$1x10^{-4}$	0.002
protein localisation	$2x10^{-4}$	0.005
establishment of protein localisation	$5x10^{-4}$	0.009
mRNA metabolic process	$9x10^{-4}$	0.017
RNA splicing	$8x10^{-3}$	0.13
cellular component organisation and biogenesis	$1x10^{-2}$	0.19
mRNA processing	$1x10^{-2}$	0.26
vesicle mediated transport	$1x10^{-2}$	0.28
RNA processing	$3x10^{-2}$	0.38
cytoskeleton organisation and biogenesis	$3x10^{-2}$	0.39
regulation of actin polymerization and/or depolymerization	$3x10^{-2}$	0.40
localisation	$3x10^{-2}$	0.47
regulation of actin filament length	$4x10^{-2}$	0.50
regulation of actin cytoskeleton organisation and biogenesis	$4x10^{-2}$	0.50
regulation of cellular component size	$4x10^{-2}$	0.50
cellular process	$4x10^{-2}$	0.50
actin cytoskeleton organisation and biogenesis	$4x10^{-2}$	0.50
regulation of mRNA processing	$4x10^{-2}$	0.50
regulation of cellular process	$4x10^{-2}$	0.50
regulation of organelle organisation and biogenesis	$4x10^{-2}$	0.50
regulation of cytoskeleton organisation and biogenesis	$5x10^{-2}$	0.50
Cellular component (CC)		
intracellular membrane bound organelle	$1x10^{-4}$	0.002
membrane bound organelle	$1x10^{-4}$	0.002
intracellular part	$2x10^{-4}$	0.004
organelle membrane	$3x10^{-4}$	0.002
intracellular organelle part	$9x10^{-4}$	0.01
organelle part	$9x10^{-4}$	0.01
cytoplasm	$1x10^{-3}$	0.02
nucleus	$3x10^{-3}$	0.04
endomebrane system	$3x10^{-3}$	0.05
cytoplasmic part	$2x10^{-2}$	0.25
Molecular function (MF)		
protein binding	$4x10^{-3}$	0.007
GTP binding	$7x10^{-3}$	0.01
guanyl ribonucleotide binding	$9x10^{-3}$	0.01
guanyl nucleotide binding	$9x10^{-3}$	0.01
GTPase activity	$3x10^{-2}$	0.45

4B4.2.3 Enriched single GO annotation terms after twenty-four hours

Enriched single annotation terms for each of the three biological domains of the GO annotation (BP, MF and CC) were identified in the genes differentially expressed at twenty-four hours. Nine biological process terms, three cellular component terms and eleven molecular function annotation terms were enriched in the genes differentially expressed at twenty-four hours. These are shown in table 14. No Kegg pathways were enriched at 24 hours.

Table 4B.2: The individual GO annotation terms from each of the three biological domains (BP, CC and MF) enriched in the genes differentially expressed after twenty-four hours of treatment with PTH. The p-value and FDR (false detection rate) are included for each term.

Biological process (BP)	*P* value	FDR
gamete generation	$8x10^{-3}$	0.15
negative regulation of transcription	$2x10^{-2}$	0.30
sexual reproduction	$2x10^{-2}$	0.30
negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	$2x10^{-2}$	0.40
amino acid biosynthetic process	$3x10^{-2}$	0.45
negative regulation of cellular process	$3x10^{-2}$	0.45
negative regulation of biological process	$4x10^{-2}$	0.50
reproductive structure development	$5x10^{-2}$	0.50
gonad development	$5x10^{-2}$	0.50
Cellular component (CC)		
nucleus	$5x10^{-3}$	0.08
intracellular membrane bound organelle	$3x10^{-2}$	0.39
membrane bound organelle	$3x10^{-2}$	0.39
Molecular function (MF)		
nucleotide binding	$4x10^{-4}$	0.007
purine nucleotide binding	$1x10^{-2}$	0.20
active transmembrane transporter activity	$2x10^{-2}$	0.30
purine ribonucleotide binding	$2x10^{-2}$	0.31
ribonucleotide binding	$2x10^{-2}$	0.31
xenobiotic transporter activity	$2x10^{-2}$	0.31
xenobiotic-transporting ATPase activity	$2x10^{-2}$	0.31
multidrug transporter activity	$3x10^{-2}$	0.40
nucleoside-triphosphatase activity	$4x10^{-2}$	0.52
adenyl nucleotide binding	$4x10^{-2}$	0.50
pyrophosphatase activity	$5x10^{-2}$	0.50

4B4.3 Enriched clusters of functionally related GO annotation terms

This was carried out separately for the genes differentially expressed at four and twenty-four hours. Subsequently, comparison was made of the results from both time points to identify clusters of GO annotation terms enriched at both time points.

4B.4.3.1 Clusters of functionally related GO annotation terms enriched at four hours

Five clusters of functionally related GO terms were identified as being enriched in the genes differentially expressed at four hours. Three of these clusters showed enrichment scores >1.3 and two showed enrichment scores <1.3 but had individual annotation terms with p-values <0.05. The five enriched annotation clusters, listed as cluster 4A to 4E, are shown in Table 4B.3.

4B.4.3.2 Clusters of functionally related GO annotation terms enriched at twenty-four hours

Six clusters of related GO terms were identified as being enriched in the list of genes differentially expressed at twenty four hours. Three of these clusters had enrichment scores >1.3 and four showed enrichment scores <1.3, but had individual annotation terms with p-values <0.05. The six enriched annotation clusters, listed as cluster 24A to 24F, are shown in Table 4B.4.

Table 4B.3: The five annotation clusters (4A to 4E) enriched in the genes differentially expressed at four hours. The enrichment score for each cluster and the GO terms found in each cluster are shown. The differentially expressed genes found in each cluster and their direction of change is shown.

	GO terms (Biological domain)	ES	Genes changed (direction of change)
4A	Macromolecule localisation (BP) Protein localisation(BP) Establishment of protein localisation (BP) Protein transport (BP)	3.5	COPG, RHOB, RFIP1,TOMM22, RAB8A, TMED2, CANX, LIN7C, NUP50, PTPN11, NUTF2, RDX, RAB5B, NCBP1 (Down) and ARF6 (Up)
4B	mRNA metabolic process (BP) mRNA processing (BP) RNA processing (BP)	2.4	SNRNP40, SRRM2, HSPA1B, NCBP1, SRPK1, SPOP, ROD1, SFRS1 (Down)
4C	GTP binding (MF) Guanyl ribonucleotide binding (MF) Guanyl nucleotide binding (MF) GTPase activity (MF)	2.1	CEBPA, RHOB, EIF2S3, RAB8A, ARHGAP5, RAB5B (Down) ARF6 (Up)
4D	Regulation of cellular process (BP) Regulation of biological process (BP) Biological regulation (BP)	1.2	JMJD1B, CEBPA, GSR, ELL2, DNAJB6, STAT2, PPAP2B, RAB8A, ICAM1, HSPA1B, SKP2, LIN7C, CTBP1, NFAT5, PTPN11, RAB5B, UTP20, RDX, NCBP1, RHOB, ARFIP1, KLF3, NLK, CD9, NDRG3, GPC3, GSN, PDCD4, SRPK1, RBBP4, ATF2, HOXA7, SELT (Down)ARF6, MAFF (Up)
4E	Regulation of actin polymerization and/or depolymerization (BP) Regulation of actin filament length (BP) Regulation of actin cytoskeleton organization and biogenesis (BP) Regulation of cytoskeleton organization and biogenesis (BP) Cell Leading edge (CC) Cell projection (CC) Cellular component organization and biogenesis (BP)	1.1	RDX, CNN3, GSN, ACTB (Down) ARF6 (Up)

Table 4B.4: The six annotation clusters (24A to 24F) enriched at twenty-four hours, along with the enrichment score for each cluster. The GO terms found in each cluster, the differentially expressed genes found in each cluster and their direction of change is shown.

	GO terms (Biological domain)	ES	Genes changed (direction of change)
24A	Purine nucleotide binding (MF) Ribonucleotide binding (MF) Purine ribonucleotide binding (MF) Adenyl nucleotide binding (MF) ATP binding (MF) Adenyl ribonucleotide binding (MF)	1.6	SGK1, GNL2, WNK1, DYNC1LI2, ARL4C, RALA, DARS, ABCB1, NOX1, HSPA1B, PRKCI, RFC3, MAP4K4, MSH2, ABCG2 (Down)
24B	Negative regulation of transcription (BP) Negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process (BP) Negative regulation of cellular metabolic process (BP) Negative regulation of metabolic process (BP)	1.5	ZNF148, LRRFIP1, EREG, SOX9, ID1, NRIP1 (Down)
24C	Nucleoside-triphosphatase activity (MF) Pyrophosphatase activity (MF) Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides (MF) Hydrolase activity, acting on acid anhydrides (MF)	1.4	ARL4C, ATP1B1, RFC3, ABCB1, MSH2, GNL2, ABCG2 (Down)
24D	Gamete generation (BP) Sexual reproduction (BP) Reproduction (BP)	1.1	ZNF148, BRD2, EREG, SOX9, PAFAH1B1, NRIP1 (Down)
24E	Negative regulation of cellular process(BP) Negative regulation of biological process (BP) Regulation of cellular process(BP)	1.1	ZNF148, GJA1, HSPA1B, PRNP, MSH2, WNK1, EREG, SOX9, ID1, NRIP1, DKK1, LRRFIP1, HOXA7, DUSP6, MYC (Down)
24F	Cell development (BP) Cellular developmental process (BP) Cell differentiation (BP)	0.8	HSPA1B, SGK1, GJA1, SOX9, PHLDA2, PRKCI, PRNP, EREG, PHLDA1, PDCD2, , EFNB2, PAFAH1B1 (Down)

4B.4.3.3 Clusters of functionally related GO annotation terms enriched at both four and twenty-four hours

The annotation terms enriched in response to PTH treatment at both four and twenty four hours were identified. Clusters of annotation terms that were enriched to any degree at both time points were identified, even if they did not meet the enrichment and significance criteria used for individual time points. This process identified five annotation clusters (V-Z). Only one of these clusters (Z) was identified as enriched at a single time point, the other four clusters though enriched at both time points have lower enrichment scores. The five enriched annotation clusters, listed as clusters V-Z are shown in Table 4B.5.

Table 4B.5 The five annotation clusters (V-Z) enriched at both four and twenty-four hours.
The enrichment score for each cluster at both time points is shown along with the GO terms included in each cluster. The differentially expressed genes found in each cluster and their direction of change is shown.

	GO terms (Biological domain)	Four hours		Twenty four hours	
		ES	Genes changed (direction of change)	ES	Genes changed (direction of change)
V	Apoptosis (BP) Programmed cell death (BP) Death (BP) Cell death (BP)	0.5	CROP, PDCD4, HSPA1B, DNAJB6, RTN3, RHOB, ARF6 (Down)	0.8	HSPA1B, GJA1, SGK1, PRNP, PHLDA1, PDCD2 (Down)
W	Protein modification process (BP) Biopolymer modification (BP) Post-translational protein modification (BP)	0.3	SKP2, CTBP1, SRPK1, BTRC, NLK, SPOP, OGT, STT3A, DUSP5 (Down)	0.1	SGK1, PRKCI, WNK1, STT3A, DUSP6 (Down)
X	Phosphate metabolic process (BP) Phosphorus metabolic process (BP) Phosphorylation (BP) Protein amino acid phosphorylation (BP)	0.1	CTBP1, SRPK1, NLK, DUSP5 (Down)	0.3	SGK1, PRKCI, WNK1, EREG, DUSP6 (Down)
Y	Amino acid and derivative metabolic process (BP) Amine metabolic process (BP) Nitrogen compound metabolic process (BP) Carboxylic acid metabolic process (BP) Organic acid metabolic process (BP)	0.3	TTR, CTBP1, GATM, PSPH (Down)	0.4	ASNS, DARS, PSPH (Down)
Z	Regulation of cellular process (BP) Regulation of biological process (BP) Biological regulation (BP)	1.1	ELL2, DNAJB6, RAB8A, ICAM1, HSPA1B, SKP2, LIN7C, CTBP1, NFAT5, RAB5B, UTP20, RDX, RHOB, ARFIP1, KLF3, NLK, CD9, NDRG3, GPC3, GSN, PDCD4, SRPK1, RBBP4, ATF2, HOXA7, SELT (Down) ARF6, MAFF (Up)	0.2	ZNF148, GJA1, UTS2, PRNP, WNK1, EREG, ID1,NRIP1, HSPA1B, DKK1, LRRFIP1, HOXA7, DUSP6, MYC (Down)

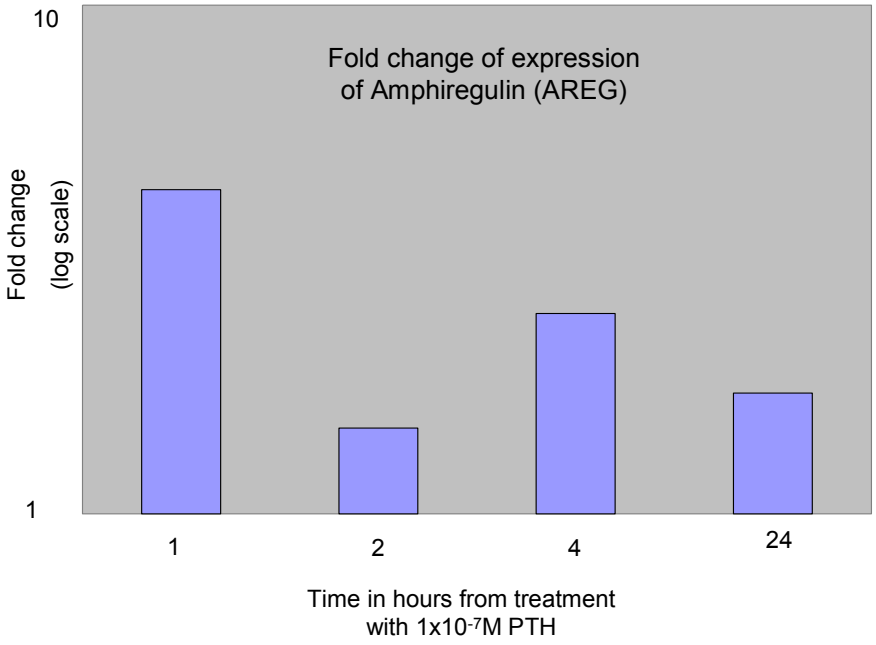
4B4.4 Semi-quantitative real time PCR for selected micro-array targets

To validate the micro-array studies, expression of eight (AREG, CYP1A1, STT3A, PDCD4, CTBP1, GSN, DKK1 and GJA1) of the differentially expressed genes was investigated by semi-quantitative real time PCR. Expression of each of the genes was compared to an untreated control and fold change calculated. Relative expression was studied at the time points at which the gene was differentially expressed in the array study (4 and 24 hours). Additionally relative expression at other time points was investigated, based on the predicted time course of PTH activity. A fold change of > +/- 1.5 fold was considered to indicate differential expression.

4B4.4.1 Amphiregulin (AREG)

Expression of Amphiregulin mRNA showed increased relative expression at one hour (+4.3 fold), two hours (+1.5 fold), four hours (+2.5 fold) and twenty four hours (+1.7 fold). This is shown graphically in Figure 4B.1. These results are consistent with the array study at four hours but are in contrast with the results at twenty fours hours, in which AREG showed decreased expression.

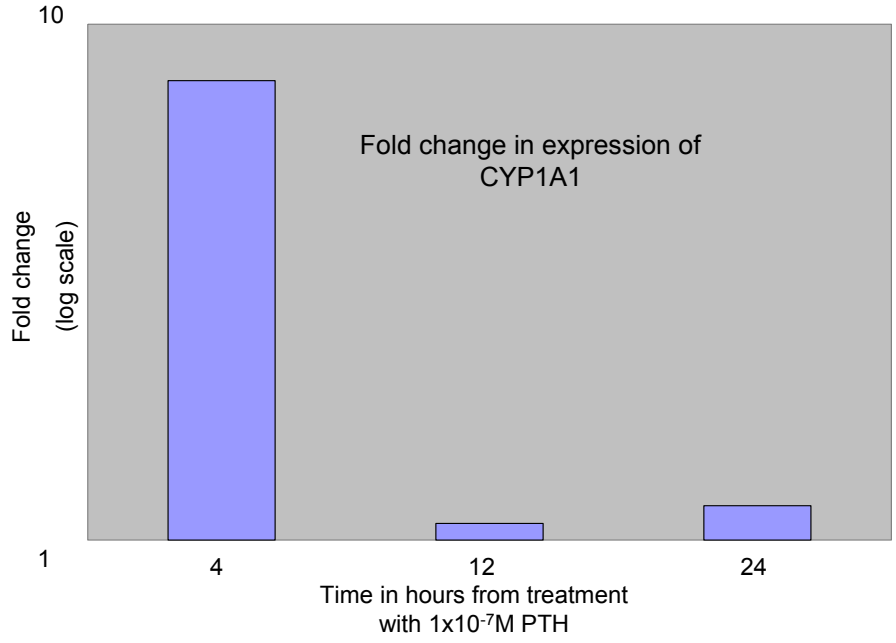
Figure 4B.1: Fold change in expression of Amphiregulin at a series of time points following PTH treatment.



4B4.4.2 Cytochrome P450, family 1, subfamily A, polypeptide1 (CYP1A1)

Expression of CYP1A1 mRNA showed increased relative expression at four hours (+22.3 fold) and twenty four hours (+1.7 fold). At twelve hours relative expression was +1.2 fold. These results are consistent with the array study at four hours but are in contrast with the results at twenty fours hours, in which CYP1A1 showed decreased expression. The magnitude of the change at four hours is however larger than that seen in the array study (+22.3 vs. +3.2 fold).

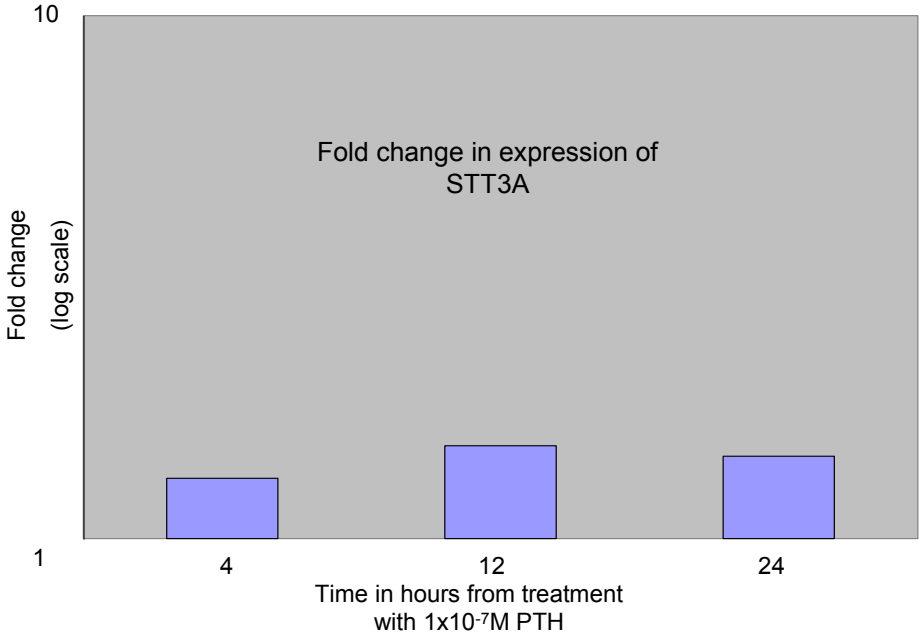
Figure 4B.2: Fold change in expression of CYP1A1 at a series of time points following PTH treatment.



4B4.4.3 STT3, subunit of the oligosaccharyl transferase complex, homolog A (STT3A)

Expression of STT3A mRNA was increased at twelve hours (+1.5 fold). At four hours relative expression was +1.3 fold and at twenty four hours was +1.4 fold. This contrasts with the array studies which showed decreased expression at four and twenty four hours.

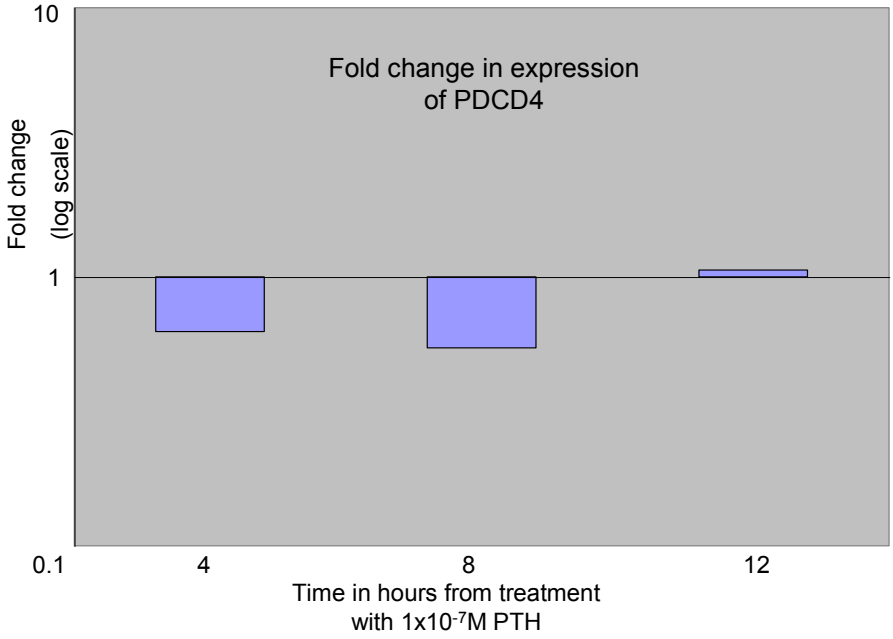
Figure 4B.3: Fold change in expression of STT3A at a series of time points following PTH treatment.



4B4.4.4 Programmed cell death 4 (PDCD4)

Expression of PDCD4 mRNA was decreased at four hours (-1.6 fold) and eight hours (-1.8 fold) and unchanged at twelve hours. This is consistent with the findings in the array study.

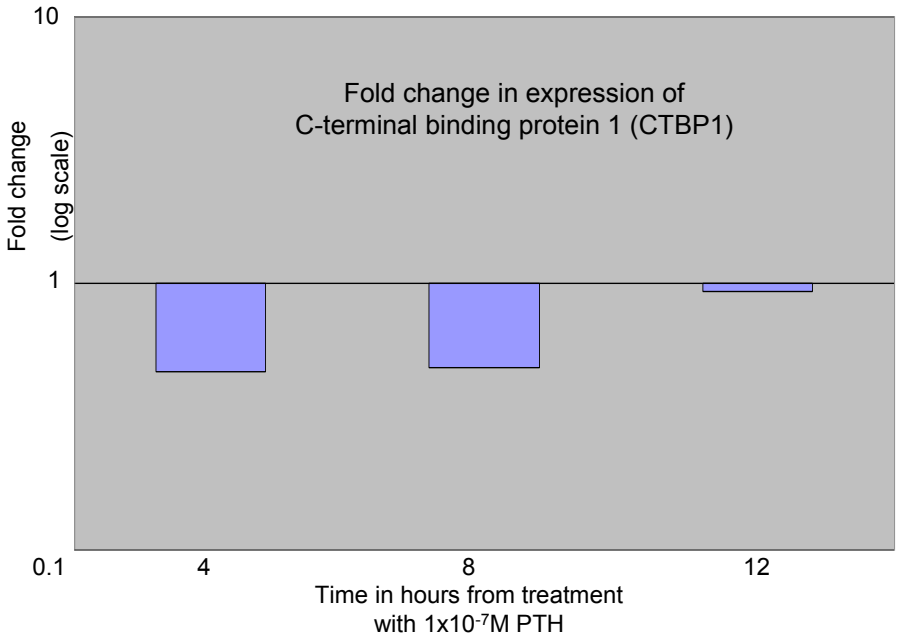
Figure 4B.4: Fold change in expression of PDCD4 at a series of time points following PTH treatment.



4B.4.4.5 C-terminal binding protein 1 (CTBP1)

Expression of CTBP1 mRNA was decreased at four hours (-2.2 fold) and eight hours (-2.1 fold). At twelve hours relative expression was -1.1 fold. This result is consistent with the findings in the array study.

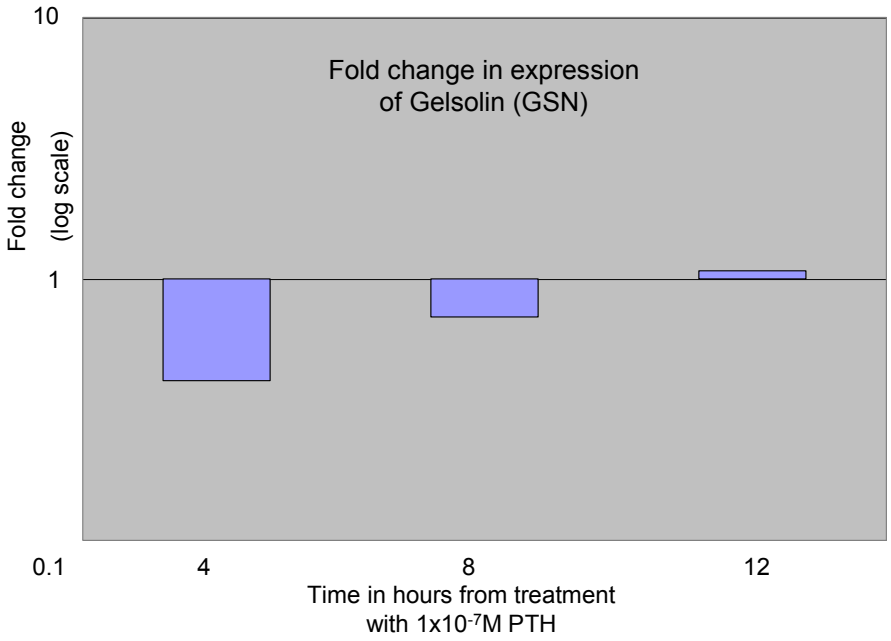
Figure 4B.5: Fold changes in expression of CTBP1 at a series of time points following PTH treatment.



4B.4.4.6 Gelsolin (GSN)

Expression of GSN mRNA was decreased at four hours (-2.5 fold). At eight and twelve hours, expression was altered by -1.4 and +1.1 fold respectively. This is consistent with findings in the array study.

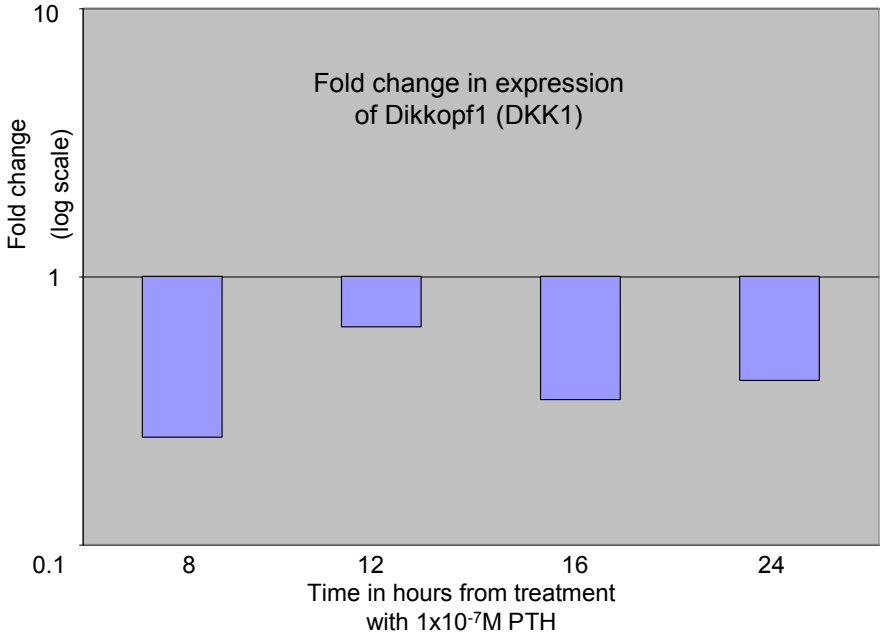
Figure 4B.6: Fold change in expression of GSN at a series of time points following PTH treatment.



4B.4.4.7 Dickkopf homolog 1 (DKK1)

Expression of DKK1 mRNA was decreased at eight (-3.4 fold), twelve (-1.5 fold), sixteen (-2.9 fold) and twenty four (-2.4 fold) hours. This is consistent with findings in the array study.

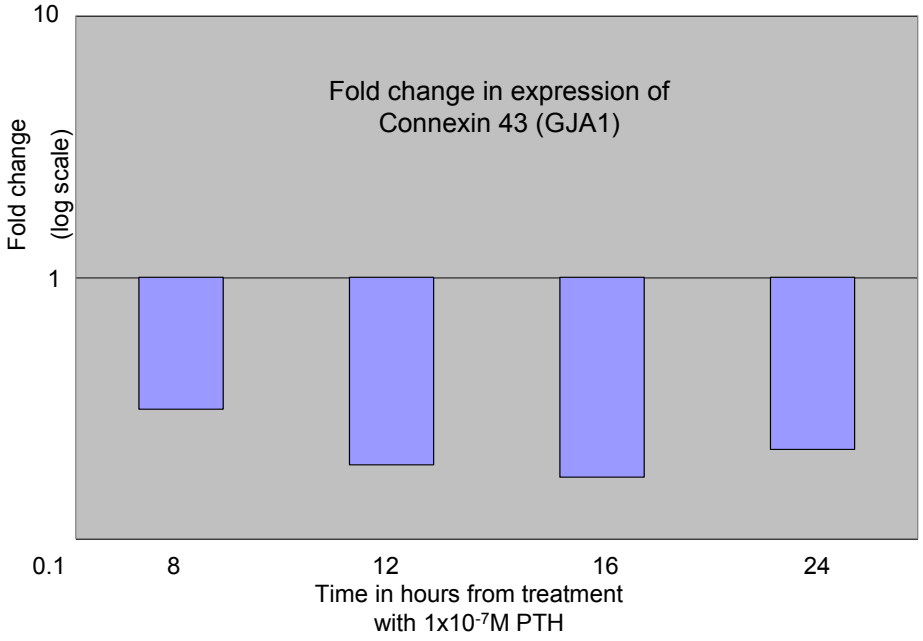
Figure 4B.7: Fold change in expression of DKK1 at a series of time points following PTH treatment.



Gap junction associated 1 (GJA1)

Expression of GJA1 mRNA was decreased at eight (-3.2 fold), twelve (-5.2 fold), sixteen (-5.8 fold) and twenty four (-4.5 fold) hours.

Figure 4B.8 Fold changes in expression of GJA1 at a series of time points following PTH treatment.



4B.4.4.9 Summary of real-time PCR results

For five of the six genes differentially expressed at four hours and two out of the five genes differentially expressed at twenty-four hours, the findings of the real-time PCR and array studies were consistent, although the fold changes were larger in the PCR study. One gene STT3A was not changed at either four or twenty-four hours in the PCR study in contrast to the array results. For two genes AREG and CYP1A1, differentially expressed at twenty-four hours, the findings in the PCR studies were the opposite of those in the array study. A summary of the real time PCR results for all eight of the genes are shown in Table 4B.6.

Table 4B.6: Summary of the real-time PCR results for all eight genes. This shows the fold change at each time point studied. The results from the array studies are shown for comparison. H=hours, nc=no change

Gene	Change in array		1H	2 H	4 H	8 H	12 H	16 H	24 H
	4H	24H							
AREG	+1.5	-1.6	+4.3	+1.5	+2.5	-	-	-	+1.7
CYP1A1	+3.2	-5.2	-	-	+22.3	-	+1.2	-	+1.7
STT3A	-1.9	-1.3	-	-	+1.3	-	+1.5	-	+1.4
PDCD4	-1.8	nc	-	-	-1.6	-1.8	1	-	-
CTBP1	-1.3	nc	-	-	-2.2	-2.1	-1.1	-	-
GSN	-1.5	nc			-2.5	-1.4	+1.1	-	-
DKK1	nc	-1.4	-	-	-	-3.4	-1.5	-2.9	-2.4
GJA1	nc	-1.4	-	-	-	-3.2	-5.2	-5.8	-4.5

4B.5 Discussion

4B.5.1 Function of PTH regulated genes

In this study DAVID (Database for Annotation, Visualization and Integrated Discovery) was used to identify enriched biological function in the genes differentially expressed in response to PTH. It is suggested that the results produced by DAVID be seen as a scoring system with statistical terms (eg p-values) indicating likely involvement of the enriched biological phenomenon, but that these results should be judged in terms of known or predicted biology (Huang da, Sherman et al. 2009). The results of the functional annotation are therefore discussed in the context of the known functions of PTH and a predicted role in colorectal tumorigenesis. The stringency of the selection criteria for both the DAVID tools used were increased from the default settings; the aim of this was to increase the identification of relevant functional annotation.

The enriched single annotation terms for each of the three GO annotation term domains (BP, MF and CC) are included (Tables 4B.1 and 4B.2) as this is the most commonly presented format and allows for comparison with other studies. However, for attempting to identify the function of PTH, the clusters of enriched functionally related annotation terms demonstrates PTH function with greater clarity. There are a number of enriched annotation clusters suggesting a role for PTH in colorectal neoplasia. Rho-GTPase signalling (Cluster 4C) regulates various processes in colorectal tumorigenesis and expression of components of these signalling pathways are commonly altered (Karlsson, Pedersen et al. 2009). PTH down-regulates genes involved in cell differentiation (Cluster 24F). This suggests a role in

preventing differentiation, which is of relevance to tumorigenesis and suggests a possible role for PTH in the normal colon. Inhibition of differentiation is consistent with a known physiological function of signalling via PTHR1, which inhibits differentiation of keratinocytes (Foley, Longely et al. 1998).

Perhaps the most interesting enriched cluster indicates that PTH is involved in remodelling of the actin cytoskeleton and cell migration (Cluster 4E). The altered genes in cluster 4E have been implicated to varying degrees in tumorigenesis. Gelsolin and Radixin show decreased expression in neoplastic compared to normal colonic mucosa (Gay, Estornes et al. 2008; Segditsas, Sieber et al. 2008) and decreased expression of CNN3 is found in tumours with metastases (Bertucci, Salas et al. 2004). Reduction of Gelsolin expression using siRNA induces epithelial-mesenchymal transition, increased motility and invasion (Tanaka, Shirkoohi et al. 2006). Though changes in expression of ARF6 in colorectal neoplasia have not been demonstrated, it has been shown to promote epithelial cell motility through the downstream activation of phospholipase D and the Rac1 GTPase (Santy and Casanova 2001) and regulates melanoma cell invasion (Tague, Muralidharan et al. 2004). Remodelling of the actin cytoskeleton is part of epithelial-mesenchymal transition (EMT) which is a process by which tumour migration and invasion occurs (Thiery, Acloque et al. 2009). PTH stimulates the migration of osteosarcoma cell lines in-vitro (Yang, Hoang et al. 2007; Berdiaki, Datsis et al. 2010). Although PTH itself has not been implicated in EMT, activation of PTHR1 by PTHRrP has been shown to induce EMT in kidney (Ardura, Rayego-Mateos et al. 2010) and during embryonic

development (van der Heyden, Veltmaat et al. 2000). This provides support for the evidence provided here that PTH may promote migration, invasion and EMT.

A cluster of genes involved in apoptosis and programmed cell death (Cluster V) was weakly enriched at both time points. It is not clear whether this indicates a pro or anti-apoptotic effect of PTH. All the differentially expressed genes in this cluster were down-regulated and include genes with both pro-apoptotic (PDCD2, PDCD4, RTN3, RHOB and PHLDA1) (Liu, Cerniglia et al. 2001; Neef, Kuske et al. 2002; Fan, Chan et al. 2004; Zhang, Ozaki et al. 2006; Lee, Lee et al. 2009) and anti-apoptotic activity (DNAJB6 and SGK1) (Dehner, Hadjihannas et al. 2008). Based on the majority of the genes being pro-apoptotic and down regulated this suggests that PTH acts to prevent apoptosis. Indeed, in bone Parathyroid Hormone results in the altered expression of genes with anti-apoptotic activity (Onyia, Helvering et al. 2005). This is consistent with its predicted role in tumorigenesis but it is not clearly the case. There is evidence that PTH has pro-apoptotic activity and since this work was carried out, a study demonstrating PTH induced apoptosis in Caco2 has been published (Calvo, Gentili et al. 2008). Of note, the authors of this study acknowledge that PTH can have both pro or anti-apoptotic effects depending on the cellular context. Their experiments were carried out with cells 70% confluent in comparison to the 7days post-confluent cells used in this study. This may explain the differences between these two studies and would require further work to clarify the effect of PTH on apoptosis in the colon.

The one enriched KEGG pathway, the Wnt signalling pathway, is of significant interest as it is commonly deregulated in colorectal tumorigenesis, with constitutive activation of β-catenin/TCF4 mediated signalling found in the majority of colorectal tumours (Kinzler and Vogelstein 1996). It has also been shown in a number of studies that Parathyroid hormone acts by modulating the wnt signalling pathway in bone (Reppe, Stilgren et al. 2006; Li, Liu et al. 2007). Parathyroid hormone activates the wnt signalling pathway in bone by two methods: activation of β-catenin by direct interaction of PTHR1 with Dishevelled-1 (Romero, Sneddon et al. 2010) and increased β-catenin expression, with evidence of increased activity of a wnt responsive reporter gene (Kulkarni, Halladay et al. 2005). Of the differentially expressed genes in the Wnt signalling pathway, Nemo like kinase phosphorylates TCF4, resulting in reduction of its DNA binding ability and decreased β-catenin/TCF4 mediated transcription (Ishitani, Ninomiya-Tsuji et al. 1999). Similarly CTBP1 binds TCF4 reducing its transcriptional activity (Cuilliere-Dartigues, El-Bchiri et al. 2006). Mutations in TCF4 are commonly found in micro-satellite unstable tumours and result in loss of CTBP1 binding sites, with consequential loss of CTBP1 mediated inhibition of TCF mediated transcription (Cuilliere-Dartigues, El-Bchiri et al. 2006). BTRC binds β-catenin and targets it for destruction (Spiegelman, Slaga et al. 2000). Decreased expression of these three negative regulators of the canonical wnt signalling pathway, suggests that PTH activates the canonical wnt signalling pathway in colonic cells.

Downstream targets of wnt signalling have been well characterised and of the differentially expressed genes at four hours, six down-regulated genes

(GSN, RHOB, TMOD3, DUSP5, FBLN1 and PPAB2B) show decreased expression in response to wnt signalling (van de Wetering, Sancho et al. 2002; Segditsas, Sieber et al. 2008). This would suggest that at four hours the altered expression of the wnt signalling pathway components results in the activation of wnt signalling and altered expression of wnt target genes. At twenty-four hours the situation is less clear, with down-regulation of three genes (SLC26A2, SGK1 and ABCG2) known to be down regulated in response to wnt signalling (Segditsas, Sieber et al. 2008). However, there are five down-regulated genes (PHLDA1, SOX9, SLC12A2, TGIF and MYC) that are known to be up-regulated in response to wnt signalling (van de Wetering, Sancho et al. 2002; Segditsas, Sieber et al. 2008). These wnt target genes include Myc which is recognised to be a key mediator of wnt signalling (Sansom, Meniel et al. 2007). The results presented here suggest that activation of the wnt signalling pathway is an early response to PTH. And in the absence of continued PTH stimulation, the effect on wnt signalling decreases with time. Consistent with this is the observed increase in expression of the wnt signalling target ornithine-decarboxylase (ODC) (Van der Flier, Sabates-Bellver et al. 2007). Expression of ODC in the colorectal cell line Lovo was up-regulated with a peak after two hours treatment with PTH(Yu, Seitz et al. 1992).

The majority of the enriched annotation clusters seen at both four and twenty-four hours are of a general nature. Enrichment of these would suggest that PTH: signals via altering transcription (4B, 24B), has a regulatory role in some biological processes (4D, 24B, 24E and Z), is involved in protein localisation (4A) and a number of metabolic processes (24A, 24C, W, X and

Y). These are general annotation terms with no overlap between these annotation terms and those found to be enriched in the genes differentially expressed in colorectal cancers (Chan, Griffith et al. 2008) or adenomas (Sabates-Bellver, Van der Flier et al. 2007). Comparison with the annotation terms enriched in genes differentially expressed in response to PTH treatment of bone, identified a number of related annotation terms, including anti-apoptosis, cell migration and catalytic activity (Onyia, Helvering et al. 2005). This suggests there is some overlap between the function of PTH in the bone and in the colon.

4B.5.2Real time PCR

The changes seen in the array studies were predominantly replicated in the real time PCR studies and the fold changes seen were larger than the array changes, a common finding. There were discrepant findings between array and real-time PCR studies for the expression of STT3A which was down regulated at both time points in the array but unchanged in the real time PCR study. For CYP1A1 and AREG, the changes in the array and real time PCR at four hours were consistent, both showing increased expression; but at twenty-four hours the array study showed decreased and the real-time PCR showed increased expression. There are a number of possible explanations; this includes the possibility that either of the two methods have given an incorrect result. In the absence of other supporting data there is no hierarchy in the validity of array and real time results. In this study there were only two replicates in the array study at twenty-four hours so it may be argued that these results are less valid. However the most likely explanation only became

apparent after post-hoc analysis of the array data. This shows changes in expression of cytokeratin 8 (KRT8) which was used as the control gene in the PCR study. Cytokeratin 8 was down regulated by PTH at twenty-four hour by -1.22 fold and unchanged at four hours, though it was not identified as differentially expressed using rank product. These changes are probably large enough to explain the discrepancies for CYP1A1, AREG and STT3A at twenty four hours in the PCR study. The use of KRT8 as a control gene was routine in the laboratory at that time (Caldwell, Jones et al. 2004). It was not known to be regulated by PTH and as it was not in the differentially expressed genes it was used in this study.

Having observed this finding, it may be considered ideal to repeat the real time PCR studies with a different control gene. However this was not logistically possible at the stage it became apparent. The change in KRT8 was only seen at twenty-four hours with no discrepancy between the micro-array and real time PCR results at four hours. Thus it can be argued that the micro-array results at four hours have been validated. Though, this is not the case for the twenty four hour point. It seems likely based on these observations that where there is a discrepancy that the array results are more likely to be valid than the real-time PCR results.

4B.5.3 Future studies

The enriched annotation terms in the genes differentially expressed in response to PTH, suggest functions of PTH also implicated in colorectal neoplasia. Future studies investigating hypotheses generated by this study would include functional studies investigating migration and invasion of PTH treated colonic cells. As deranged wnt signalling is a ubiquitous feature of

colonic neoplasia the effect of PTH on wnt regulated genes and functions, would be of interest in further determining the function of PTH in the colon.

4B.5.4Conclusion

The functions of PTH regulated genes in the colon have been identified. This has highlighted the wnt signalling pathway and regulation of the cytoskeleton and cell migration with relevance to colorectal neoplasia. The observations that PTH stimulates differential expression of genes in-vitro leads to the question: does PTH influence colonic epithelial cells in-vivo and if there is any relationship to the findings found in-vitro? These are the questions addressed in the following chapters. Firstly, the influence of serum PTH and secondly, the effect of dietary calcium supplementation on gene expression in the normal rectal mucosa were investigated.

Chapter 5

INVESTIGATION OF THE INFLUENCE OF DIETARY CALCIUM SUPPLEMENTATION ON SERUM BIOCHEMISTRY IN SUBJECTS UNDERGOING A STUDY OF GENE EXPRESSION IN RECTAL MUCOSA

5.1: Aims:

1. Determine the baseline serum levels of PTH, 25OH-vitamin D and calcium in subjects undergoing a study to asses the effect of these factors on gene expression in normal rectal mucosa.
2. Determine the changes in the levels of serum PTH, 25OH-vitamin D and calcium in these subjects in response to dietary calcium supplementation

5.2: Introduction

Having observed that PTH induces changes in gene expression in colonic cells in-vitro, the next step was to investigate whether PTH had an effect on gene expression in colonic mucosa in-vivo. The results of these in-vivo studies are described in the proceeding chapters.

The aims of this chapter are firstly, to describe the study methodology and the subjects involved. Secondly, to investigate the baseline biochemical data of the patients taking part, particularly serum levels of PTH, calcium and 25-OH Vitamin D and to describe the effect of dietary calcium supplementation on these parameters. The results of this chapter will be used in the next chapters investigating gene expression in the rectal mucosa of these subjects. The importance of measuring these biochemical parameters is that serum levels of both 25OH-Vitamin D and PTH have been

associated with colorectal neoplasia, as discussed in chapter one. Also both PTH (Chapter 4) and vitamin D (Palmer, Sanchez-Carbayo et al. 2003) have been shown to influence gene expression in colonic cells in-vitro. If the serum levels of these factors are directly relevant to the disease process, it would seem likely that there would be a relationship between these biochemical parameters and gene expression in the rectal epithelium. Similarly, it is predicted that there would be changes in both the relevant biochemical factor and gene expression associated with calcium supplementation.

5.3 Methods

5.3.1 Study overview

A descriptive biological study was carried out. This investigated the influence of serum biochemical factors and supplemental dietary calcium on the colonic epithelium of patients susceptible to colorectal neoplasia. The main objective was to identify the transcriptional changes in rectal mucosa in a population susceptible to neoplasia, associated with;

1. Serum biochemical factors, particularly PTH;
2. Dietary calcium supplementation.

The aim was to recruit twenty subjects with a history of colorectal adenomas to a crossover trial, six weeks of dietary calcium supplementation and six weeks of no treatment. Rectal biopsies were taken at the beginning and after each six week period. Blood samples were taken to measure Vitamin D, PTH and calcium. RNA was extracted from the biopsies and Micro-array expression analysis was carried out on the biopsy samples. Ethical approval was obtained from the Local Research and Ethics Committee and the study was carried out in the Wellcome Trust Clinical Research Facility at University Hospital, Birmingham.

5.3.2 Rationale

The number of patients selected for the study (20) was pragmatic, based on feasibility and a number that is likely to produce biological variability in the assessed parameters (eg serum PTH and 25OH-Vitamin D). The study was carried out on patients with a past history of adenomas, because they are known to be susceptible to colorectal cancer, they therefore have a „predisposed' colonic epithelium. They also formed the study group in the

Randomised Controlled Trials of calcium supplementation. The rectum is easily accessible to obtain biopsies and is representative of the colon as a whole (Terpstra, van Blankenstein et al. 1987).

The efficacy of calcium in adenoma prevention has been established and the daily dose used in this study (1.5g), is in between the doses used in the two randomised controlled adenoma prevention trials (1.2g and 2.0g) (Baron, Beach et al. 1999; Bonithon-Kopp, Kronborg et al. 2000). The six week period of calcium supplementation was also pragmatic. However, other studies of calcium supplementation have shown an effect on rectal mucosa after as little as one week (Lans, Jaszewski et al. 1991) and after up to four months supplementation(Holt, Wolper et al. 2001). The crossover study design in which dietary calcium will be varied means each patient could potentially contribute three data points: entry (baseline), low and high calcium intake.

5.3.3 Recruitment

Volunteer subjects were recruited from patients with a history of colonic adenomas, who were under colonoscopic surveillance at University Hospital Birmingham. They were invited to take part in the study by letter. Subjects who showed an interest in taking part in the study were invited to a preliminary appointment. This provided the investigator with the opportunity to ensure the patients met inclusion and exclusion criteria and enabled the patients to get more information before they made a decision to take part in the study. The inclusion criteria were a past history of treated adenomatous polyps. Exclusion criteria included: a history of malignancy, sarcoidosis, renal impairment, hypercalcaemia or bleeding disorders. Pre-menopausal

women were excluded, as were those taking digoxin, non-steroidal anti-inflammatory, anti-platelet or anti-coagulant drugs. Patients taking any over the counter vitamin or mineral supplements were not excluded from the study providing they were willing to stop taking them for the duration of the study. If the patient wished to proceed and met the inclusion/exclusion criteria, written consent was obtained.

5.3.4 Study protocol

The study lasted twelve weeks and following the initial recruitment visit the subjects attended on three occasions: at the beginning (visit A), after 6 weeks (visit B) and at the end of the study, 12 weeks (visit C). On each visit the subjects underwent:

-Venous blood sampling for PTH, calcium and Vitamin D levels.

-Rigid sigmoidoscopy and biopsy of rectal mucosa.

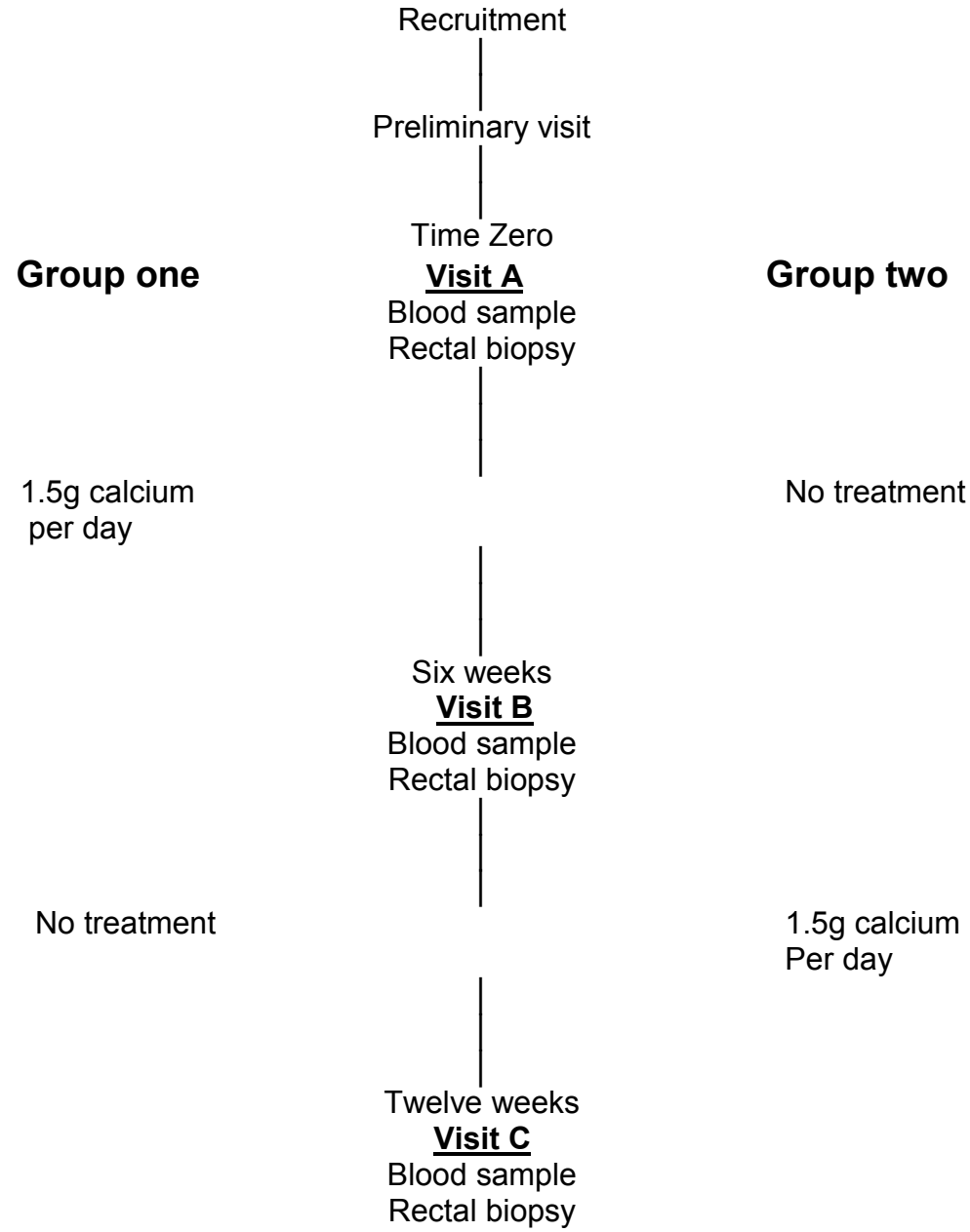
All of the visits were carried out in the morning to reduce variability due to diurnal variation in any of the biochemical parameters. The dietary calcium supplementation consisted of a daily dose of three1.25g tablets of calcium carbonate per day, as Calcichew® (Shire). Each tablet provided 500mg of calcium, giving a total daily dose of 1.5g of calcium. The subjects were encouraged to take one tablet three times a day with meals, though this was not compulsory. The correct number of tablets for the six week period was provided for each subject. Compliance with the calcium supplementation was assessed by direct questioning and counting of returned pills.

This was a crossover study and the patients were assigned randomly by selection of pre-sealed envelopes to one of two groups.

-Group one had no intervention for the first six weeks and then crossed over to take calcium carbonate for the second six week period.

-Group two took calcium for the first six weeks and crossed over to no intervention for the second six weeks.

Figure 5.1 Flow diagram for the study



5.3.5 Protocol for sigmoidoscopic biopsy of rectal epithelium

Rigid sigmoidoscopy was carried out with the patient in the left lateral position. The posterior wall of the lower rectum was visualised and biopsies were taken from this region. The biopsies were snap frozen in liquid nitrogen. Up to four biopsies were taken depending on the presence of excess bleeding and patient tolerance.

5.3.6 Collection of serum samples

Blood samples were all taken in the morning to avoid differences due to diurnal variation. Venous blood was taken in a routine manner from the ante-cubital fossa. Three samples of 5-10mls each were taken. Samples were centrifuged and the serum separated within 4 hours and then protected from the light (Vitamin D samples). All the samples were stored at -80°C. Biochemical analysis of all study samples from the eleven subjects who completed the study was undertaken as a batch at the end of the study.

5.3.7 Analysis of serum samples

Analysis of the serum samples was undertaken in the NHS biochemistry laboratory at The University Hospital of Coventry and Warwickshire. Serum PTH was measured using the ElectroChemiLuminescence Immunoassay (ECLIA) (Roche). Serum 25OH-Vitamin D was measured using the IDS enzyme immunoassay (Boldon, UK) and serum calcium was measured on a modular analyzer (Roche).

5.3.8 Statistics

The mean serum levels of PTH, 25OH-Vitamin D and calcium were compared before and after calcium supplementation. Both 25OH-Vitamin D and calcium are normally distributed at the at the population level unlike PTH which has a skewed distribution (Jorde, Saleh et al. 2005). Therefore paired t-tests were carried out for calcium and 25OH-Vitamin D using the online utility: http://www.graphpad.com/quickcalcs/ttest2.cfm.

As PTH is not normally distributed the serum levels of PTH were compared before and after calcium supplementation using the Mann-Whitney U test. This was calculated using the online utility: http://www.holah.karoo.net/Mann-WhitneyU-test.xls

5.4 Results

5.4.1 Subjects

Only thirteen subjects were recruited to the study; this was despite over eighty letters of invitation being sent out. Of the thirteen who started the study two subjects withdrew after the initial visit, one because of ill health and one for personal reasons. The remaining eleven subjects completed the study. Nine of the eleven were male and two female, the mean age was 65, range (61-69). Of the eleven subjects who completed the study, nine were randomised to group one and two to group two (both the subjects who withdrew had been randomised to group two). Compliance with the calcium supplementation regime was 94.8% overall and was >90% for all the subjects. This data is shown in table 5.1.

Table 5.1 Subject demographics
This table shows the age and sex of the volunteers, the study group to which they were randomised and their compliance with calcium supplements.

Subject	Age	Sex	Group	Compliance (%)
1	62	M	1	100
2	66	M	1	100
3	61	M	1	90
4	64	M	2	Withdrew
5	69	F	2	Withdrew
6	66	M	1	95
7	62	F	2	92
8	68	F	1	97
9	65	M	2	93
10	69	M	1	96
11	68	M	1	91
12	66	M	1	91
13	67	M	1	98

5.4.2 Biochemistry results

The biochemical results for all three time points are shown in Table 5.2 (PTH), Table 5.3 (25OH-Vitamin D) and Table 5.4 (calcium). Note that subjects 4 and 5 withdrew before completing the study.

5.4.2.1 Baseline biochemical data

The baseline biochemical data, prior to calcium supplementation for the eleven subjects who completed the study is presented below. This is of interest as the samples were taken prior to intervention with calcium.

5.4.2.1.1Serum PTH

At baseline mean serum PTH was 3.5 pmol/L, median PTH was 3.3pmol/L (Range 2.6-5.2 pmol/L). The normal range is 1.1-4.2 pmol/L and two subjects (6 and 8) had high PTH values outside the normal range, see Table 5.2.

Table 5.2 Serum PTH levels (pmol/L) for each of the three visits.
This table show the PTH levels for the eleven subjects who completed the study. Subjects 7 and 9 were in group two and took calcium supplements between visits B and C. The remaining nine subjects who were in group one took the calcium supplements between visits A and B.

Subject	parathyroid hormone (pmol/L)		
	Visit A	Visit B	Visit C
1	3.1	3.1	2.8
2	2.9	2.3	1.9
3	3.7	2.7	4.2
6	4.7	3.7	4.5
7	3.0	3.1	2.5
8	5.2	4.5	4.5
9	2.5	3.6	3.1
10	3.1	2.4	3.0
11	3.3	2.9	4.2
12	2.6	2.2	2.4
13	3.6	2.6	2.8

5.4.2.1.2 Serum 25OH-Vitamin D

At baseline, mean serum 25OH-Vitamin D was 34.7ug/L, with a standard deviation of 21 and a range of 14.1-88.5ug/L. These results are shown in Table 5.3. The normal range is 10-60ug/L and none of the subjects had a value below the lower limit. One subject (9) had a value above the upper limit; this sample was collected in the summer and this result is likely to represent high exposure to UVB.

Table 5.3 Serum 25OH-Vitamin D levels (ug/L) for each of the three visits.
This table show serum 25OH-Vitamin D levels for the eleven subjects who completed the study. Subjects 7 and 9 were in group two and took calcium supplements between visits B and C. The remaining nine subjects who were in group one took the calcium supplements between visits A and B.

Subject	25OH-Vitamin D (ug/L)		
	Visit A	Visit B	Visit C
1	38.6	33.5	27.2
2	43.6	33.2	23.8
3	41.1	38.0	35.1
6	14.1	16.1	25.0
7	26.1	26.2	42.9
8	22.6	27.3	35.4
9	45.3	88.5	73.2
10	22.2	21.2	22.5
11	22.5	39.9	28.5
12	16.8	21.3	31.2
13	45.9	42.2	37.4

5.4.2.1.3 Serum calcium

At baseline mean serum calcium was 2.30mmol/L, with a standard deviation of 0.09 and a range of 2.18-2.53mmol/L. These results are shown in Table 5.4. The normal range is 2.10-2.58mmol/L and all of the subjects had a value within this range. This includes subjects 6 and 8 who had PTH values outside of the normal range. This indicates that they had secondary rather than primary hyperparathyroidism.

Table 5.4 Serum calcium levels (mmol/L) for each of the three visits
This table shows the serum calcium levels (mmol/L) for the eleven subjects who completed the study. Subjects 7 and 9 were in group two and took calcium supplements between visits B and C. The remaining nine subjects who were in group one took the calcium supplements between visits A and B.

Subject	Serum Calcium		
	Visit A	Visit B	Visit C
1	2.35	2.28	2.31
2	2.3	2.35	2.41
3	2.35	2.28	2.24
6	2.27	2.33	2.36
7	2.53	2.48	2.59
8	2.32	2.29	2.33
9	2.32	2.28	2.39
10	2.22	2.22	2.23
11	2.18	2.33	2.20
12	2.32	2.32	2.30
13	2.21	2.21	2.22

5.4.2.2 Change in biochemical parameters in response to calcium supplementation

The changes in biochemical parameters in response to six weeks calcium supplementation are shown in Table 5.5. For the nine subjects in group 1 the comparison is between visits B and A, for those in group 2 the comparison is between visits C and B.

Table 5.5 Changes in biochemical parameters after six weeks calcium supplementation
This table shows the changes in the level of serum PTH, 25OH-vitamin D and calcium after six weeks of dietary calcium supplementation.

Subject	Change in PTH	Change in 25OH-Vit D	Change in calcium
1	0	-5.1	-0.07
2	-0.6	-10.4	+0.05
3	-1.0	-3.1	-0.07
6	-1.0	+2.0	+0.06
7	-0.6	+16.7	+0.11
8	-0.7	+4.7	-0.03
9	-0.5	-15.3	+0.11
10	-0.7	-1.0	0
11	-0.4	+17.4	+0.15
12	-0.4	+4.5	0
13	-1.0	-3.7	0
Mean change	-0.63	+0.6	-0.03

5.4.2.2.1 Serum PTH

After six weeks of calcium supplementation, serum PTH was decreased in ten and unchanged in one subject. The range of the change in PTH was 0 to -1.0pmol/L. At baseline the median PTH value was 3.3pmol/L (Range 2.6-5.2 pmol/L) and after calcium supplementation was 2.7pmol/L (Range 2.2-4.5 pmol/L). A Mann-Whitney U-test was performed to compare the difference between serum PTH levels before and after calcium supplementation (Table 5.6), this showed a highly significant difference at the 5% level ($p<0.05$).

Table 5.6 Statistical comparison of mean serum PTH, before and after calcium supplementation

Group	Baseline	Calcium
Median	3.3	2.7
Range	2.6-5.2	2.2-4.5
N	11	11

U = 27.5
Significant at 5% level

5.4.2.2.2 Serum 25OH-Vitamin D

After six weeks of calcium supplementation, serum 25OH-Vitamin D was decreased in six and increased in five subjects. The range of this change was -15.3 to +17.4ug/L. At baseline, mean 25OH-Vitamin D was 34.7ug/L and after six weeks calcium supplementation was 35.3, representing an increase in the mean of 0.6ug/L (95%CI=-7.4 to +6.2), equivalent to a 2% increase in 25OH-Vitamin D. A paired t-test was performed to compare the differences of the mean serum 25OH-Vitamin D before and after calcium supplementation (Table 5.7), this showed no significant difference (p=0.85).

Table 5.7 Statistical comparison of mean serum 25OH-Vitamin D, before and after calcium supplementation SD=Standard Deviation, SEM=Standard Error of Mean

Group	Baseline	Calcium
Mean	34.7	35.3
SD	21.0	15.5
SEM	6.3	4.7
N	11	11

t = 0.2004
Degrees of freedom = 10
Two-tailed P value = 0.85

5.4.2.2.3 Serum calcium

After six weeks of calcium supplementation, serum calcium was decreased in three, increased in five and unchanged in three subjects. The range of the change in calcium was -0.07 to +0.15mmol/L. At baseline mean serum calcium was 2.31mmol/L and after calcium supplementation was 2.33 mmol/L representing a mean decrease in serum calcium of -0.03mmol/L (95%CI -0.078 to +0.021), which is equivalent to a 1% reduction in mean serum calcium level. A paired t-test was performed to compare the differences of the mean serum calcium before and after calcium supplementation, this showed no significant difference (p=0.24).

Table 5.8 Statistical comparison of mean serum Calcium, before and after calcium supplementation SD=Standard Deviation, SEM=Standard Error of Mean

Group	Baseline	Calcium
Mean	2.30	2.33
SD	0.083	0.102
SEM	0.025	0.030
N	11	11

t = 1.2630
Degrees of freedom = 10
Two-tailed P value = 0.24

These results demonstrate that six weeks dietary calcium supplementation significantly reduced serum PTH but had no effect on serum 25OH-Vitamin D or calcium.

5.5: Discussion

In this study the main finding was that dietary calcium supplementation for six weeks resulted in a significant reduction in serum PTH levels with no significant effect on serum 25OH Vitamin D or calcium. That calcium supplementation suppresses PTH is not a novel observation. Calcium supplementation results in a reduced serum level of PTH after a single dose (Thomas, Need et al. 2008), after six weeks supplementation (Saleh, Jorde et al. 2003) or following two years supplementation (Reid, Ames et al. 2008). The magnitude of the change in PTH level is also similar to that found in these studies. The effect of calcium on the level of serum PTH was not measured in either of the adenoma prevention studies (Baron, Beach et al. 1999; Bonithon-Kopp, Kronborg et al. 2000). But based on the observations above it seems reasonable to expect that serum PTH was lower in the calcium treated group.

Calcium supplementation had no effect on the serum level of 25OH-Vitamin D, which is consistent with the finding of the polyp prevention study. In that study, there was no difference in 25OH-Vitamin D level between the calcium and placebo group after three years of calcium supplementation (Grau, Baron et al. 2003). It is therefore not a novel observation, that calcium suppresses PTH, with no effect on 25OH-Vitamin D. However, its importance relates to the interpretation of the results of the rectal mucosal gene expression studies described in the following chapters.

One criticism of this study could be that serum 1,25OH-Vitamin D was not measured. There are a number of reasons for this; the studies of vitamin D and colorectal cancer have shown an inverse association with 25OH-Vitamin D but not 1,25OH-Vitamin D (Tangrea, Helzlsouer et al. 1997;

Feskanich, Ma et al. 2004). Similarly, the relationship observed between serum levels of vitamin D and the efficacy of calcium in preventing adenomas, was related to higher serum levels of 25OH-Vitamin D, with no relationship to 1,25OH-Vitamin D (Grau, Baron et al. 2003). Further evidence that calcium does not act by increasing the level of serum 1,25OH-Vitamin D is found in the polyp prevention study. In this study there was a significant reduction in the level of 1, 25OH-Vitamin D at the end of the study in the calcium treated group, with no effect in the placebo group (Grau, Baron et al. 2003).

The baseline biochemical results for PTH show some features of note. The serum PTH levels show a wide range of values and two out of the eleven subjects had values above the upper limit of normal. They both had normal serum calcium levels indicating that they had secondary rather than primary hyperparathyroidism. Secondary hyperparathyroidism is common, with around 6% of healthy subjects having a level outside the normal range (Saleh, Jorde et al. 2006). In the general population parathyroid hormone is not normally distributed, but has a positively skewed distribution. Factors that contribute to the level of serum PTH include serum 25OH-Vitamin D level and dietary calcium intake (Saleh, Jorde et al. 2006), body mass index (Kamycheva, Sundsfjord et al. 2004), age (Jorde, Bonaa et al. 1999) and renal function (Stein, Flicker et al. 2001). However none of the factors above satisfactorily explains the causes of raised serum PTH. In most cases it is multi-factorial and incompletely explained by known risk factors (Saleh, Jorde et al. 2006).

Conclusion

In this study the baseline biochemical data for the subjects in this study have been reported and the suppressive effect of six weeks dietary calcium supplementation on serum PTH has been demonstrated.

Chapter 6A

INVESTIGATION INTO THE RELATIONSHIP BETWEEN SERUM BIOCHEMICAL PARAMETERS AND GENE EXPRESSION IN NORMAL RECTAL MUCOSA

6A.1 Primary aims:

1. Determine the gene expression profile in the normal rectal mucosa of individuals at baseline prior to any intervention

2. Investigate the relationship between biochemical parameters (PTH, 25OH-Vitamin D and calcium) and gene expression in normal rectal mucosa.

3. Identify individual genes whose expression correlates with the serum levels of 25OH-Vitamin D, PTH and calcium.

6A.1.1 Secondary aims:

1. Carry out functional annotation of genes showing correlation with biochemical parameters.

6A.2 Introduction

In chapter two it was demonstrated that colonic cells in-vitro are sensitive to PTH. It is therefore hypothesised that normal colorectal mucosa is sensitive to variation in serum PTH levels. With this in mind it is predicted that there will be inter-individual variation in gene expression in normal rectal mucosa and that the observed variation is at least in part due to inter-individual variation in serum parathyroid hormone levels. In studies of patients with primary hyperparathyroidism before and after parathyroidectomy, the in-vivo transcriptional targets of PTH have been identified in a classical PTH target,

bone (Reppe, Stilgren et al. 2006) and two non classical PTH targets, muscle and haematopoietic cells (Reppe, Stilgren et al. 2007). These studies indicate that PTH alters gene expression in non classical PTH targets, supporting the hypothesis that PTH may influence gene expression in the colon. Biopsies of normal rectal epithelium are easily obtainable and have been used here to investigate the influence of biochemical factors on gene expression. Previous studies of normal rectal mucosa have been used to investigate the influence of various agents implicated in the prevention of colorectal neoplasia (Holt, Atillasoy et al. 1998) and features associated with the risk of colorectal neoplasia (Ponz de Leon, Roncucci et al. 1988); but only one study has used micro-arrays to study gene expression in normal mucosa before and after intervention (Glebov, Rodriguez et al. 2006). As it has been demonstrated that both Vitamin D (Palmer, Sanchez-Carbayo et al. 2003) and calcium (Chakrabarty, Radjendirane et al. 2003) alter gene expression in-vitro, the relationship of these biochemical factors to gene expression in rectal mucosa will also be studied.

6A.3: Methods

6A.3.1 Subjects

Six subjects were investigated in the studies carried out in this chapter. The subjects used in this study were selected from those described in the previous chapter. The criteria for selecting which subjects would be studied were:

1. The availability of enough good quality (10ug) RNA. See table 6A.1
2. Serum levels of 25OH-Vitamin D and PTH at baseline, specifically half of the subjects had high (>29ug/L) 25-OH-Vitamin D levels and half had levels below this.
3. In view of the studies described in the next chapter; subjects in which a decrease in PTH was seen in response to calcium supplementation.

Table 6A.1 Concentration and purity of RNA extracted from the rectal mucosal biopsies used in the studies described in chapters 6 and 7.
The sample numbers anonymously identify the volunteers, A and B refer to samples taken at base line and after calcium supplementation.

Sample	RNA concentration (ng/µl)	RNA purity (260/280nm ratio)
3A	1293	2.08
3B	1343	2.1
7A	1012	2.09
7B	1149	2.0
8A	1157	2.1
8B	1591	2.09
10A	1610	2.08
10B	1614	2.09
11A	1424	2.1
11B	2102	2.09
13A	1864	2.08
13B	3271	2.02

There were two females and four males in the six subjects selected. Their baseline biochemical data and demographics are shown in Table 6A.2.

6A.3.2 Micro-array analysis of gene expression in normal rectal mucosa

Gene expression profiling was performed on the baseline samples of normal rectal mucosa from the six subjects using the Affymetrix Human genome U133A 2.0 gene chip. RMA (Irizarry, Bolstad et al. 2003) was used to calculate gene expression level.

6A.3.3 Identification of genes correlated with biochemical parameters

It was hypothesised that there was a linear relationship between serum PTH and gene expression, with gene expression dependent on the serum PTH level. A similar approach was taken for calcium and 25OH-Vitamin D. Using the data from the six baseline samples, Pearson's product-moment correlation coefficient r was calculated for each probe on the gene chip. Serum PTH levels were log2 transformed to normalise the data, as normally distributed data is a requirement for the calculation of r. The population distribution of PTH is important in this context and this has been studied, with the majority of studies indicating that PTH is positively skewed (Jorde, Saleh et al. 2005). The sample size for this study is too small to determine whether PTH is normally distributed, but inspection of distribution plots of PTH suggest a right skew and for both of these reasons the PTH values were log transformed, these values are shown in Table 6A.2. The values for calcium and 25OH-Vitamin D were not log transformed as at a population level these are normally distributed.

Table 6A.2 Demographics and baseline biochemical values for the six subjects studied in this chapter.

Subject	Age	Sex	PTH pmol/l	PTH pmol/l (log2)	25OH-Vitamin D ug/L	Calcium mmol/L
3	61	M	3.7	1.89	41.1	2.35
7	62	F	3.1	1.63	26.2	2.48
8	68	F	5.2	2.38	22.6	2.32
10	69	M	3.1	1.63	22.2	2.22
11	68	M	3.3	1.72	22.5	2.18
13	67	M	3.6	1.85	45.9	2.21

Initial analysis of the Pearson's correlation coefficient for all of the genes identified was carried out using MSExcel. This identified a large number of genes for which expression correlated with PTH. The majority of these genes were negatively correlated, with a smaller number showing positive correlation with PTH. Visualisation of x,y regression plots for some of this data indicated that a number of the genes showing correlation with PTH were expressed at very low levels, with a single outlying value resulting in significant correlation. Examination of the present/absent calls was carried out using Gene Chip Operating System (GCOS). The results of this are shown in table 6A.3, this demonstrates a binomial distribution, with around 2./3 of genes called as present in the majority of the samples and just under a 1/3 called as absent in the majority with a smaller number called as present in an intermediate number.

Table 6A.3 Relationship between number of subjects and the proportion of genes called as present using GCOS
The binomial distribution can be seen.

Number of samples in which gene expression was called as present	Number of genes
0	4010
1	440
2	231
3	274
4	295
5	889
6	7201

The low expressing genes showing correlation were not surprisingly called as absent. As the correlation for these genes may be spurious, it was decided to limit further analysis of correlation to genes called as present in at least two out of the six samples. This resulted in 8890 genes being studied, representing around 2/3 of the genes on the chip. Following initial explorative data-analysis, SAM (Significance Analysis of Micro-arrays) was used to identify those genes significantly correlated with PTH.

6A.3.4 Significance analysis of micro-arrays (SAM)

To identify genes correlated with serum PTH and determine their statistical significance, the SAM approach (Tusher, Tibshirani et al. 2001) was used. The RMA data was used with Pearson's correlation with each of the biochemical parameter as the response variable, with six hundred permutations. The output from SAM is the (d)-score which is the outcome of the gene specific t-test and the q-value which is the lowest false discovery rate (FDR) at which the gene is called significant. In this study a q-value of <1% was considered significant.

6A.3.5 Regression plots

Regression plots were constructed for genes of interest using MSExcel. Log2 transformed values for PTH and micro-array RMA data were plotted on the x and y axes respectively. Log2 transformed array data was used as that is the format used by SAM

6A.3.6 Functional annotation of PTH correlated genes using DAVID

The list of correlated genes was uploaded to DAVID (Database for Annotation, Visualization and Integrated Discovery (http://david.abcc.ncifcrf.gov/) (Dennis, Sherman et al. 2003; Huang da, Sherman et al. 2009). The background to and the methods employed by DAVID were described in section 4B.3.1. The enriched annotation terms were identified using two different utilities available on DAVID and for each analysis the background was set as Affymetrix HG U133A 2.0.

1. Functional annotation chart

   This identifies enriched terms within the gene list and performs a "term centric singular enrichment analysis". The default settings were deployed apart from using an EASE score of 0.01 (instead of 0.1) to identify enriched annotation terms.

2. Functional annotation clustering

   This clusters functionally similar enriched terms into groups, performing a "term centric modular enrichment analysis". Default settings were employed apart from stringency being increased to high. Clusters of terms with enrichment scores of >2, which is equivalent to non-log scale value of 0.01, were identified.

6A.4 Results

6A.4.1 Correlation of gene expression level with serum biochemical parameters

The expression of genes in the normal rectal mucosa from the six baseline samples was determined using the Affymetrix HG U133A 2.0 array. The SAM (significance analysis of micro-arrays) utility was used to identify statistically significant genes, for which expression correlates with the measured serum biochemical parameter; Parathyroid hormone (PTH), 25OH-Vitamin D and calcium.

6A.4.1.1 Parathyroid hormone (PTH)

Using a q-value of 1%, the expression of 2693 genes was negatively correlated with serum PTH levels. That is, at higher levels of PTH, expression of these genes was lower and at lower levels of PTH, expression of these genes was higher. There were no statistically significant genes for which expression correlated positively with PTH identified by this method. Initial analysis of all of the genes present on the chip identified some positively correlated genes. Limiting the analysis to the "expressed" genes (>2/6 present call) for the reasons discussed in the methods, meant some of these were removed and the others were not subsequently identified as statistically significant and are not further analysed here.

6A.4.1.2 25OH-Vitamin D

There were no genes for which expression correlated with serum levels of 25OH-vitamin D, with a q value<10%.

6A.4.1.3 Calcium

There was only one gene in which expression correlated with the serum levels of calcium with a q-value of less than 10%. This single gene was VIP (vasoactive intestinal peptide), which had a d-score of -6.4 and a q value of 0. Of note the analyses for calcium and 25OH-vitamin D were carried out on all the genes and the "present" genes with no difference in result and no genes were correlated with a q-value up to 10%.

This analysis demonstrates that there are a large number of genes for which expression level correlates with serum PTH level. This contrasts with the situation for calcium and 25OH-Vitamin D. As correlation does not equal causation further studies will be needed for it to be argued that serum PTH is a significant determinant of gene expression in the rectal mucosa. However, in analysing the set of genes showing negative correlation with PTH it is presumed that the relationship is functional and that the expression of these genes is in part regulated by PTH. Therefore identifying the function of this set of genes may provide evidence for the biological actions of PTH in the normal rectal mucosa.

6A.4.2 Functional annotation of the genes which showed correlation with serum PTH

The online utility DAVID was used to identify the GO biological annotation terms enriched in the set of PTH correlated genes. As described in the methods, two features of DAVID were used. First, enriched single GO annotation terms were identified. Secondly, from within these enriched single annotations terms, enriched clusters of functionally related GO annotation terms were identified.

6A.4.2.1 Enriched single GO annotation terms in the PTH correlated genes

Enriched single annotation terms for each of the three biological domains of the GO annotation (BP, MF and CC), were identified. There are sixty-four biological process terms, forty two cellular component and twenty-four molecular function annotation terms enriched in the PTH correlated genes. These are shown in Tables 6A.4, 6A.5 and 6A.6.

Table 6A.4 The 63 GO biological process annotation terms enriched in the 2693 genes negatively correlated with PTH
The p-value and false detection rate (FDR) for each term is shown.

Biological process (BP)	*P* value	FDR %
intracellular transport	6E-12	3E-08
protein transport	1E-11	4E-08
establishment of protein localization	2E-11	4E-08
protein catabolic process	1E-10	2E-07
protein localization	5E-10	5E-07
macromolecule catabolic process	1E-09	9E-07
cellular protein catabolic process	1E-09	9E-07
proteolysis involved in cellular protein catabolic process	1E-09	9E-07
vesicle-mediated transport	2E-09	1E-06
modification-dependent protein catabolic process	5E-09	2E-06
modification-dependent macromolecule catabolic process	5E-09	2E-06
cellular macromolecule catabolic process	1E-08	5E-06
cellular macromolecule localization	1E-08	5E-06
cellular protein localization	3E-08	1E-05
intracellular protein transport	4E-08	1E-05
small GTPase mediated signal transduction	1E-07	5E-05
ubiquitin-dependent protein catabolic process	1E-07	5E-05
regulation of apoptosis	6E-07	2E-04
regulation of cell death	6E-07	2E-04
regulation of programmed cell death	8E-07	2E-04
negative regulation of apoptosis	8E-07	2E-04
negative regulation of programmed cell death	1E-06	4E-04
negative regulation of cell death	2E-06	4E-04
cell death	3E-06	8E-04
death	5E-06	1E-03
macromolecular complex assembly	5E-06	1E-03

apoptosis	7E-06	1E-03
Golgi vesicle transport	8E-06	1E-03
programmed cell death	1E-05	2E-03
anti-apoptosis	1E-05	2E-03
intracellular signaling cascade	1E-05	2E-03
enzyme linked receptor protein signaling pathway	1E-05	2E-03
induction of apoptosis	2E-05	3E-03
induction of programmed cell death	2E-05	3E-03
macromolecular complex subunit organization	3E-05	3E-03
membrane organization	3E-05	4E-03
protein complex biogenesis	3E-05	4E-03
protein complex assembly	3E-05	4E-03
protein targeting	4E-05	5E-03
transmembrane receptor protein tyrosine kinase signaling pathway	5E-05	6E-03
nitrogen compound biosynthetic process	6E-05	7E-03
regulation of cell proliferation	9E-05	1E-02
G1/S transition of mitotic cell cycle	1E-04	1E-02
negative regulation of macromolecule metabolic process	1E-04	2E-02
positive regulation of protein kinase cascade	2E-04	2E-02
dephosphorylation	3E-04	3E-02
positive regulation of I-kappaB kinase/NF-kappaB cascade	3E-04	3E-02
positive regulation of signal transduction	3E-04	3E-02
chromatin remodeling	4E-04	4E-02
regulation of I-kappaB kinase/NF-kappaB cascade	4E-04	4E-02
positive regulation of cell death	4E-04	4E-02
cofactor biosynthetic process	4E-04	4E-02
protein amino acid dephosphorylation	4E-04	4E-02
phosphorus metabolic process	4E-04	4E-02
phosphate metabolic process	4E-04	4E-02
positive regulation of programmed cell death	5E-04	4E-02
induction of apoptosis by extracellular signals	5E-04	5E-02
negative regulation of gene expression	6E-04	5E-02
positive regulation of apoptosis	6E-04	5E-02
ER to Golgi vesicle-mediated transport	6E-04	5E-02
protein import	6E-04	5E-02
endosome transport	6E-04	5E-02
steroid hormone receptor signaling pathway	7E-04	5E-02
cellular macromolecular complex assembly	8E-04	6E-02
cellular response to insulin stimulus	9E-04	7E-02

Table 6A.5 The 42 GO cellular component annotation terms enriched in the 2693 genes negatively correlated with PTH.

The p-value and false detection rate (FDR) for each term is shown.

Cellular component (CC)	*P* value	FDR %
Golgi apparatus	3E-15	2E-12
endoplasmic reticulum	2E-13	8E-11
endomembrane system	6E-13	2E-10
nuclear lumen	2E-10	4E-08
membrane-enclosed lumen	5E-10	7E-08
organelle membrane	8E-10	1E-07
organelle lumen	9E-10	1E-07
intracellular organelle lumen	1E-09	1E-07
nucleoplasm	1E-08	8E-07
Golgi apparatus part	9E-08	6E-06
endosome	1E-07	8E-06
cytosol	1E-07	1E-05
cell fraction	2E-07	1E-05
vacuole	4E-07	2E-05
endoplasmic reticulum membrane	2E-06	6E-05
nuclear envelope-endoplasmic reticulum network	1E-06	7E-05
nucleoplasm part	2E-06	7E-05
vesicle	5E-06	1E-04
insoluble fraction	1E-05	5E-04
membrane fraction	2E-05	5E-04
cytoplasmic vesicle	4E-05	1E-03
Golgi membrane	7E-05	2E-03
nucleolus	7E-05	2E-03
lytic vacuole	7E-05	2E-03
lysosome	7E-05	2E-03
membrane-bounded vesicle	9E-05	2E-03
nuclear envelope	1E-04	2E-03
trans-Golgi network	1E-04	3E-03
endoplasmic reticulum part	2E-04	6E-03
cytoplasmic membrane-bounded vesicle	2E-04	6E-03
focal adhesion	3E-04	6E-03
vacuolar membrane	3E-04	7E-03
cell-substrate junction	3E-04	7E-03
adherens junction	4E-04	9E-03
apical part of cell	4E-04	9E-03
apical plasma membrane	5E-04	1E-02
histone acetyltransferase complex	6E-04	1E-02
cell-substrate adherens junction	6E-04	1E-02
anchoring junction	6E-04	1E-02
perinuclear region of cytoplasm	7E-04	1E-02
vacuolar part	9E-04	1E-02
protein serine/threonine phosphatase complex	1E-03	1E-02

Table 6A.6 The 24 GO molecular functions annotation terms enriched in the 2693 genes negatively correlated with PTH.
The p-value and false detection rate (FDR) for each term is shown.

Molecular Function (MF)	*P* value	FDR %
enzyme binding	2E-07	2-04
phosphoprotein phosphatase activity	1E-05	9E-03
GTPase activity	1E-05	7E-03
ubiquitin protein ligase binding	1E-05	6E-03
protein serine/threonine phosphatase activity	3E-05	1E-02
phosphatase activity	4E-05	1E-02
transcription factor binding	4E-05	9E-03
ligase activity, forming carbon-nitrogen bonds	4E-05	8E-03
GTP binding	6E-05	1E-02
SMAD binding	8E-05	1E-02
protein C-terminus binding	9E-05	1E-02
acid-amino acid ligase activity	1E-04	2E-02
protein dimerization activity	1E-04	2E-02
guanyl ribonucleotide binding	1E-04	2E-02
guanyl nucleotide binding	1E-04	2E-02
transcription coactivator activity	2E-04	3E-02
transcription cofactor activity	2E-04	3E-02
transcription activator activity	3E-04	3E-02
purine ribonucleotide binding	3E-04	3E-02
ribonucleotide binding	4E-04	3E-02
small conjugating protein ligase activity	4E-04	3E-02
purine nucleotide binding	4E-04	4E-02
signal sequence binding	4E-04	4E-02
transcription repressor activity	7E-04	5E-02

6A.4.2.2 Enriched clusters of functionally related GO annotation terms

Fifteen enriched clusters of functionally related annotation terms were identified using the criteria described in the methods. These fifteen enriched clusters are shown in Table 6A.7. The findings of particular note include: four clusters (5, 6, 7 and 14) of enriched annotation terms related to apoptosis and cell death. These include terms indicating the induction of apoptosis and both the positive and negative regulation of apoptosis, as well as the process itself. There are two clusters of enriched annotation terms (1 and 4) relating to the localisation of macromolecules and proteins; and another (10), indicating the

formation of these protein and macromolecule complexes. A cluster of annotation terms relating to focal adhesion and adherens junctions (15), was enriched. Apoptosis, localisation and cell-cell adhesion are features of differentiated crypt top cells (Kosinski, Li et al. 2007) suggesting that PTH may be a negative regulator of these biological activities and cellular components. Consistent with this is the finding that two single annotation terms “apical part of cell” and “apical plasma membrane” are also enriched (See Table 6A.5).

The biological significance of the other enriched clusters is less clear as they have a general role, though both cluster 9 and 11 may indicate the secretory function of mature enterocytes. There is also some overlap between the annotation terms enriched in the genes negatively correlated with PTH in this study and those up-regulated by PTH in-vitro, as discussed below.

Table 6A.7 The fifteen clusters of annotation terms enriched in the 2693 genes negatively correlated with PTH.
The enrichment score (ES) for each is cluster is shown.

Cluster	GO terms (Biological domain)	ES
1	protein transport (BP) establishment of protein localization (BP) protein localization (BP)	9.8
2	nuclear lumen (CC) membrane-enclosed lumen (CC) organelle lumen(CC) intracellular organelle lumen (CC)	8.2
3	protein catabolic process (BP) macromolecule catabolic process (BP) cellular protein catabolic process (BP) proteolysis involved in cellular protein catabolic process (BP) modification-dependent macromolecule catabolic process(BP) modification-dependent protein catabolic process (BP) cellular macromolecule catabolic process (BP)	7.7
4	cellular macromolecule localization (BP) cellular protein localization (BP) intracellular protein transport (BP)	6.4

5	regulation of apoptosis (BP) regulation of cell death (BP) regulation of programmed cell death (BP)	6.2
6	negative regulation of apoptosis (BP) negative regulation of programmed cell death(BP) negative regulation of cell death (BP)	5.8
7	cell death (BP) death (BP) apoptosis (BP) programmed cell death (BP)	5.2
8	endoplasmic reticulum membrane (CC) nuclear envelope-endoplasmic reticulum network (CC) endoplasmic reticulum part (CC)	5.1
9	vacuole (CC) lysosome (CC) lytic vacuole(CC)	4.9
10	macromolecular complex assembly (BP) macromolecular complex subunit organization (BP) protein complex biogenesis (BP) protein complex assembly (BP)	4.0
11	vesicle (CC) cytoplasmic vesicle (CC) membrane-bounded vesicle (CC) cytoplasmic membrane-bounded vesicle (CC)	3.9
12	phosphoprotein phosphatase activity (MF) dephosphorylation (BP) protein amino acid dephosphorylation (BP)	3.8
13	GTP binding (MF) guanyl nucleotide binding (MF) guanyl ribonucleotide binding (MF)	3.2
14	induction of apoptosis (BP) induction of programmed cell death (BP) positive regulation of cell death (BP) positive regulation of programmed cell death(BP) positive regulation of apoptosis (BP)	2.6
15	focal adhesion (CC) cell-substrate junction (CC) adherens junction (CC) cell-substrate adherens junction (CC)	2.2

6A.4.2.3 Comparison with functional annotation of genes differentially expressed in response to PTH in-vitro

Comparison with the functional annotation identified in the PTH regulated genes in-vitro identified a number of features in common (Compare Tables 4B.4, 4B.5 and 4B.6 with table 6A.7). These are clusters 13 and 4C, which includes GTP binding and GTPase activity; clusters 1 and 4A, which include protein localisation and establishment of protein localisation and clusters 5 and V, which include apoptosis and cell death.

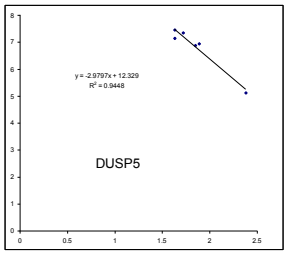
6A.4.3 Relationship of genes correlated with PTH to genes differentially expressed in response to PTH in-vitro

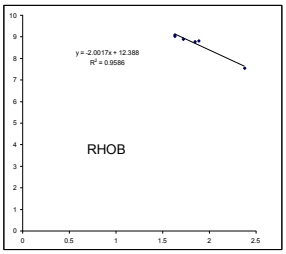
Having identified a group of genes negatively correlated with PTH in-vivo, it was hypothesised that there would be similarity with those genes differentially expressed in response to PTH in-vitro. Out of the one hundred and twenty-four genes differentially expressed in response to PTH in-vitro (at either four or twenty-four hours), forty eight were also negatively correlated with serum PTH. These forty-eight genes are shown in Table 6A.8.

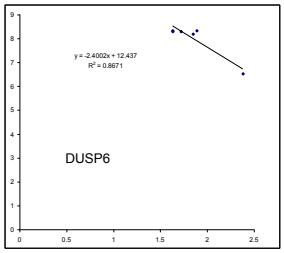
Regression plots for some of the genes that are negatively correlated and down regulated by PTH in-vitro are shown in Figure 6A.1. These plots graphically demonstrate the relationship between serum PTH and gene expression level.

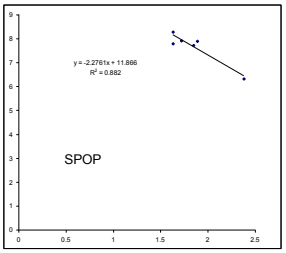
Figure 6A.1 Regression plots comparing gene expression and serum PTH

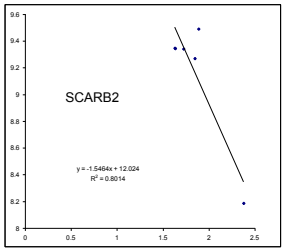
These genes are examples of genes that are negatively correlated with PTH in-vivo and also down-regulated by PTH in-vitro. X=PTH, Y=Expression level, both are log2 transformed values.

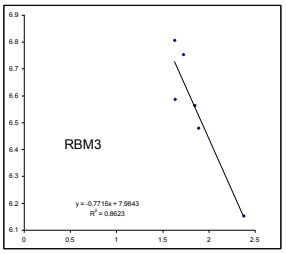


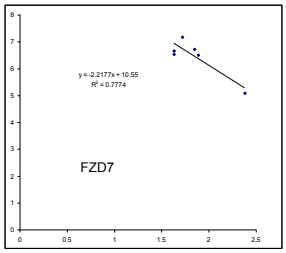












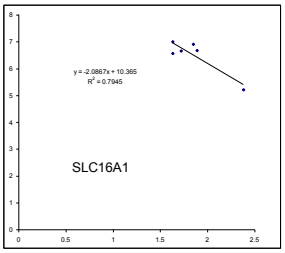


Table 6A.8 The 48 genes that negatively correlate with serum PTH and were down-regulated by PTH in-vitro.
The SAM output is shown along with the Pearson's correlation coefficient r. q=False detection rate and d value is the degree of difference from the expected variance.

Gene	SAM data		Pearson's correlation coefficient (r)
	d	q	
DUSP5	-3.8	0	-0.97
RHOB	-3.5	0	-0.98
DUSP6	-2.8	0	-0.97
SPOP	-2.5	0.1	-0.94
SCARB2	-2.5	0.1	-0.90
RBM3	-2.4	0.1	-0.95
KDM3B	-2.4	0.1	-0.91
FZD7	-2.2	0.3	-0.88
SLC16A1	-2.2	0.3	-0.74
ABCG2	-2.1	0.3	-0.83
AREG	-2.0	0.3	-0.88
PHLDA2	-2.0	0.3	-0.92
RALA	-2.0	0.3	-0.80
APLP2	-2.0	0.3	-0.93
ABCB1	-1.9	0.3	-0.80
PHLDA1	-1.9	0.3	-0.86
SGK1	-1.9	0.3	-0.78
PDCD2	-1.9	0.3	-0.93
RTN3	-1.8	0.3	-0.90
ROD1	-1.8	0.3	-0.84
PAFAH1B1	-1.7	0.3	-0.85
YTHDF2	-1.7	0.3	-0.88
CEACAM5	-1.7	0.3	-0.94
GSR	-1.7	0.5	-0.85
NLK	-1.7	0.5	-0.87
CTBP1	-1.6	0.5	-0.87
TMOD3	-1.6	0.5	-0.80
NUP98	-1.6	0.5	-0.89
GATM	-1.6	0.5	-0.87
SLC25A32	-1.5	0.5	-0.86
EFNB2	-1.5	0.6	-0.89
ATP1B1	-1.5	0.6	-0.86
RFC3	-1.5	0.6	-0.83
GJA1	-1.4	0.6	-0.76
LRRFIP1	-1.4	0.6	-0.74
FBLN1	-1.4	0.6	-0.78
RBBP4	-1.4	0.6	-0.87
MAP4K4	-1.3	0.6	-0.86

ACTB	-1.3	0.9	-0.79
CALM1	-1.3	0.9	-0.83
EREG	-1.3	0.9	-0.59
BRD2	-1.3	0.9	-0.89
ARHGAP5	-1.3	0.9	-0.80
PTPN11	-1.3	0.9	-0.82
RAB8A	-1.3	0.9	-0.97
SS18	-1.3	0.9	-0.85
ARL4C	-1.2	0.9	-0.77
BRD4	-1.2	0.9	-0.87

6A.4.4 Relationship of genes correlated with PTH to position in the colonic crypt

It was noted that there were a number of genetic markers expressed in differentiated colonic cells in the genes negatively correlated with PTH including; CDKN1A, GPA33, PRSS3, CEACAM1, BMP2 and EZR.

This suggests the possibility that PTH negatively regulates differentiation of colonic cells. The statistical significance was not assessed in this analsyses, but will be investigated in the next chapter.

6A.5 Discussion

In this study it was identified that some of the inter-individual variation in gene expression correlated with serum PTH level, with the expression of a large number of genes showing negative correlation with serum PTH levels. In contrast, no correlation was found between gene expression and serum 25OH-Vitamin D levels and only a single gene showed significant correlation with serum calcium. These findings would suggest that serum PTH level may be a significant determinant of gene expression in normal rectal mucosa, particularly in view of the large number of genes showing negative correlation with PTH. It is acknowledged that correlation does not equal causation and this will be addressed in further studies in this thesis. There are however, some interesting data provided in this chapter to suggest that the relationship between PTH and gene expression may be functional. Firstly, both the in-vitro and in-vivo studies of PTH are consistent; in both studies PTH down-regulated gene expression. The in-vivo study identified only genes showing negative correlation and in the in-vitro study the majority (>90%) of genes were down-regulated in response to PTH.

Secondly, there were a large number of genes showing correlation with PTH and these were enriched with interesting functional annotation terms. The use of SAM enabled the identification of genes which were statistically significantly correlated with serum PTH. This produced a large list of genes, which can be difficult to analyse, but all of these genes met the stringent 1% FDR cut off and it was felt appropriate to analyse all of these genes. In analysing such a list it is desirable to identify the biological processes enriched within the whole group rather than carry out singular functional

analysis of each gene in the list. DAVID is well suited to this, being able to carry out functional annotation of up to 3000 genes. A large number of functional annotation terms were highly statistically enriched in the group of genes negatively correlated with PTH. This large number of enriched annotation terms is more significant than would be expected were this a random set of genes. The annotation terms enriched in the genes negatively correlated with PTH include features consistent with mature colonic cells. Particularly, the regulation and process of apoptosis; and structural features such as adherens junctions and the apical parts of cell membranes were enriched. This suggests that PTH negatively regulates genes found in differentiated colonic crypt cells.

6A.5.1 Caveats and limitations

The decision to investigate only those genes identified as present in >2/6 samples seems reasonable, though the cut-off may be arbitrary. Not all genes are expressed at the mRNA level in every tissue with a number of tissue specific (including colonic) transcripts (Axelsen, Lotem et al. 2007).
In investigating the relationship between biochemical parameters and gene expression in the rectal mucosa, this study has a number of strengths. The individuals studied had a past history of adenomatous polyps and therefore had a pre-disposed rectal epithelium but had no current neoplasia. The patients had not received any intervention prior to this study and biopsies and serum samples were taken simultaneously, thus any direct influence of biochemical factors would be apparent. The main weakness is the small number of samples and the skewed distribution of the PTH values. The main negative finding was the absence of any relationship between serum 25OH-

Vitamin D and gene expression. It may be suggested that this is because of the small number of samples. However, it has been argued in this thesis that the relationship between the serum level of 25OH-Vitamin D and colorectal neoplasia seen in other studies is secondary, representing the relationship between PTH and colorectal neoplasia. Therefore the lack of correlation between gene expression and 25OH-Vitamin D, in contrast with the strong correlation with PTH is consistent with the hypothesis underlying this thesis. There may be an unrecognised alternative explanation for the observed difference between individuals unrelated but coincident with serum PTH levels and in aiming to improve the study a larger number of samples may have provided further clarity on this point.

6A.5.2 Conclusion

This study identified a large number of genes for which expression showed significant negative correlation with serum levels of PTH. These negatively correlated genes have interesting associated biology. There is the suggestion of a relationship between the genes correlated with PTH and those regulated by PTH in-vitro and genetic markers of colonic differentiation. Further evidence showing a relationship between recognised biological processes and the genes identified in this study would add support to the notion that these correlations are real. In the second part of this chapter, such a comparison will be made using gene set enrichment analysis (GSEA).

Chapter 6B
GENE SET ENRICHMENT ANALYSIS OF GENES CORRELATED WITH SERUM PTH IN NORMAL RECTAL MUCOSA

6B.1 Aim

1. Identify gene sets which are enriched in the genes whose expression correlates with PTH, using gene set enrichment analysis (GSEA).

6B.2 Introduction

In chapter 6A it was demonstrated that the expression of a large number of genes was negatively correlated with serum PTH level. This suggests that the variation between individuals in gene expression in the rectal mucosa is due at least in part to variation in serum PTH level. Functional annotation of these genes indicated a number of interesting biological features of the correlated genes. It is hypothesised that the genes correlated with PTH in-vivo would include the genes identified as PTH targets in Chapter 4A. To address this hypothesis further, an alternative approach was used that identified the sets of genes enriched in the genes correlated with PTH. This Gene Set Enrichment Analysis (GSEA) approach (Subramanian, Tamayo et al. 2005) additionally allows the study of other sets of genes that are hypothesised to be regulated by PTH in the colon. These include genes altered in colorectal adenomas and cancer compared to normal mucosa, markers of colonic proliferation and differentiation. It is also hypothesised that there will be overlap with the sets of genes regulated by PTH in other tissues including bone (Reppe, Stilgren et al. 2006), muscle and haematopoietic cells (Reppe, Stilgren et al. 2007). This approach may add further to the understanding of the biology of the genes for which expression correlates with

serum PTH and therefore the consequences of PTH signalling on the colorectal epithelium.

6B.3 Methods

6B.3.1 Analysis of micro-array data

A gene set approach was used to study the same samples analysed in chapter 6A.

6B.3.2 Gene set enrichment analysis (GSEA)

Gene set enrichment analysis is a computational method used to identify biological activity from the results of micro-array studies (Subramanian, Tamayo et al. 2005). In this method, the relationship between pre-defined gene sets and the biological classes under investigation are compared. Rather than identify a list of up or down differentially expressed genes, as was done in Chapter Four, the whole dataset is interrogated to identify any enriched gene sets. This is done by applying a ranking metric (eg fold change) to the micro-array data based on its class or correlation to phenotype. In addition to allowing a class comparison, eg comparing before and after a treatment, the GSEA programme allows the calculation of Pearson's correlation coefficient r and genes are ranked by their correlation to phenotype. In this study the correlation to serum PTH (log2) was used as the ranking metric and the genes were ranked by their correlation to serum PTH levels.

The rank position of genes from each pre-defined gene set within the ranked data is determined and an enrichment score is calculated by walking down the ranked list and increasing a rank-sum statistic when a gene is in the gene set and decreasing it when it is not. The enrichment score calculates the difference between the observed rankings and that which would be expected from a random distribution, this is normalised to the gene set size to

give a normalised enrichment score (NES). The NES is the principal statistical outcome used for determining the outcome of GSEA. The significance of the results is determined by reiterative permutation using either random phenotype permutations or gene set permutations and the calculation of a false detection rate (FDR). The real results that outperform the iterative permutations are considered significant. The method is more fully described in (Subramanian, Tamayo et al. 2005).

The advantages of GSEA over single gene techniques are its ability to identify biological meaning where the changes in individual genes are small (Mootha, Lindgren et al. 2003). Using GSEA, all genes in the dataset are analysed, not just those above a certain threshold. The use of pathways and gene sets allows more clear definition of biological processes than single genes and is more reproducible. The GSEA enrichment programme is available as a Java executable desktop application (Subramanian, Kuehn et al. 2007) and was used in this analysis.

The biological information derived from GSEA is highly dependent on the gene sets employed and their relationship to the biological processes being studied. A database of gene sets, (MSIGdB), has been established for use with GSEA and is available at http://www.broadinstitute.org/gsea/msigdb/. For this study the C2 curated gene sets (version 2.5) were used, this consists of a total of 1892 gene sets. The C2 gene sets consist of two types of gene sets; canonical pathways (CP) derived from online pathway databases (eg KEGG) and chemical and genetic perturbations (CGP) derived from publications in Pubmed. Additionally, GSEA allows the use of user generated gene sets, either constructed or experimentally determined by them. This

enables the user to investigate the relationship between specific biology relevant to the study and investigate specific hypotheses.

6B.3.2.1 User defined gene sets

Seventeen user defined gene sets were employed in the analysis. Twelve colorectal specific gene sets and five PTH related gene sets were developed to investigate specific biological questions. These are discussed below and shown in Table 6B.1. Three gene sets were created from the experimental data in chapter two; **caco2_pth4** and **caco2_24h** consist of the genes down-regulated in response to PTH at four and twenty four hours respectively; and **caco_2pthall** contains all the genes down-regulated at both time points. There were too few genes to create a list of genes up-regulated in response to PTH. The other additional gene sets were derived from published studies. There were four gene sets related to changes found in colorectal neoplasia, **adenoma_up** and **adenoma_down** consist of genes changed in adenomas compared to normal mucosa (Segditsas, Sieber et al. 2008); and **crc_down** and **crc_up** consist of genes changed in colorectal cancer compared to normal mucosa (Cardoso, Boer et al. 2007). Genes differentially expressed at the top and bottom of the colonic crypt, **crypt_base** and **crypt_top** have been identified using SAM analysis (Kosinski, Li et al. 2007). The genes showing a fold change >2 following transfection of a colorectal cell line with a dominant negative TCF4, **dn_tcf4_up** and **dn_tcf_dn,** representing the effects of abrogation of wnt signalling. (van de Wetering, Sancho et al. 2002). Colorectal cancer cells positive for CD133 have the features of cancer initiating stem cells and differentially express a number of genes **cd133_stemcells** (Ieta, Tanaka et al. 2008). The parathyroid hormone

related gene sets were from two studies of patients with primary hyperparathyroidism and gene expression was compared in three different tissues before and after parathyroidectomy. **pth_bone_dn** and **pth_bone_up** are from studies of bone, **pth_haem_dn** and **pth_haem_up** from studies of haematopoietic cells and **pth_muscle_up** from studies of muscle (Reppe, Stilgren et al. 2006; Reppe, Stilgren et al. 2007). There were too few genes down-regulated in muscle to provide a gene set and the **pth_bone_dn** gene set was limited to the top 500 down-regulated genes.

6B.3.2.2 Parameters used for GSEA

In this study GSEA was run with the following parameters. The expression datasets used were the RMA data from the six baseline samples. The genes were ranked by their correlation with serum PTH, log2 transformed PTH values were used for the reasons discussed in section 6A.3.3. The C2 curated set of genes from the MSigDB was used. This consists of a total of 1892 gene sets. Additionally, the seventeen user defined gene sets described above were used. The gene sets were filtered to exclude sets with >500 or <25 genes, this left a total of 1048 gene sets within this size range. The ranking metric used was Pearson. Gene set permutation was used and the number of permutations set at 1000, as recommended. A FDR of<0.05 was considered significant; however, usually the most highly ranked gene sets are the ones of most interest, even if those lower down the ranking have a FDR of <0.05.

Table 6B.1The user defined gene sets used in the gene set enrichment analysis. The gene set name, the number of genes in the set, the biology represented and the source of the gene set are shown.

Gene set name	Number of genes	Description of biology
caco2_pth4	77	Genes down-regulated in response to PTH after 4 hours treatment of Caco2
caco2_pth24	61	Genes down-regulated in response to PTH after 24 hours treatment of Caco2
caco2_pthall	134	Genes down-regulated in response to PTH treatment of Caco2 at both 4 and 24 hours
crypt_base	290	Genes identified as differentially expressed at the crypt base using SAM (Kosinski, Li et al. 2007)
crypt_top	406	Genes identified as differentially expressed at the crypt top using SAM (Kosinski, Li et al. 2007)
dn_tcf4_up	287	Genes up-regulated by dominant-negative TCF with fold change >2 in both replicates(van de Wetering, Sancho et al. 2002)
dn_tcf_dn	101	Genes down-regulated by dominant-negative TCF with fold change >2 in both replicates(van de Wetering, Sancho et al. 2002)
adenoma_up	194	Genes up-regulated in adenomas compared to normal mucosa (Segditsas, Sieber et al. 2008)
adenoma_down	474	Genes up-regulated in adenomas compared to normal mucosa (Segditsas, Sieber et al. 2008)
crc_down	60	Genes down-regulated in colorectal cancer compared to normal mucosa (Cardoso, Boer et al. 2007)
crc_up	68	Genes up-regulated in colorectal cancer compared to normal mucosa (Cardoso, Boer et al. 2007)
cd133_stemcells	39	A genetic markers of colonic stem cells identified by presence of CD133 (Ieta, Tanaka et al. 2008)
pth_bone_dn	488	Genes down-regulated by PTH in bone (Reppe, Stilgren et al. 2006)
pth_bone_up	500	Genes up-regulated by PTH in bone (Reppe, Stilgren et al. 2006)
pth_haem_dn	123	Genes down-regulated by PTH in haematopoietic cells (Reppe, Stilgren et al. 2007)
pth_haem_up	38	Genes up-regulated by PTH in haematopoietic cells (Reppe, Stilgren et al. 2007)
pth_muscle_up	168	Genes up-regulated by PTH in haematopoietic cells (Reppe, Stilgren et al. 2007)

6B.4Results

6B.4.1Gene set enrichment analysis

Gene set enrichment analysis was performed to identify gene sets enriched in the genes strongly correlated with PTH. The primary outcomes are the normalised enrichment score (NES) and the false detection rate (FDR). Gene sets with FDR of <0.05 were identified as significant.

6B.4.2 Enriched gene sets

There were 1049 gene sets meeting the size criteria. There were one hundred and four gene sets enriched in the negatively correlated phenotype and twenty-one enriched in the positively correlated phenotype with a FDR <0.05.

6B.4.2.1Gene sets enriched in negatively correlated with PTH phenotype

Gene sets enriched in the negatively correlated phenotype can be considered as though they were negatively correlated with PTH. The gene sets with the highest enrichment scores are most likely to represent the biology of the negatively correlated with PTH phenotype. The top twenty-five enriched gene sets are listed in Table 6B.2. These top twenty-five enriched gene sets can be divided into a number of groups, including three gene sets derived from studies of the colon. These three gene sets should be considered the most relevant findings as they are derived from the tissue on which this study was carried out. The most highly enriched gene set is **crypt_top** which contains genes up-regulated at the top of normal colonic crypts compared to the base of the crypt (Kosinski, Li et al. 2007). It has a significantly higher enrichment score than all of the gene sets with a score of 3.75, with the next highest score being 2.45. The **crypt_top** gene set contains genes involved in cell

adhesion, cell maturation, apoptosis and inhibition of cell proliferation, including antagonists of myc. These genes also encode proteins involved in the function of mature intestinal cells (Kosinski, Li et al. 2007). A gene set **crc_down** representing the genes most commonly down-regulated in colorectal cancer (Cardoso, Boer et al. 2007) was also prominently enriched. The other two enriched colonic gene sets are derived from in-vitro studies. Interestingly, the gene set containing all the genes down-regulated by PTH in caco2, **caco2_pthall** in chapter two was significantly enriched in the negatively correlated genes. Enrichment plots for these three gene sets are shown in Figure 6B.1.

The finding that these three gene sets (**crypt_top, caco2_pthall, crc_down**) are significantly enriched provides information on the function of the genes correlated with serum PTH in the colorectal epithelium. These findings suggest that PTH down-regulates the expression of genes found in differentiated colonic cells and genes commonly found to be down-regulated in colorectal cancer. There is also overlap between the genes regulated by PTH in-vitro and those correlated with PTH in-vivo. These three are the only gene sets in the top twenty-five enriched sets derived from studies of the colon and therefore are the most pertinent to the biological function of the PTH correlated genes. The additional information derived from the other gene sets is therefore only likely to be complementary.

The remaining twenty-two enriched gene sets are displayed in Table 6B.3. There are two canonical pathway (CP) and twenty chemical and genetic perturbations (CGP) gene sets. A number of the gene sets are from the same source and have overlapping gene sets. The biological relevance of

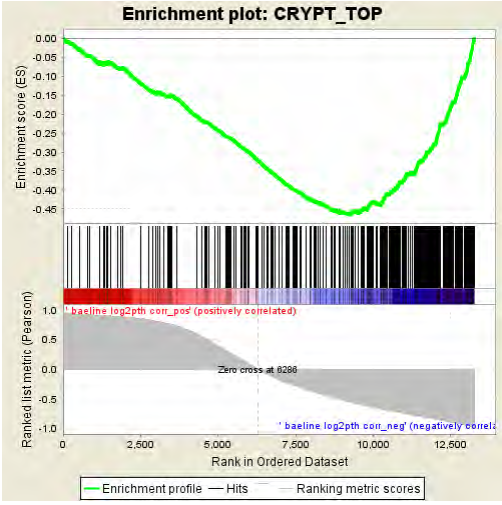
these gene sets is not as clear as for those derived from the colon. They however are likely to involve similar overlapping biological processes.

Table6B.2 The top twenty-five ranked gene sets in the negatively correlated with PTH phenotype.
They are ranked by NES. NES= normalised enrichment score. P-value=nominal p-value, not corrected for multiple testing. FDR=false detection rate.

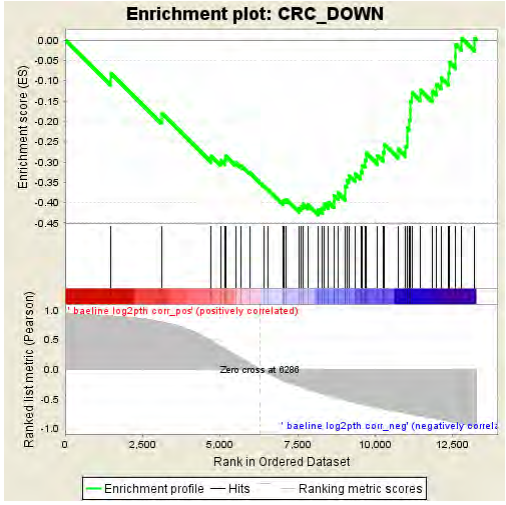
Gene set	NES	p-value	FDR
CRYPT_TOP	-3.75	0	0
AGUIRRE_PANCREAS_CHR12	-2.40	0	8.1E-04
HEARTFAILURE_ATRIA_DN	-2.45	0	0.001
FALT_BCLL_DN	-2.45	0	0.001
RNA_TRANSCRIPTION_REACTOME	-2.40	0	0.001
HSC_INTERMEDIATEPROGENITORS_SHARED	-2.39	0	0.001
CRC_DOWN	-2.38	0	0.002
FLECHNER_KIDNEY_TRANSPLANT_REJECTION_DN	-2.36	0	0.002
UVC_HIGH_ALL_DN	-2.35	0	0.001
FALT_BCLL_IG_MUTATED_VS_WT_DN	-2.35	0	0.002
HSC_LATEPROGENITORS_ADULT	-2.29	0	0.003
HCC_SURVIVAL_GOOD_VS_POOR_DN	-2.28	0	0.003
ROME_INSULIN_2F_UP	-2.27	0	0.003
BRCA1_OVEREXP_UP	-2.27	0	0.003
CACO2_PTHALL	-2.26	0	0.003
HSC_LATEPROGENITORS_SHARED	-2.25	0	0.003
HSC_INTERMEDIATEPROGENITORS_ADULT	-2.24	0	0.003
HSC_LATEPROGENITORS_FETAL	-2.22	0	0.003
GOLDRATH_HP	-2.23	0	0.003
CAMPTOTHECIN_PROBCELL_DN	-2.21	0	0.004
FLECHNER_KIDNEY_TRANSPLANT_WELL_UP	-2.21	0	0.004
NOUZOVA_CPG_H4_UP	-2.19	0	0.004
IDX_TSA_UP_CLUSTER5	-2.17	0	0.004
HSC_INTERMEDIATEPROGENITORS_FETAL	-2.17	0	0.005

Figure 6B.1 Enrichment plots for three colorectal gene sets that were enriched in the genes negatively correlated with PTH. The top portion of the plot shows the running ES for the gene set as the analysis walks down the ranked list. The score at the peak of the plot (the score furthest from 0.0) is the ES for the gene set. The middle portion indicates where each of the genes in the set occurs in the ranked list. The bottom portion of the plot shows the value of the ranking metric (pearson correlation factor r +1 to -1). The gene sets shown here are a) **crypt_top**, b) **crc_down** c) **caco2_pthall**.

a)



b)



c)

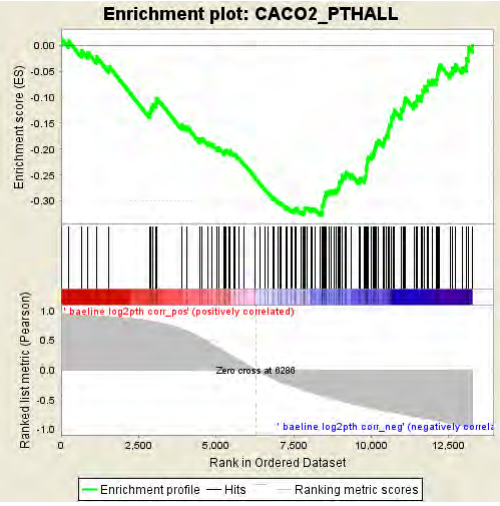

Enrichment plot: CACO2_PTHALL
Enrichment score (ES)
0.00
-0.05
-0.10
-0.15
-0.20
-0.25
-0.30
' baeline log2pth corr_pos' (positively correlated)
Zero cross at 6286
' baeline log2pth corr_neg' (negatively correl
Ranked list metric (Pearson)
1.0
0.5
0.0
-0.5
-1.0
0
2,500
5,000
7,500
10,000
12,500
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Table6B.3 The twenty two gene sets that were enriched in the negatively correlated with PTH phenotype. The four gene sets derived from colonic tissue are not listed (see text)**.** These gene sets are all from the MSigDb C2 collection; gene sets from the same source publication are listed together. The tissue and related disease state are described and where relevant the source publication. CP=canonical pathway, CGP=chemical and genetic perturbation.

Gene Set	Collection	Tissue origin	Involved biology and source publication
HSC_INTERMEDIATEPROGENITORS_SHARED HSC_INTERMEDIATEPROGENITORS_ADULT HSC_INTERMEDIATEPROGENITORS_FETAL HSC_INTERMEDIATEPROGENITORS_SHARED HSC_LATEPROGENITORS_ADULT HSC_LATEPROGENITORS_FETAL	CGP	Murine haematopoietic stem cells (HSC)	Identification of gene expression profiles of various stages of HSC development (Ivanova, Dimos et al. 2002)
FLECHNER_KIDNEY_TRANSPLANT_WELL_UP FLECHNER_KIDNEY_TRANSPLANT_REJECTION_DN	CGP	Human kidney transplant	Genes differentially expressed between well functioning and rejected kidneys (Flechner, Kurian et al. 2004)
FALT_BCLL_DN FALT_BCLL_IG_MUTATED_VS_WT_DN	CGP	Human B-cell lymphoma	Genes down-regulated in BCLL and in BCLL with a mutation in IGVH (Falt, Merup et al. 2005)
AGUIRRE_PANCREAS_CHR12	CGP	Human pancreatic adenocarcinoma	Genes from chromosome 12 amplified in pancreatic cancer(Aguirre, Brennan et al. 2004)
HEARTFAILURE_ATRIA_DN	CGP	Explanted failing human hearts	Genes down-regulated in failing human atria (Kaab, Barth et al. 2004)
RNA_TRANSCRIPTION_REACTOME	CP	Human	Genes involved in transcription
UVC_HIGH_ALL_DN	CGP	Human fibroblasts	Gene expression changes due to exposure UVC radiation, increased expression of cell cycle arrest and apoptosis associated genes was the

			main finding(Gentile, Latonen et al. 2003).
HCC_SURVIVAL_GOOD_VS_POOR_DN	CP	Human hepato-cellular carcinoma	Genes highly expressed in hepatocellular carcinoma with good survival
ROME_INSULIN_2F_UP	CGP	Human skeletal muscle	Genes up-regulated after 2 hours in response to insulin (Rome, Clement et al. 2003)
BRCA1_OVEREXP_UP	CGP	BRCA1 negative human breast cancer cell line	Genes up-regulated in response to expression of BRCA1 (Welcsh, Lee et al. 2002)
GOLDRATH_HP	CGP	CD8+ T-cells	Genes down-regulated in proliferating T-cells in response to lymphopenia(Goldrath, Luckey et al. 2004)
CAMPTOTHECIN_PROBCELL_DN	CGP	Human Pro-B cells	Genes down-regulated in response to the chemotherapy agent camptothecin (Brachat, Pierrat et al. 2002)
NOUZOVA_CPG_H4_UP	CGP	Leukaemia cell line NB4	Genes expressed in response to treatment with retinoic acid. (Nouzova, Holtan et al. 2004)
IDX_TSA_UP_CLUSTER5	CGP	Human fibroblasts	Genes up-regulated in fibroblasts stimulated to differentiate into adipocytes (Burton, Nagarajan et al. 2004)

6B.4.2.2 Gene sets enriched in positively correlated with PTH phenotype

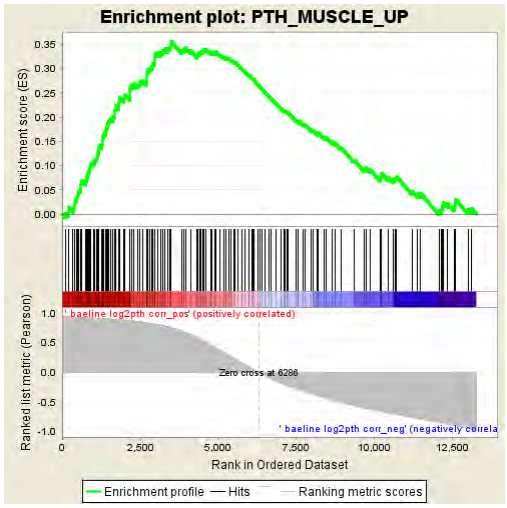
The twenty one gene sets enriched in the positively correlated with phenotype are show in Table6B.4. This is considerably fewer gene sets than was found enriched in the negatively correlated with PTH phenotype. Of these gene sets none are derived from colonic tissue, though two of them (**pth_bone_up** and **pth_muscle_up**) are the transcriptional targets of PTH in bone and muscle. Enrichment plots for **pth_bone_up** and **pth_muscle_up** are shown in Figure 6B.2. The remaining eighteen enriched gene sets are shown in Table 6B.4 with a description of their biology.

Table 6B.4 The twenty one gene sets enriched in the positively correlated with serum PTH phenotype, with a FDR of <0.05.
NES= normalised enrichment score. FDR=false detection rate.

Gene set	NES	p-value	FDR
HSA04080_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	+2.62	0	0
GPCRDB_CLASS_A_RHODOPSIN_LIKE	+2.42	0	2.7E-04
REFRACTORY_GASTRIC_UP	+2.34	0	0.002
PTH_MUSCLE_UP	+2.28	0	0.002
HSA04940_TYPE_I_DIABETES_MELLITUS	+2.23	0	0.004
HUMAN_TISSUE_LIVER	+2.24	0	0.004
GPCRS_CLASS_A_RHODOPSIN_LIKE	+2.16	0	0.006
MYOD_BRG1_UP	+2.08	0	0.008
MONOAMINE_GPCRS	+2.09	0.002	0.008
PEPTIDE_GPCRS	+2.11	0	0.008
MOREAUX_TACI_HI_VS_LOW_UP	+2.1	0	0.008
HSA04020_CALCIUM_SIGNALING_PATHWAY	+2.11	0	0.009
STRIATED_MUSCLE_CONTRACTION	+2.06	0	0.009
MOREAUX_TACI_HI_IN_BMPC	+2.01	0	0.01
KANG_TERT_DN	+1.99	0	0.01
TESTIS_EXPRESSED_GENES	+1.99	0	0.02
IGF_VS_PDGF_UP	+1.92	0	0.02
PTH_BONE_UP	+1.91	0	0.02
SHIPP_DLBCL_CURED_UP	+1.86	0.002	0.05
BCNU_GLIOMA_MGMT_48HRS_DN	+1.82	0	0.05

Figure 6B.2 Enrichment plots for the two PTH related gene sets that were enriched in the genes positively correlated with PTH. The top portion of the plot shows the running ES for the gene set as the analysis walks down the ranked list. The score at the peak of the plot (the score furthest from 0) is the ES for the gene set. The middle portion indicates where each of the genes in the set occurs in the ranked list. The bottom portion of the plot shows the value of the ranking metric (pearson correlation factor r +1 to -1). a) **pth_muscle** _up b) **pth_bone_down**

a)



b)

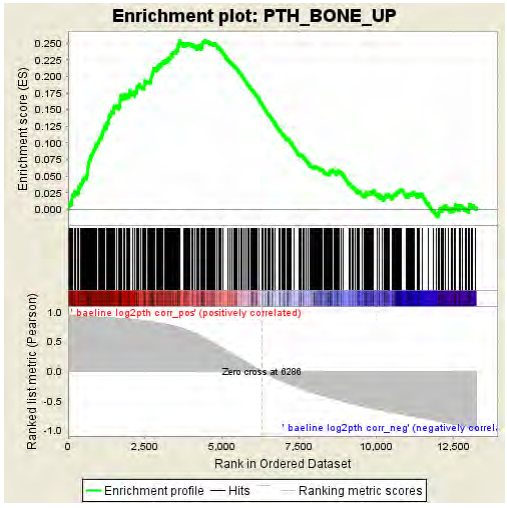


Table6B.5 Nineteen gene sets that were enriched in the positively correlated with PTH phenotype not discussed in the text
These gene sets are all from the MSigDb C2 collection; gene sets from the same source publication are listed together. The tissue and related disease state are described and where relevant the source publication. CP=canonical pathway, CGP=chemical and genetic perturbation.

Gene Set	Collection	Tissue origin	Involved biology and source publication
HSA04080_NEUROACTIVE_LIGAND_RECEPTOR _INTERACTION GPCRDB_CLASS_A_RHODOPSIN_LIKE GPCRS_CLASS_A_RHODOPSIN_LIKE MONOAMINE_GPCRS PEPTIDE_GPCRS	CP	Human	G-protein coupled receptors
MOREAUX_TACI_HI_VS_LOW_UP MOREAUX_TACI_HI_IN_BMPC	CGP	Human multiple myeloma cells (MMC)	Gene expression profiles comparing MMC's with low expression of TACI (Moreaux, Cremer et al. 2005)
REFRACTORY_GASTRIC_UP	CGP	Human gastric cancer	Genes enriched in gastric cancers resistant to treatment with 5FU(Kim, Choi et al. 2004)
HSA04940_TYPE_I_DIABETES_MELLITUS	CP	Human	Genes involved in type I diabetes mellitus
HUMAN_TISSUE_LIVER	CGP	Human and murine liver	The transcriptome of mammalian liver (Su, Cooke et al. 2002)
MYOD_BRG1_UP	CGP	Human fibroblasts	Genes up-regulated in fibroblasts in response to expression of Myo-D (de la Serna, Ohkawa et al. 2005)
HSA04020_CALCIUM_SIGNALING_PATHWAY	CP	Human	Genes involved in calcium signaling pathway
STRIATED_MUSCLE_CONTRACTION	CP	Human muscle	Genes involved in striated muscle contraction
KANG_TERT_DN	CGP	Human stroma	Genes down-regulated in response to

			expression of the catalytic subunit of telomerase in adipose derived stromal cells (Kang, Putnam et al. 2004)
TESTIS_EXPRESSED_GENES	CP	Human testis	Testis related genes curated from the GNF normal tissue compendium
IGF_VS_PDGF_UP	CGP	Human myoblasts	Genes differentially expressed between IGF versus PDGF treated myoblasts (Kuninger, Kuzmickas et al. 2004)
SHIPP_DLBCL_CURED_UP	CGP	Human B-cell lymphoma	Genes highly expressed in B-cell lymphoma that was cured rather than refractory (Shipp, Ross et al. 2002)
BCNU_GLIOMA_MGMT_48HRS_DN	CGP	Human glioma cell line expressing MGMT	Genes down-regulated following treatment with alkylating chemotherapy agent BCNU (Bandres, Andion et al. 2005)

6B.4.3 Outcome for the user defined gene sets

The twelve user defined gene sets selected specifically for use in this study are of particular interest due to their tissue and disease process specific nature, so their outcomes are described here. Of theses twelve gene sets, three of them (**crypt_top**, **crc_down** and **caco2_pthall**) were enriched in the top twenty-five PTH negatively correlated gene sets, as described above. A further two gene sets (**caco2_pth24** and **caco2_pth4**) were enriched in the negatively correlated with PTH phenotype with a FDR of <0.05. This is unsurprising as these two gene sets together make up the **caco2_pthall** gene set. The relevance of these findings has been discussed above. Another gene set of interest (**dn_tcf_up**) showed a low level enrichment in the negatively correlated with PTH phenotype, with a FDR of 0.15. This gene set consists of the genes up-regulated in response to expression of a dominant-negative TCF4 in the Lovo colorectal cancer cell line and represents the genes negatively regulated by the wnt signalling pathway. There is overlap between the genes found in the **crypt_top** and **dn_tcf_up** sets (Kosinski, Li et al. 2007). Two gene sets **dn_tcf_dn** and **cd133_stemcells** were enriched in the positively correlated with PTH phenotype, both showed low level enrichment with a FDR of >0.05.

The other four gene sets (**adenoma_up**, **adenoma_down**, **crc_up** and **crypt_base**) showed very low level enrichment with high FDR, suggesting that there is no relationship between these gene sets and either negative or positive correlation with PTH phenotype

6B.5 Discussion

The findings of this gene set enrichment analysis provide information on the biological function of the genes for which expression correlated with serum PTH. This second analytical approach was taken to provide consistent evidence in view of the difficulties of interpreting micro-array studies. Both the single gene and gene set approach are considered valid in trying to identify biological meaning from micro-array studies (Allison, Cui et al. 2006). The use of GSEA may also identify relevant biological processes where the relationship is weaker and takes account of all the genes in the dataset; in contrast to the single gene approach, which only analyses those identified as significant. This was not particularly relevant for the negatively correlated genes in this study, as there were a large number of highly significantly genes identified, but may be of interest for the positively correlated genes. More useful in this study, was that GSEA also allows the investigation of specific hypotheses by the use of user generated gene sets.

Both of the approaches used provide evidence of a functional role for the PTH correlated genes, with overlap of the findings from the two methods. This is despite small differences in the methods used to identify genes correlated with PTH. In particular SAM uses log2 transformed micro-array data and GSEA uses natural data, but the RMA data was used for both. For GSEA, all of the dataset was used; in contrast in the single gene SAM approach only those genes identified as present in >2/6 samples were used. Both analytical approaches were consistent in demonstrating that the majority of the interesting biological features were present in the genes negatively correlated with PTH, with relatively few gene sets enriched in the genes

positively correlated with PTH. This again suggests that PTH acts as a transcriptional repressor.

6B.5.1 Colorectal related gene sets

Of the two methods of analysis, GSEA perhaps provided the clearest description of the biology associated with the genes correlated with PTH. In the GSEA, the set of genes differentially expressed at the top of the colonic crypt (**crypt_top**) was highly significantly enriched in the genes negatively correlated with PTH. The enriched functional annotation terms found in the genes identified by the SAM analysis were also consistent with this finding as they contain a number of biological features of differentiated crypt top cells; particularly apoptosis and structural features such as adherens junction and the apical of cell membranes. The genes in **crypt_top** are found in differentiated post-mitotic cells at the top of the colonic crypt. This suggests that PTH down-regulates the genes found in differentiated colonic cells and therefore inhibits differentiation. Differentiation of colonic epithelial cells occurs as a consequence of decreased Wnt pathway signalling with evidence for this from both in-vitro (Mariadason, Bordonaro et al. 2001) and in-vivo studies (Sansom, Reed et al. 2004). There is also overlap between the genes found in differentiated colonic cells and those negatively regulated by wnt signalling (Kosinski, Li et al. 2007). These findings suggest that PTH may stimulate the wnt signalling pathway to alter gene expression in rectal mucosa. Parathyroid hormone has been shown to stimulate the wnt signalling pathway (Kulkarni, Halladay et al. 2005). Recently it has been shown that the PTH receptor, PTHR1 interacts with the wnt effector molecule dishevelled and

that PTH activates β-catenin, resulting in nuclear localisation independent of a wnt signal (Romero, Sneddon et al. 2010). Consistent with this, the gene set representing genes negatively regulated by Wnt signalling (**dn_tcf4_up**) is also weakly enriched (NES -1.47) in the negatively correlated with PTH phenotype. Less clearly relevant, is that the gene set representing genes positively regulated by wnt signalling (**dn_tcf_dn**) is found in the positively correlated with PTH phenotype, but its enrichment is very weak and non-significant. The observation that they are enriched in opposite directions along with the findings for the **crypt_top** gene set suggests that PTH modulates the Wnt signalling pathway in the rectal mucosa; this effect would appear to be predominantly to influence the expression of genes negatively regulated by wnt signalling.

A role in colorectal tumorigenesis is suggested by the observation that the **crc_down** gene set was enriched in the negatively correlated with PTH phenotype. This suggests that PTH negatively regulates the expression of genes commonly down regulated in colorectal cancer. The converse was not true, with no effect on genes commonly up-regulated in colorectal cancer. It is perhaps surprising that there was no relationship with genes altered in adenomas. Gene expression changes in adenomas represent the early events in the development of colorectal neoplasia and may therefore be expected to be those seen in normal mucosa. The reason for this is not clear, but may relate to the gene sets studied. The **crc_down** gene set was derived from a meta-analysis of other studies, with only genes consistently changed in three or more studies were included. It also contained relatively few genes compared to those found in the **adenoma_up** and **adenoma_down** gene

sets which were derived from only one study. A role for PTH in the development of colorectal neoplasia would suggest an effect on colonic stem cells as these have been shown to be the cells of origin of colorectal cancer (Barker, Ridgway et al. 2009). Parathyroid hormone is known to regulate the haematopoietic stem cell niche (Calvi, Adams et al. 2003) and has been shown to mobilise bone-marrow stem cells (Ballen, Shpall et al. 2007). Parathyroid hormone is not known to regulate colonic stem cells, though wnt signalling is (Korinek, Barker et al. 1998), which suggests that PTH could influence stem cells via modulation of Wnt signalling. The **cd133_stemcells** gene set is a set of genes found in colonic stem cells (Ieta, Tanaka et al. 2008) and is weakly enriched in the positively correlated with PTH phenotype. That it is only weakly enriched may indicate the absence of an effect or may be due to the fact that stem cells form only a small part of the crypt with only minor variance in stem cell related gene expression between individuals. To determine whether PTH influences colonic stem cells would require other studies.

6B.5.2 Parathyroid hormone regulated gene sets

The observation that the set of genes down-regulated by PTH in-vitro (**caco2_pth_all**) is also enriched in the genes negatively correlated with serum PTH is consistent with the proposed hypothesis. Overlap between the same biological processes in independent micro-array experiments is unusual (Fortunel, Otu et al. 2003). Particularly as; though caco2 is used as a model of the normal mucosa, it remains a malignant cell line with the features of such. The experiments on Caco2 also employed a different micro-array with

fewer genes represented, thus limiting the potential observable overlap. It is also interesting that there is overlap between the gene sets of PTH stimulated genes from bone (**pth_bone_up**) and muscle (**pth_muscle_up**) in the genes positively correlated with PTH. More intriguing, is the observation that a number of the gene sets enriched in the negatively correlated with PTH phenotype shown in Table 6B.2 represent biological processes in which PTH is implicated. For instance, it is known that PTH increases the proliferation rate and size of the haematopoietic stem cell pool (Whitfield 2005). PTH also inhibits adipocyte differentiation (Rickard, Wang et al. 2006). High serum levels of PTH are a predictor of graft failure after renal transplantation (Roodnat, van Gurp et al. 2006) and are high in patients with heart failure (Alsafwah, Laguardia et al. 2008). Whether the relationship between these findings is related to serum PTH is not clear. However similar transcriptional targets of PTH in different tissues could explain these observations as PTH acts in multiple tissue types by stimulating the same receptor PTHR1. This activates the same signalling pathways with some recognised generic downstream targets (Qiu, Qin et al. 2003). So, although there may be tissue specific features, overlapping transcriptional targets between tissues can be expected. This provides further support for the notion that the genes correlated with PTH represent real PTH targets and supports the proposed hypothesis that PTH acts directly on the colorectal epithelium to alter gene expression.

The information derived from the user defined gene sets, particularly those derived from studies of the colon, provides greater understanding of the biological processes of the enriched genes. The biological relevance of the

other gene sets is less clear. However, they are likely to represent similar overlapping biological processes as found in the colon (eg apoptosis).

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6B.5.3 Conclusion

This study identified a large number of gene sets which were enriched in the genes significantly negatively correlated with serum levels of PTH. These negatively correlated gene sets have interesting biology, consisting of; genes found in differentiated crypt top cells which are negatively regulated by wnt signalling, genes down-regulated in colorectal cancer and genes down-regulated by PTH in-vitro. Acknowledging the limitations of the data, it seems possible to infer that this relationship is functional and that serum PTH level determines gene expression in normal rectal mucosa.

However, it is important to acknowledge that correlation alone does not equal causation. It is predicted that modulating serum PTH would result in changes in the same genes, gene sets and biological functions as are correlated with PTH. Therefore the next step is to identify the genes differentially expressed in response to modulating serum PTH by dietary calcium supplementation.

Chapter 7A

IDENTIFICATION OF GENES DIFFERENTIALLY EXPRESSED IN NORMAL RECTAL MUCOSA IN RESPONSE TO MODULATING SERUM PTH BY DIETARY CALCIUM SUPPLEMENTATION

7A.1 Primary aim

1) Identify genes differentially expressed in the rectal mucosa of individuals taking dietary calcium supplementation using oligo-nucleotide micro-arrays.

7A.1.1 Secondary aims

1) Functional annotation of the genes differentially expressed in response to calcium supplementation
2) Determine the relationship between the differentially expressed genes and:

a) Genes altered in colorectal neoplasia.

b) Genes expressed in differentiated cells from the top of the colonic crypt

c) Genes expressed in proliferating cells from the base of the colonic crypt

d) Genes differentially expressed in response to PTH stimulation of Caco2

7A.2 Introduction

In previous chapters it has been demonstrated that parathyroid hormone influences gene expression in the colonic epithelium both in-vitro and in-vivo. In Chapter Five, dietary calcium supplementation was shown to significantly suppress serum PTH levels. Dietary calcium supplementation has been

shown to reduce the incidence of colorectal neoplasia in randomised controlled trials (Weingarten, Zalmanovici et al. 2008) but its mechanism of action is unknown. The hypothesis behind this study is that the effect of calcium on normal rectal epithelium is mediated by suppression of PTH. The aim of the experiments described in this chapter is to identify genes differentially expressed before and after six weeks of calcium supplementation using oligo-nucleotide micro-arrays. A similar study was carried out to determine the effect of the COX-2 inhibitor Celecoxib on gene expression in the colorectal epithelium, with significant changes in gene expression found (Glebov, Rodriguez et al. 2006).

It is hypothesised that calcium will result in changes in gene expression similar to those seen in response to PTH in chapter four; and that these changes will be consistent with its known anti-neoplastic action.

7A.3 Methods

7A.3.1 Subjects

The same six subjects were studied in this chapter as in chapter six and their demographics are shown in Table 6A.1. The study protocol was described in section 5.3.

7A.3.2 Micro-array analysis of gene expression in normal rectal mucosa

Gene expression profiling was performed on the baseline and calcium treated normal rectal mucosa samples from the six subjects studied in the previous chapter, using the Affymetrix Human genome U133A 2.0 gene chip.
Genes that were differentially expressed following dietary calcium supplementation were identified. These genes were then analysed by a descriptive analysis, performing functional annotation and comparing them to known biological processes.

7A.3.3 Identification of differentially expressed genes

To identify differentially expressed genes two approaches were taken.

7A.3.3.1 GCOS>3

For this approach, the GCOS (Gene chip operating software, Affymetrix ®) was used; the method is described in section 2.6.2. A gene was recognised as differentially expressed if it was similarly changed in three or more of the six subjects.

7A.3.3.1 Rank product (RP)

The rank products method was described in section 2.6.1. In this study it was used with a FDR of 10% and matched pair wise comparisons. This approach provides a measure of statistical significance for the differentially expressed genes, unlike GCOS>3.

7A.3.4 Functional annotation of differentially expressed genes using DAVID

The background to this approach and the methods employed by DAVID were described in section 4B.3.1. The enriched annotation terms were identified using two different utilities available on DAVID.

7A.3.4.1 Functional annotation chart

This identifies enriched terms within the gene list and performs a “term centric singular enrichment analysis”. The default settings were deployed apart from using an EASE score of 0.05 (instead of 0.1) to identify enriched annotation terms.

7A.3.4.2 Functional annotation clustering

This clusters functionally similar enriched terms into groups, performing a “term centric modular enrichment analysis”. Default settings were employed apart from stringency being increased to high. Clusters of terms with enrichment scores of >1.3, which is equivalent to non-log scale value of 0.05, were identified. For each analysis the background was set as Affymetrix HGU-133A 2.0.

7A.4 Results

7A.4.1 Identification of genes differentially expressed in response to calcium supplementation

To identify genes that were differentially expressed in response to calcium, comparison was made between the rectal mucosa samples taken at baseline and those taken after six weeks of calcium supplementation. The results of the two methods used (GCOS>3 and RP) are described separately and then the genes identified as differentially expressed by both methods are reported.

7A.4.1.1 Differentially expressed genes identified by GCOS>3

Two hundred and thirty three (233) genes were changed in half or more (3-6/6) of the subjects. There were a greater number of genes that were up-regulated in response to calcium supplementation, with 156 genes increased and 77 genes having decreased expression. The 156 genes in which expression was increased are shown in Appendix 1 and the 77 genes showing decreased expression are shown in Appendix 2. Out of the 13,480 genes on the chip, 3479 showed a change in expression in at least one pair wise comparison. For the remaining 10,001 genes no change in expression was seen in any of the six pair wise comparisons. For the majority of the 3479 changed genes, this change occurred in only one of the six subjects with 2262 (65%) genes showing altered expression in only one subject. The number of genes showing significant changes in expression in 2/6 subjects is 635, in 3/6 is 199, in 4/6 is 26, in 5/6 is 5 and in 6/6 is 2. Overall, and at each level of change more genes were up-regulated than down regulated, as is shown in Table 7A.1.

Table 7A.1 The frequency with which genes were identified as changed using GCOS.
A threshold of being changed in 3 or more subjects was used to identify a gene as being differentially expressed. There are 156 genes with increased expression and seventy seven genes with decreased expression in >3 subjects.

Number of subjects showing change in gene expression	Number of genes with increased expression	Number of genes with decreased expression
6	2	0
5	5	0
4	19	7
3	130	70
2	373	262
1	1285	977

7A.4.1.2 Differentially expressed genes identified by Rank Product (RP)

Using the rank product method there were seventy six genes differentially expressed in response to calcium supplementation. As with the GCOS >3 method there were more genes up-regulated in response to calcium, with 68 genes up-regulated and 8 genes down regulated. The up-regulated genes are shown in Appendix 3 and the down-regulated genes in Appendix 4.

7A.4.1.3 Genes found to be differentially expressed by both methods

One hundred and seventy nine genes were identified as differentially up-regulated in response to calcium by either GCOS>3 or RP, of these forty-five genes were identified as differentially up-regulate by both methods. Therefore, there were one hundred and ten genes identified as up-regulated by GCOS>3 only and twenty three genes identified as up-regulated by RP only. There were six genes that were identified as differentially down-regulated by both methods. Therefore, there were seventy one genes identified as down-regulated by GCOS>3 only and two genes identified as down-regulated by RP only.

7A.4.1.4 Fold change

The magnitude of the fold change in expression level for each of the differentially expressed genes is shown in Appendices 1-4. It is of note that for some genes there are differences between the GCOS and RP values due to the different pre-processing methods used (GCOS vs RMA). There is however no major differences in the values.

GCOS>3

The majority of differentially expressed genes showed an increase in expression of less than +1.5 fold, with only fifteen genes increased greater than this. The maximum increase in expression is +2.1 fold. Only two genes were decreased in expression > -1.5 fold and the maximum decrease in expression is -1.6 fold.

Rank product

Similar results were seen using rank-product with twenty five genes showing an increase in expression > +1.5 and only one gene showing a decrease in expression > -1.5 fold. The maximum increase in expression is +2.1 fold and the maximum decrease is -1.7 fold.

7A.4.2 Determination of the biological correlates of the genes differentially expressed in response to calcium

The biological function of the genes differentially expressed in response to calcium supplementation genes was assessed in five ways;

1. Investigation of the relationship between the differentially expressed genes and markers of specific cell types found in normal colonic mucosa.
2. Functional annotation of the differentially expressed genes using DAVID and the gene ontology (GO).

3. Investigation of whether any of the differentially expressed genes are also differentially expressed in colon neoplasia.

4. Investigation of the changes in expression in response to calcium of the genes differentially expressed in caco2 response to PTH in-vitro.

7A.4.3 The effect of calcium supplementation on the expression of genetic markers of specific cell types

The colonic epithelium is made up of three cell types; colonocytes (absorptive cells), entero-endocrine cells and mucus secreting goblet cells (Crosnier, Stamataki et al. 2006). These three cell types are derived from multi-potent stem cells situated at the crypt base. The epithelium is supported by stroma with peri-cryptal myofibroblasts forming part of the stem cell niche (Humphries and Wright 2008). There are a number of genetic markers specific to each of these cell types. The effect of calcium on the expression of marker genes for each of these cell types was studied.

7A.4.3.1 Colonocytes (Absorptive cells)

It was observed that a number of the genes up-regulated in response to calcium were markers of differentiated colonocytes, including; CEACAM1, HPGD, ABCG2, EZR, MXD1, TMPRSS2 and BCL10. Comparison was made between the genes differentially expressed in response to calcium with those differentially expressed in differentiated cells at the top of the colonic crypt (Kosinski, Li et al. 2007). Of the genes identified as up-regulated by calcium using GCOS>3; 68/156 (44%) are differentially expressed in differentiated crypt top cells. The number for the RP analysis was 38/68 (55%). This suggests that calcium results in the increased expression of genes found in differentiated crypt cells.

7A.4.3.2 Entero-endocrine cells

Calcium resulted in the down-regulation (GCOS>3) of a number of markers of intestinal enteroendocrine cells. These down regulated genes are Peptide YY (PYY), Glucagon (GCG), Somatostatin (SST), Chromogranin A (CHGA), Chromogranin B (CHGA), Tryptophan hydroxylase (TPH1), Secretogranin 5 (SCG5) and insulinoma-associated 1 (INSM1) (Bezencon, Furholz et al. 2008).

7A.4.3.3 Goblet Cells

There was no clear direction of effect of calcium on goblet cell markers. Calcium supplementation resulted in the down regulation of two well defined markers of goblet cells Mucin 2 (MUC2) and trefoil factor 3 (TFF3) but also up-regulation of other goblet cell genes; glucosaminyl (N-acetyl) transferase 3 (GCNT3), mucin 13 (MUC13) and mucin 4 (MUC4).

7A.4.3.4 Stem cells

A number of markers for normal colonic and colonic cancer stem cells have been described (LGR5, ITGB1, CD133) (Todaro, Francipane et al. 2010). None of these were identified as differentially expressed in response to calcium.

7A.4.3.5 Stromal cells

Four of the calcium up-regulated genes (GREM1, TAGLN, MYL9 and CNN1) were identified in the cluster of genes expressed in the stroma at the crypt base (Kosinski, Li et al. 2007). A number of other genes up-regulated in response to calcium are also expressed predominantly in stroma rather than epithelium. These include FHL1, MYH11, COL1A2, COL6A3 (Smith, Culhane et al. 2009), ACTG2, COL5A1, MGP, PLN, SPARC, PLEKHC1, IGFBP5,

MFAP5, CAV1 (Li, Madison et al. 2007), DES and CALD1 (Powell, Adegboyega et al. 2005). Only one gene Gremlin 2 (GREM2) expressed in the stroma was found to be decreased in response to calcium.

Overall these data suggest that calcium increases the expression of genes found in differentiated colonocytes and the peri-crypt stroma and decreases the expression of genes found in enteroendocrine cells with no clear effect on goblet cells.

7A.4.4 Functional annotation of differentially expressed genes

DAVID was use to identify the GO biological annotation terms enriched in the genes differentially expressed in response to calcium. The list of genes used in this analysis consisted of all the genes identified as differentially expressed by either GCOS>3 or RP. There were one hundred and seventy-nine up-regulated and eighty down-regulated genes. Firstly enriched single GO annotation terms were identified. Secondly from within these enriched single annotation terms enriched clusters of functionally related GO annotation terms were identified to clarify the biology associated with the differentially expressed genes.

7A.4.4.1 Enriched GO annotation terms in the genes up-regulated by calcium

Enriched single annotation terms for each of the three biological domains of the GO annotation (BP, MF and CC) were identified. There are one hundred and seventeen biological process terms, thirty four cellular component and

twelve molecular function annotation terms enriched in the 179 genes up-regulated by calcium. These are shown in Appendices 5, 6 and 7.

7A.4.4.2 Enriched clusters of functionally related GO annotation terms in the genes up-regulated by calcium

Twenty one enriched clusters of functionally related annotation terms were identified. These twenty one enriched annotation clusters are shown in Table 7A.2 with their enrichment scores. The clusters of enriched annotation terms are described first, as these grouped terms provide the clearest description of the biology of genes up-regulated by calcium. Any additional interesting enriched single annotation terms are then described.

This analysis of the enriched clusters indicates that the genes differentially up-regulated in response to calcium have a role in a number of processes. These include the control of cell migration and locomotion (clusters 1, 5 and 20), regulation of kinase and phosphate mediated signalling (clusters 2, 7, 10 and 13), immune processes (clusters 11, 17 and 18), apoptosis (clusters 19 and 21), cell-matrix adhesion (cluster 22) and functions of specialised colonic cells (cluster 9). The relevance of some of these enriched annotation clusters to the colon is unclear as some of them relate to the development of specialised tissue other than intestine (clusters 3, 14 and 15).

In the single enriched annotation terms there are a number of terms that are features of differentiated colonic cells (eg „epithelial cell differentiation', „regulation of cell proliferation', „apoptosis', „cell adhesion', „adherens junction', „apical plasma membrane' and „anion transmembrane transporter activity') and stromal cells („proteinaceous extra-cellular matrix',

„anchoring junction', 'contractile fiber' „regulation of smooth muscle cell proliferation' and „extra-cellular matrix organization'). This finding is consistent with the observation above of increased expression of genes found in differentiated crypt top cells and the peri-crypt stroma.

Table 7A.2 The twenty one clusters of annotation terms enriched in the 179 genes differentially up regulated in response to calcium. The enrichment score (ES) is shown and for each for each annotation cluster the genes which contributed to the enrichment of the annotation cluster are listed.

Cluster	GO terms (Biological domain)	ES
1	regulation of cell migration (BP) regulation of locomotion (BP) regulation of cell motion (BP)	3.5
2	negative regulation of protein kinase activity (BP) negative regulation of kinase activity (BP) negative regulation of transferase activity (BP) negative regulation of catalytic activity (BP)	3.2
3	epidermal cell differentiation (BP) epidermis development (BP) ectoderm development (BP)	3.2
4	sterol biosynthetic process (BP) cholesterol biosynthetic process (BP) sterol metabolic process (BP) steroid biosynthetic process (BP)	2.9
5	negative regulation of cell migration (BP) negative regulation of locomotion (BP) negative regulation of cell motion (BP)	2.0
6	negative regulation of transcription factor activity (BP) negative regulation of DNA binding (BP) negative regulation of binding (BP) regulation of transcription factor activity (BP) regulation of binding (BP) regulation of DNA binding (BP)	2.0
7	regulation of MAP kinase activity (BP) regulation of phosphorylation (BP) regulation of phosphorus metabolic process (BP) regulation of phosphate metabolic process (BP) regulation of protein kinase activity (BP) regulation of kinase activity (BP) regulation of transferase activity (BP)	1.9
8	response to glucose stimulus (BP) response to monosaccharide stimulus (BP) response to hexose stimulus (BP) response to carbohydrate stimulus (BP)	1.6
9	chloride ion binding (MF)	1.3

	anion binding (MF) chloride channel activity (MF) anion channel activity (MF)	
10	negative regulation of cytokine-mediated signaling pathway (BP) regulation of cytokine-mediated signaling pathway (BP) regulation of peptidyl-tyrosine phosphorylation (BP)	1.5
11	regulation of leukocyte activation (BP) regulation of cell activation (BP) regulation of lymphocyte activation (BP)	1.5
12	response to nutrient (BP) response to extracellular stimulus (BP) response to nutrient levels (BP)	1.5
13	positive regulation of phosphorylation (BP) positive regulation of phosphorus metabolic process (BP) positive regulation of phosphate metabolic process (BP) positive regulation of signal transduction (BP) positive regulation of multicellular organismal process (BP) positive regulation of cell communication (BP)	1.4
14	blood vessel development (BP) vasculature development (BP) blood vessel morphogenesis (BP)	
15	ossification (BP) bone development (BP) skeletal system development (BP)	1.4
16	endoplasmic reticulum membrane (CC) nuclear envelope-endoplasmic reticulum network (CC) endoplasmic reticulum part (CC)	1.3
17	regulation of leukocyte chemotaxis (BP) regulation of leukocyte migration (BP) regulation of chemotaxis (BP) regulation of behaviour (BP)	1.3
18	toll-like receptor signaling pathway (BP) pattern recognition receptor signaling pathway (BP) positive regulation of innate immune response (BP) innate immune response-activating signal transduction (BP) activation of innate immune response (BP) immune response-activating signal transduction (BP) immune response-regulating signal transduction (BP) regulation of innate immune response (BP) activation of immune response (BP) positive regulation of immune effector process (BP) regulation of immune effector process (BP)	1.3
19	anti-apoptosis (BP) negative regulation of apoptosis (BP)	1.3

	negative regulation of programmed cell death (BP) negative regulation of cell death (BP)	
20	positive regulation of cell migration (BP) positive regulation of cell motion (BP) positive regulation of locomotion (BP)	1.3
21	apoptosis (BP) programmed cell death (BP) cell death (BP) death (BP)	1.3
22	focal adhesion (CC) cell-substrate adherens junction (CC) cell-substrate junction (CC)	1.3

7A.4.4.3 Enriched GO annotation terms in the genes down-regulated by calcium

Enriched single annotation terms for each of the three biological domains of the GO annotation (BP, MF and CC) were identified. There are eight biological process terms, five cellular component and nine molecular function annotation terms enriched in the 80 genes down-regulated by calcium. These are shown in appendices 8, 9 and 10.

7A.4.4.4 Enriched clusters of functionally related GO annotation terms in the genes down-regulated by calcium

Three enriched clusters of functionally related annotation terms were identified as enriched in the genes down-regulated by calcium. These three enriched annotation clusters are shown in Table 7A.3 with the enrichment scores and the differentially expressed genes contributing to the enrichment. This analysis suggests that the down-regulated genes are involved in the processes of antigen processing, cytokine activity and metal ion binding.

Table 7A.3 The three clusters of annotation terms enriched in the 80 genes differentially down-regulated in response to calcium. The enrichment score (ES) is shown and for each for each annotation cluster the genes which contributed to the enrichment of the annotation cluster are listed.

Cluster	GO terms (Biological domain)	ES
1	MHC class II receptor activity (MF) MHC class II protein complex (CC) antigen processing and presentation of peptide or polysaccharide antigen via MHC class II (BP) MHC protein complex (CC) antigen processing and presentation (BP)	2.3
2	chemokine activity (MF) chemokine receptor binding (MF) cytokine activity (MF) taxis (BP) chemotaxis (BP) locomotory behavior (BP)	1.9
3	integral to plasma membrane (CC) intrinsic to plasma membrane (CC) plasma membrane part (CC)	1.8

7A.4.5Relationship of differentially expressed genes to colorectal neoplasia

The relationship between the genes differentially expressed in response to calcium and colorectal neoplasia was investigated by reviewing the literature. The genes differentially expressed in colorectal neoplasia and also differentially expressed in response to calcium (both GCOS>3 and RP) and the relevant studies are shown in Tables 7A.4 and 7A.5.

7A.4.5.1 GCOS>3 Up-regulated genes

Of the 156 genes showing increased expression in response to calcium supplementation, forty eight have been reported to have decreased expression in colorectal neoplasia, twenty genes have shown increased expression and for eighty eight no changes in expression have been reported.

7A.4.5.2 GCOS>3 Down-regulated genes

Of the 79 genes showing decreased expression in response to calcium supplementation, twelve have been reported to have increased expression and twenty-two to have decreased expression in colorectal neoplasia.

7A.4.5.3 Rank-product Up-regulated genes

Of the sixty-eight genes up-regulated in response to calcium, twenty six have been reported to have decreased expression in colorectal neoplasia, ten have increased expression and for thirty two no changes in expression have been reported.

7A.4.5.4 Rank-product Down-regulated genes

Of the eight genes down-regulated in response to calcium, five are reported to be down-regulated and one to have increased expression in colorectal neoplasia.

Table 7A.4 The genes differentially up-regulated by calcium which have also been shown to be differentially expressed in colorectal neoplasia. RED=GCOS>3 only, BLUE=RP only, BLACK=GCOS>3 and RP.

Study type	Genes up-regulated in response to Calcium which are also changed in colorectal neoplasia	
	Up-regulated in colorectal neoplasia	Down-regulated in colorectal neoplasia
Micro-array; Normal mucosa vs Cancer	KIAA0101 (Notterman, Alon et al. 2001) MAFF(Friederichs, Rosenberg et al. 2005) AREG (Croner, Foertsch et al. 2005) COL1A2, SPARC, S100P, MMP12 (Cardoso, Boer et al. 2007) COL1A1, HSPD1 (T. Komori and Monden 2004) RRM2 (Grade, Hormann et al. 2007)	HPGD, CEACAM1, FHL1, CEACAM7, KLF4, MYL9, MYH11, CNN1, SLC26A3 (Cardoso, Boer et al. 2007), CLCA4 (Grade, Hormann et al. 2007) TMPRSS2, PDE9A, SLC4A4, DSC2, MUC4 (N Ancona and S Liuni 2006) MUC13, IFI27 (Lin YM 2002), TXNIP (T. Komori and Monden 2004) GUCA2B, TAGLN, PLN, THBS1 (Birkenkamp-Demtroder, Olesen et al. 2005) PAFAH1B1, TNS1, CXADR, MGC14376, PTPRC (Hlubek, Brabletz et al. 2007) BMP2 (Wiese, Auer et al. 2007), CALD1, DES (Bertucci, Salas et al. 2004) SFN (Kitahara, Furukawa et al. 2001), IL6ST (Nosho, Yamamoto et al. 2005) CRIP1 (Martinez, Bhattacharya et al. 2005), GPD2 (Bianchini, Levy et al. 2006)
Micro-array; Advanced vs early cancer	IGFBP5 (Koehler, Bataille et al. 2004) TM4SF1(Kleivi, Lind et al. 2007)	CFTR (Groene, Mansmann et al. 2006) DDX3X (Fritzmann, Morkel et al. 2009)
Micro-array; Normal mucosa vs adenoma	OLFM4 (SEUNGKOO LEE 2006) DUOX2, (Kita, Hikichi et al. 2006)	DHRS9, TRIM15, FLNA, PALLD, EFNA1, COL6A2 (Nikola A. Bowden 2007) CD177 (Kita, Hikichi et al. 2006) CLIC5, GREM1, CXCL9, MFAP5, SGK1, ZBTB16 (Segditsas, Sieber et al. 2008) MXD1 (Sabates-Bellver, Van der Flier et al. 2007)
PCR; Normal mucosa vs cancer	FHL2 (Wang, Yang et al. 2007) SLC6A14 (Gupta, Miyauchi et al. 2005)	KLF6 (Reeves, Narla et al. 2004), SPRED2 (Lü, Xu et al. 2008) ABCG2 (Gupta, Martin et al. 2006), MGP (Fan, Sheu et al. 2001)
PCR; Normal mucosa vs adenoma	CYP3A5 (Bergheim, Bode et al. 2005)	-
Protein expression; Normal mucosa vs cancer	MCL1 (Krajewska, Moss et al. 1996) CAV1 (Bender, Reymond et al. 2000) FAS (Zhu, Liu et al. 2005) RARRES3 (Shyu, Jiang et al. 2003)	-

Table 7A.5. The genes differentially down-regulated by calcium which have also been shown to be differentially expressed in colorectal neoplasia. RED=GCOS>3 only, BLUE=RP only, BLACK=GCOS>3 and RP. Of the 80 genes identified as down regulated by calcium using either GCOS>3 or RP, 13 are up regulated in colorectal neoplasia and 24 are down regulated.

Study type	Genes down-regulated in response to Calcium which are also changed in colorectal neoplasia	
	Up-regulated in colorectal neoplasia	Down-regulated in colorectal neoplasia
Micro-array; Normal mucosa vs Cancer	IGL@ (Friederichs, Rosenberg et al. 2005) MT1H, MT2A (Giacomini, Leung et al. 2005) PTTG1 (Hlubek, Brabletz et al. 2007) CCNA2 (N Ancona and S Liuni 2006) PIGR (Chen, Zhang et al. 2006) IGHG1 (Grade, Hormann et al. 2007)	MT1G, PCK1 (Birkenkamp-Demtroder, Olesen et al. 2005), PYY, CKB, FXYD3, SST, GCG, CHGA, SL26A2, MUC2 (Cardoso, Boer et al. 2007) DNASE1L3, INSL5, HAPLN1 (Croner, Foertsch et al. 2005) IGHA1 (Koehler, Bataille et al. 2004)
Micro-array; Advanced vs early cancer	TFF3, BAMBI (Fritzmann, Morkel et al. 2009)	-
Micro-array; Normal mucosa vs adenoma	HLA-DPA1, IL1R2 (Salahshor, Goncalves et al. 2005)	HBB, (Salahshor, Goncalves et al. 2005) MT1F, IGLJ3, HLA-DQA1 (SEUNGKOO LEE 2006) INSM1, MT1M, ABCA8 (Kita, Hikichi et al. 2006) MS4A1, CXCL13 (Segditsas, Sieber et al. 2008)
PCR; Normal mucosa vs cancer	IL8 (Rubie, Frick et al. 2007)	GALNT12 (Guo, Chen et al. 2004)
Protein expression; Normal mucosa vs cancer	NDRG1(Koshiji, Kumamoto et al. 2007)	-

7A.4.6 Relationship between genes differentially expressed in response to calcium supplementation and genes regulated by PTH

To address the hypothesis that calcium acts by modulating PTH levels the overlap between the genes differentially expressed in response to calcium and those regulated by PTH in-vitro was studied. Firstly the effect of calcium supplementation on serum PTH levels in the six subjects was studied.

7A.4.6.1 Influence of calcium supplementation on Serum PTH

It was demonstrated in chapter three that calcium supplementation for six weeks resulted in a statistically significant suppression of PTH in the eleven subjects studied. This analysis was repeated for the six subjects studied here. In each subject PTH decreased following calcium supplementation, baseline and post calcium supplementation serum PTH results are shown in Tables 5.2 and 5.5. There was mean decrease in PTH of 0.73pmol/L (95%CI 0.49 to 0.98). A Mann-Whitney U test was performed to compare the difference between serum PTH levels before and after calcium supplementation, this showed a difference significant at the 5% level:

pmol	Before calcium	After calcium
Median PTH (pmol/L)	3.45	2.65
Range	3.1-5.2	2.4-4.5
N	6	6

U= 5

7A.4.6.2 Effect of calcium supplementation on the genes changed in response to PTH in-vitro

The effect of calcium supplementation on the genes differentially expressed in response to PTH in-vitro was studied. The majority of genes differentially expressed in response to PTH were down-regulated and the 133 down-

regulated genes from the two time points (4 and 24 hours) were studied together. For each of these 133 genes the outcomes in response to calcium in-vivo were analysed. The following features were identified for each gene; fold-change, number of subjects in which change in expression occurred using GCOS and those identified as differentially expressed by GCOS>3 and RP. These features are shown in table 7A.6.

7A.4.6.2.1 Fold change

The majority of PTH regulated genes were up-regulated in response to calcium with only nineteen genes showing a decrease in expression. The fold-change for these nineteen genes was small with none showing a fold change greater than -1.2 fold. The fold change for the up-regulated genes was also small but there were 31 genes in which expression changed greater than +1.2 fold. The fold changes in response to calcium supplementation are shown in Table 7A.6.

7A.4.6.2.2 Number of subjects changed (GCOS)

The majority of PTH regulated genes showed an increase in expression in more subjects than decreased expression. Eight genes were increased in 3/6 subjects, twenty three were increased in 2/6 subjects and 35 were increased in 1/6 subject. In contrast one gene was decreased in 3/6 subjects and two genes were decreased in 1/6 subjects and the remaining fifty five genes were unchanged in any of the subjects.

7A.4.6.2.3 Differentially expressed genes

Using GCOS>3 there were eight PTH regulated genes that were differentially expressed in response to calcium (AREG, PAFAH1B1, DUSP6, GPD2, LIN7C, ID1, ABCG2 and SFRS3). Using RP there were six PTH regulated genes that

were differentially expressed in response to calcium (EREG, AREG, SGK1, PAFAH1B1, GPD2 and ABCG2). There was one down-regulated gene identified by GCOS>3. These genes are identified in Table 7A.6.

Table 7A.6 The effect of calcium on the 133 genes differentially expressed in response to PTH treatment of Caco2 cell line.
The genes are ranked by fold-change. Up and Dn refer to the number of subjects (out of 6) a gene was identified as up or down regulated using GCOS. The eight genes differentially expressed in response to calcium using GCOS>3 are noted with a * and those by RP are identified with a #. FC=fold change

Symbol	Description	Up	Dn	FC
EREG #	Epiregulin	2	1	+1.59
AREG* #	Amphiregulin	3	1	+1.54
PSPH	phosphoserine phosphatase	1	0	+1.47
SGK1 #	serum/glucocorticoid regulated kinase	2	2	+1.46
TMOD3	tropomodulin 3 (ubiquitous)	1	0	+1.4
PAFAH1B1*#	Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	3	0	+1.35
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	2	0	+1.34
ROD1	ROD1 regulator of differentiation 1 (S. pombe)	1	0	+1.34
DUSP6*	dual specificity phosphatase 6	3	0	+1.33
GPD2* #	glycerol-3-phosphate dehydrogenase 2	3	0	+1.33
RHOB	ras homolog gene family, member B	2	0	+1.32
MBNL2	muscleblind-like 2 (Drosophila)	1	0	+1.31
LIN7C*	lin-7 homolog C (C. elegans)	3	0	+1.28
SS18	synovial sarcoma translocation, chromosome 18	2	0	+1.28
PHLDA2	pleckstrin homology-like domain, family A, member 2	2	0	+1.27
PTPN11	protein tyrosine phosphatase, non-receptor type 11 (Noonan syndrome 1)	1	0	+1.27
GJA1	gap junction protein, alpha 1, 43kDa (connexin 43)	1	1	+1.24
NUP50	nucleoporin 50kDa	0	0	+1.24
ID1*	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	3	1	+1.23
SRPK1	SFRS protein kinase 1	1	0	+1.23
AGPS	alkylglycerone phosphate synthase	1	0	+1.22
ARFIP1	ADP-ribosylation factor interacting protein 1	1	0	+1.22
CANX	calnexin	2	0	+1.22
EML4	echinoderm microtubule associated protein like 4	1	0	+1.22

RFC3	replication factor C (activator 1) 3, 38kDa	1	0	+1.22
DUSP5	dual specificity phosphatase 5	1	1	+1.21
PHLDA1	pleckstrin homology-like domain, family A, member 1	1	0	+1.21
SCARB2	scavenger receptor class B, member 2	2	0	+1.21
ABCG2* #	ATP-binding cassette, sub-family G (WHITE), member 2	3	2	+1.2
EFNB2	ephrin-B2	2	0	+1.2
NCBP1	nuclear cap binding protein subunit 1	1	0	+1.2
HSPA1B	heat shock 70kDa protein 1B	2	0	+1.18
PRKCI	protein kinase C, iota	2	1	+1.18
SFRS3*	splicing factor, arginine/serine-rich 3	3	0	+1.18
TXNDC1	thioredoxin domain containing 1	2	0	+1.18
ACTB	actin, beta	1	0	+1.17
CALM1	calmodulin 1 (phosphorylase kinase, delta)	1	0	+1.17
NLK	nemo-like kinase	0	0	+1.17
ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	2	0	+1.16
BRD2	bromodomain containing 2	0	0	+1.16
RALA	v-ral simian leukemia viral oncogene homolog A (ras related)	2	0	+1.16
SFRS1	splicing factor, arginine/serine-rich 1	1	0	+1.16
CNN3	calponin 3, acidic	1	0	+1.15
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	1	0	+1.15
HSPA9	heat shock 70kDa protein 9	1	0	+1.15
LRRFIP1	leucine rich repeat (in FLII) interacting protein 1	2	1	+1.15
RDX	radixin	0	0	+1.15
SLC25A32	solute carrier family 25, member 32	0	0	+1.15
BRD4	bromodomain containing 4	0	0	+1.14
EIF2S3	eukaryotic translation initiation factor 2, subunit 3 gamma	2	1	+1.14
NOX1	NADPH oxidase 1	1	0	+1.14
NUP98	nucleoporin 98kDa	1	0	+1.14
CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	2	0	+1.13
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	2	1	+1.13
KLF3	Kruppel-like factor 3 (basic	1	0	+1.12
NDRG3	NDRG family member 3	0	0	+1.12
SFRS7	splicing factor, arginine/serine-rich 7, 35kDa	0	0	+1.12
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1	0	0	+1.12
ATF2	activating transcription factor 2	1	0	+1.11
GSN	gelsolin (amyloidosis, Finnish type)	2	1	+1.11

SELT	selenoprotein T	0	0	+1.11
SKP2	S-phase kinase-associated protein 2 (p45)	1	0	+1.11
TMED2	transmembrane emp24 domain trafficking protein 2	1	0	+1.11
ARL4C	ADP-ribosylation factor-like 4C	1	0	+1.1
DYNC1LI2	dynein, cytoplasmic 1, light intermediate chain 2	1	0	1.1
NOL5A	nucleolar protein 5A (56kDa with KKE/D repeat)	1	0	+1.1
PDCD2	programmed cell death 2	0	0	+1.1
SLC12A2	solute carrier family 12 (sodium/potassium /chloride transporters), member 2	2	1	+1.1
WNK1	WNK lysine deficient protein kinase 1	0	0	+1.1
MSH2	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	0	0	+1.09
ARHGAP5	Rho GTPase activating protein 5	0	0	+1.08
BTRC	beta-transducin repeat containing	0	0	+1.08
JMJD1B	jumonji domain containing 1B	0	0	+1.08
PDCD4	programmed cell death 4 (neoplastic transformation inhibitor)	2	0	+1.08
RAB8A	RAB8A, member RAS oncogene family	1	0	+1.08
YTHDF2	YTH domain family, member 2	0	0	+1.08
B3GNT2	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2	0	0	+1.07
FBLN1	fibulin 1	2	0	+1.07
PRNP	prion protein (p27-30)	0	0	+1.07
STAT2	signal transducer and activator of transcription 2	0	0	+1.07
TOMM22	translocase of outer mitochondrial membrane 22 homolog (yeast)	0	0	+1.07
FZD7	frizzled homolog 7 (Drosophila)	0	0	+1.06
RBBP4	retinoblastoma binding protein 4	0	0	+1.06
UBIAD1	UbiA prenyltransferase domain containing 1	0	0	+1.06
APLP2	amyloid beta (A4) precursor-like protein 2	1	0	+1.05
CD9	CD9 molecule	1	0	+1.05
DARS	aspartyl-tRNA synthetase	0	0	+1.05
GNL2	guanine nucleotide binding protein-like 2 (nucleolar)	0	0	+1.05
MAP4K4	mitogen-activated protein kinase kinase kinase kinase 4	0	0	+1.05
ZNF148	zinc finger protein 148	1	0	+1.05
CROP	cisplatin resistance-associated overexpressed protein	2	1	+1.04
GSR	glutathione reductase	0	0	+1.04
PVR	poliovirus receptor	0	0	+1.04

RAB5B	RAB5B, member RAS oncogene family	0	0	+1.04
FUS	fusion (involved in t(12;16) in malignant liposarcoma)	0	0	+1.03
HOXA7	homeobox A7	0	0	+1.03
RBM3	RNA binding motif (RNP1, RRM) protein 3	0	0	+1.03
ASNS	asparagine synthetase	1	0	+1.02
CTBP1	C-terminal binding protein 1	0	0	+1.02
SERPINH1	serpin peptidase inhibitor, clade H (heat shock protein 47), member 1	0	0	+1.02
SOX9	SRY (sex determining region Y)-box 9	1	0	+1.02
SPOP	speckle-type POZ protein	0	0	+1.02
ATP1B1	ATPase, Na+/K+ transporting, beta 1 polypeptide	1	1	+1.01
NRIP1	nuclear receptor interacting protein 1	1	1	+1.01
NUTF2	nuclear transport factor 2	1	0	+1.01
CKAP4	cytoskeleton-associated protein 4	1	1	1
COPG	coatomer protein complex, subunit gamma	0	0	1
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	0	0	1
ELL2	elongation factor, RNA polymerase II, 2	0	0	1
SRRM2	serine/arginine repetitive matrix 2	0	0	1
TGIF1	TGFB-induced factor (TALE family homeobox)	0	0	1
UTS2	urotensin 2	0	0	1
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	1	0	-1.01
DKK1	dickkopf homolog 1 (Xenopus laevis)	0	0	-1.01
MOCS3	molybdenum cofactor synthesis 3	0	0	-1.01
RTN3	reticulon 3	0	0	-1.01
SNRNP40	WD repeat domain 57 (U5 snRNP specific)	0	0	-1.02
STT3A	STT3, subunit of the oligosaccharyltransferase complex, homolog A	0	0	-1.02
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	1	0	-1.03
NFAT5	nuclear factor of activated T-cells 5, tonicity-responsive	0	0	-1.04
SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	1	1	-1.04
ICAM1	intercellular adhesion molecule 1 (CD54), human rhinovirus receptor	0	0	-1.05
UTP20	UTP20, small subunit (SSU) processome component, homolog (yeast)	0	0	-1.05
PPAP2B	phosphatidic acid phosphatase type 2B	0	1	-1.06
GATM	glycine amidinotransferase (L-	0	1	-1.1

	arginine:glycine amidinotransferase)			
ANKRD11	ankyrin repeat domain 11	0	0	-1.12
SULT1E1	sulfotransferase family 1E, estrogen-preferring, member 1	0	0	-1.12
GPC3	glypican 3	0	0	-1.14
TTR	transthyretin (prealbumin, amyloidosis type I)	0	0	-1.14
TAF15	TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 68kDa	0	0	-1.15
SLC26A2	solute carrier family 26 (sulfate transporter), member 2	1	3	-1.16
HNRPU	heterogeneous nuclear ribonucleoprotein U (scaffold attachment factor A)	-	-	-

7A.5 Discussion

This study has demonstrated that dietary calcium supplementation results in the differential expression of a number of genes in the rectal mucosa of the individuals studied. Firstly the findings will be discussed in general terms. Two approaches were used to identify the genes differentially expressed in response to calcium. Both of them (GCOS>3 and RP) employed a matched pair approach, allowing identification of genes that are differentially expressed in each individual following treatment with calcium. This is particularly true for the GCOS>3 approach, which only compares each individuals „before' and „after' sample. The rank-product (RP) analysis was performed alongside this because it provides a measure of statistical significance which the GCOS>3 method does not. Because of it matched pair wise comparison the GCOS>3 approach is intuitively attractive. The selection of altered in >3/6 samples is an arbitrary, though reasonable, cut-off. Notably, there was significant overlap between the genes identified by the two different methods, suggesting these changes are valid.

Another general observation is that more genes showed increased expression in response to calcium than decreased expression. This suggests either calcium induced gene expression or relief of transcriptional repression. Also, the magnitude of the fold change was relatively small with a maximum fold change of just over two. Even among the genes identified as up-regulated by calcium only 15/156 (GCOS>3) and 25/68 (RP) showed a fold change of >1.5 fold and only one gene down-regulated by calcium was decreased by a fold change of >1.5. It is perhaps not surprising that the fold changes seen are small in contrast to studies comparing normal and diseased

tissues as the normal mucosa has various homeostatic mechanisms controlling crypt kinetics. The fold changes seen are also similar to those seen in the only other micro-array study investigating the effect of a chemo-preventive agent on gene expression in rectal mucosa (Glebov, Rodriguez et al. 2006). In this study investigating the effect of Celecoxib, a two fold change in gene expression was again the maximum effect size seen.

7A.5.1 Action of calcium on the colonic epithelium

In analysing the results of these array studies the aim was to determine the biological processes that are represented by the genes differentially expressed in response to calcium. The results suggest that calcium supplementation increases the numbers of differentiated cells within the crypt. This is based on the observation of a number of genes found in differentiated cells. These include genes negatively regulated by wnt signalling including MXD1, EZR, PDE9A, ISG20, CEACAM1, TRIM31 and HPGD (van de Wetering, Sancho et al. 2002), suggesting calcium negatively regulates Wnt signalling. This is consistent with a pro-differentiation effect of calcium, as differentiation is a consequence of decreased Wnt pathway signalling (Mariadason, Bordonaro et al. 2001). The annotation terms enriched in the differentially expressed genes also demonstrated biological features of differentiated colonic cells particularly apoptosis, anion binding and anion channel activity, as well as control of migration and differentiation.

Other evidence that the effect of calcium is mediated by modulation of the Wnt signalling pathway is provided by the observation that calcium supplementation resulted in the down-regulation of a number of genetic

markers of entero-endocrine cells. Deletion of TCF4 in mice results in the absence of entero-endocrine cells, whilst mature enterocytes and goblet cells remain (Korinek, Barker et al. 1998). This suggests that the effect of calcium in reducing markers of entero-endocrine cells may be mediated by a suppressive effect on wnt signalling. Modulation of wnt signalling by calcium in combination with vitamin D has been demonstrated in a study of gene expression in colonic mucosa of mice. Calcium and vitamin D decreased expression of the wnt effectors beta-catenin and TCF (Yang, Kurihara et al. 2008).

Other signalling pathways that may be influenced by calcium include the hedgehog signalling pathway, this is suggested by the finding that calcium supplementation results in the increased expression of a number of genes found in the peri-crypt stroma, particularly myofibroblasts. An increase in the number of myofibroblasts is a consequence of activation of the hedgehog signalling pathway (van Dop, Uhmann et al. 2009). The hedgehog signalling pathway is also activated by down regulation of the wnt signalling pathway (van den Brink, Bleuming et al. 2004). Therefore, as well as down-regulating wnt signalling, calcium may also act partly by modulating hedgehog signalling. Though, there are no known targets of hedgehog signalling to compare with.

7A.5.2 Comparison with previous studies of calcium

Other studies have investigated whether calcium results in increased apoptosis in colorectal epithelium; some of these have shown no effect on apoptosis (Holt, Wolper et al. 2001; Holt, Bresalier et al. 2006), while others have suggested that calcium may enhance apoptosis (Miller, Keku et al. 2005;

Fedirko, Bostick et al. 2009). Annotation terms representing apoptosis were enriched in the differentially expressed genes, suggesting calcium does increase apoptosis.

7A.5.3 Relationship between genes differentially expressed in response to calcium and those differentially expressed in colorectal neoplasia

Calcium does alter the expression of genes involved in colorectal tumorigenesis. However, it is clear that the effect of calcium is not just to alter the expression of genes known to be differentially expressed in colorectal neoplasia in the opposite direction. Calcium up-regulates genes commonly up and down regulated in colorectal neoplasia, with similar results for the genes down-regulated by calcium.

7A.5.4 Effect of calcium on normal mucosal markers of the risk of neoplasia

Reduced apoptosis has been shown to predict current (Anti, Armuzzi et al. 2001) and future neoplasia (Keku, Amin et al. 2008). In this study, calcium resulted in the increased expression of genes involved in apoptosis, suggesting, as have others, (Miller, Keku et al. 2005; Fedirko, Bostick et al. 2009) that calcium induces apoptosis. These observations indicate that calcium supplementation influences normal mucosa in a manner predicted to reduce the incidence of neoplasia. This is consistent with its observed chemo-preventive action. It also underlines the importance of understanding the mechanism of action of calcium.

7A.5.5 Mechanism of action of calcium

The hypothesis of this thesis is that that raised PTH acts on the normal colon increasing the risk of neoplasia and that calcium acts by reducing serum PTH. There are a number of results in this chapter that support the hypothesis that calcium influences gene expression in the rectal mucosa by modulating PTH level. Firstly, the influence of calcium on gene expression was predominantly to increase the expression of certain genes and the effect of PTH both in-vitro and in-vivo was predominantly to decrease the expression of certain genes. This raises the possibility that PTH signalling results in transcriptional repression and that calcium, by reducing PTH relieves that repression. There are more genes for which expression correlates with PTH in-vivo, than are changed by calcium. However, it is not surprising that all of the genes correlated with PTH were not up-regulated by calcium as the degree of correlation does not indicate the slope of the regression line; or how much expression will change in response to a change in PTH.

There is support in the data, for the hypothesis that calcium acts by modulating PTH. Most of the genes down-regulated by PTH in-vitro are up-regulated by calcium in-vivo, if only with a small fold change. However there were only eight gene (GCOS>3), identified as differentially expressed by both PTH in-vitro and calcium in-vivo. The effect of calcium on the genes regulated by PTH in-vitro will be studied further using GSEA in the second part of this chapter.

7A.5.6 Strengths and limitations

This study has a number of strengths; it is a study of human subjects with a past history of colorectal adenomas and has employed a standardised

protocol for determining the effect of calcium on gene expression in the rectal mucosa. In identifying genes differentially expressed in response to calcium both the GCOS>3 and RP method employed a pair wise approach so that each subject acts as their own control, thus enabling identification of only those genes changed in response to calcium. The weaknesses of this study include the absence of an untreated control group. This was planned for in the study design but was not possible for logistical reasons. In a similar study investigating the effect of Celecoxib on gene expression in rectal mucosa, there was an untreated control groups. In the treated group one hundred and seventy three genes were identified as altered in expression and only fourteen in the placebo group. It would seem reasonable therefore that the majority of the changes seen in response to calcium are real.

7A.5.6 Conclusion

This study provides further evidence that dietary calcium supplementation has an effect on the rectal mucosa, with demonstrable changes in gene expression. These changes suggest that calcium induces differentiation and apoptosis, contributing evidence to support an anti-neoplastic action of calcium. These results also suggest overlap between the genes changed by calcium and PTH. In the second part of this chapter gene set enrichment analysis will be used to further address this question.

Chapter 7B

GENE SET ENRICHMENT ANALYSIS TO INVESTIGATE THE EFFECT OF DIETARY CALCIUM SUPPLEMENTATION ON GENE EXPRESSION IN NORMAL RECTAL MUCOSA

7B.1 Aim

1. Identify gene sets enriched in the genes differentially expressed in the rectal mucosa of individuals taking dietary calcium supplementation using oligo-nucleotide micro-arrays.

7B.2 Introduction

In the first part of this chapter it was demonstrated that calcium supplementation resulted in the differential expression of a number of genes. Descriptive analysis and functional annotation of these genes indicated a number of interesting biological features of these genes. It is hypothesised that calcium supplementation results in the differential expression of genes representing different biological processes. Particularly that calcium alters the genes identified as regulated by PTH in the studies described in chapters 4 and 6. To address this hypothesis further, the gene set enrichment analysis (GSEA) approach (Subramanian, Tamayo et al. 2005) will be used. As was carried out in chapter 6B; investigation of the expression of user defined gene sets will be carried out.

GSEA was performed as there were relatively few differentially expressed genes identified with small fold changes. The advantages of GSEA over single gene techniques lie in its ability to identify biological meaning where the fold changes for individual genes are small (Mootha, Lindgren et al.

2003). It also analyses all genes in the dataset not just those above a certain threshold. The use of gene sets allows more clear definition of biological processes than single genes and is more reproducible.

7B.3 Methods

7B.31 Gene set enrichment analysis (GSEA)

The gene set enrichment analysis methodology was described in section 6B.3.2. In this study, GSEA was run with the parameters described below. The RMA data from the six controls and the six treated samples were the expression datasets, a class comparison was made comparing control and treated samples. The C2 curated set of genes from the MSigDB (V2.5) was used; this consists of a total of 1892 gene sets. The gene sets were filtered to exclude sets with >500 or <25 genes, this left a total of 1048 gene sets within this size range. The default setting for ranking metric, signal to noise ratio was used. Signal to noise ratio is the difference of the mean expression between treated and control samples scaled by the standard deviation. Gene set permutation was used and the number of permutations, set at 1000 as recommended. A FDR of<0.05 was considered significant; however, the most highly ranked gene sets are usually the ones of most interest, even if those lower down the ranking have a FDR of <0.05.

In addition to the C2 gene sets, twenty user defined gene sets were used. Seventeen of these were used in the Chapter 6B and have been described in section 6B.3.2.1 and Table 6B.1. In addition three other gene sets were used. The relationship between the effect of Celecoxib (A NSAID with chemo-preventive activity) and calcium was compared by studying the gene sets **celecoxib_dn** and **celecoxib_up**. These sets contain the genes differentially expressed in normal colonic mucosa in response to celecoxib treatment (Glebov, Rodriguez et al. 2006). The influence of calcium on the genes negatively correlated with PTH in vivo was studied by the inclusion of a

gene set consisting of the top five hundred genes; **pth_neg,** found to be negatively correlated with PTH in chapter three.

7B.4Results

7B.4.1Enriched gene sets

Of the 1049 gene sets studied, 204 were enriched in the calcium treated phenotype, which are the gene sets up-regulated in response to calcium. And there were 11 enriched in the control phenotype with a FDR <0.05, representing the gene sets down-regulated in response to calcium.

7B.4.1.1Gene sets enriched in calcium treated phenotype

The 204 gene sets enriched in the treated phenotype represent those up-regulated in response to calcium. The top ranked gene sets with the highest enrichment scores represent the biology associated with the calcium treated phenotype. The top twenty-five enriched gene sets are shown in Table 7B.1. These top twenty-five enriched gene sets can be divided into a number of groups. These groups are either derived from the same or similar studies or containing genes with overlapping function. The most relevant of which are those from studies of the colon.

The most highly enriched gene set is **crypt_top**, this contains genes differentially expressed at the top of normal colonic crypts compared to the base of the crypt (Kosinski, Li et al. 2007). The **crypt_top** gene set contains markers of differentiation with genes involved in; cell adhesion, cell maturation, apotosis and inhibition of cell proliferation. This set of genes also showed significant overlap with the genes induced by a dominant negative TCF4 (Kosinski, Li et al. 2007). It is consistent with this that the gene set **dn_tcf4_up** is also significantly enriched in the genes up-regulated by calcium.

The second most enriched gene set is **caco2_pthall**, this consists of all the genes up-regulated by PTH in caco2 at both time points. The two gene sets from twenty-four **caco2_pth24** and four hours **caco2_pth4** were also separately, strongly enriched. The genes in these sets and their function were described in chapter 2; they represent down-regulated transcriptional targets of PTH in colonic epithelial cells in-vitro. The third colonic related gene set that was enriched **pth_neg**, consists of the top 500 genes that showed negative correlation with PTH. The finding that these three clusters of colorectal specific gene sets are enriched in response to calcium provides evidence on the mechanism of action of calcium. Enrichment plots for these six gene sets are shown in Figure 7B.1.

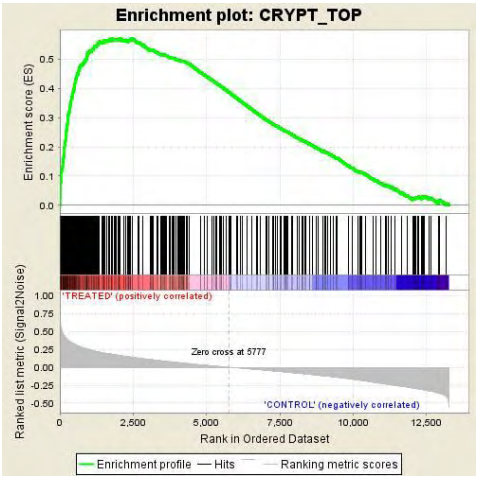
The three clusters of colon derived gene sets are the most pertinent to understanding the biological action of calcium on colon. The additional information derived from the other gene sets may be complementary, but is most likely to represent overlapping biological processes occurring in other tissues. All of the top twenty five gene sets are shown, in related clusters, in Appendix 12 along with a brief description of their biology.

Table 7B.1 The top 25 gene sets enriched in the treated by calcium phenotype. The gene sets are ranked by normalised enrichment score (NES). Size, is the number of genes in the genes set. Significance of the enrichment results are represented by p-value and false detection rates (FDR).

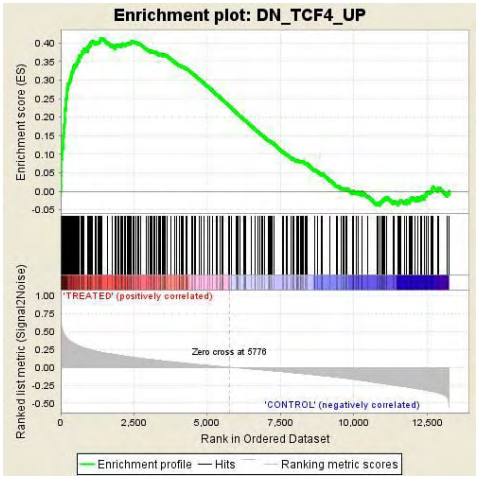
Gene set	NES	p-value	FDR
CRYPT_TOP	+3.2	0	0
CACO2_PTHALL	+3.1	0	0
REOVIRUS_HEK293_UP	+3.0	0	0
PTH-NEG	+2.9	0	0
ET743_SARCOMA_24HRS_DN	+2.8	0	0
CHEN_HOXA5_TARGETS_UP	+2.8	0	0
CACO2_PTH24	+2.8	0	0
ET743_SARCOMA_48HRS_DN	+2.7	0	0
IFNA_HCMV_6HRS_UP	+2.7	0	0
ET743_SARCOMA_DN	+2.6	0	0
CACO2_PTH4	+2.6	0	0
ET743_SARCOMA_72HRS_DN	+2.5	0	0
UVB_NHEK3_C1	+2.4	0	0
CANCER_UNDIFFERENTIATED_META_UP	+2.4	0	0
UVC_HIGH_ALL_DN	+2.4	0	0
UVB_NHEK3_ALL	+2.4	0	0
STEMCELL_COMMON_UP	+2.4	0	0
UVB_NHEK3_C0	+2.4	0	0
FSH_OVARY_MCV152_DN	+2.3	0	0
BRCA_PROGNOSIS_NEG	+2.3	0	0
DSRNA_UP	+2.3	0	0
DER_IFNB_UP	+2.3	0	0
CMV_HCMV_TIMECOURSE_ALL_UP	+2.2	0	0
DN_TCF4_UP	+2.3	0	0
FLECHNER_KIDNEY_TRANSPLANT_WELL_UP	+2.2	0	$8x10^{-5}$

Figure 7B.1(a)-(e). Enrichment plots for the six colorectal gene sets that were enriched in the genes up-regulated by calcium. The top portion of the plot shows the running ES for the gene set as the analysis walks down the ranked list. The score at the peak of the plot (the score furthest from 0.0) is the ES for the gene set. The middle portion shows a line for where each of the genes in the set occurs in the ranked set. The bottom portion of the plot shows the value of the ranking metric (signal to noise ratio) as you move down the list of ranked genes. The gene sets shown here are a) Crypt-top, b) DN_TCF4, c) caco2_pthall, d) caco2_pth24 e) caco2_pth4, f) PTH_NEG

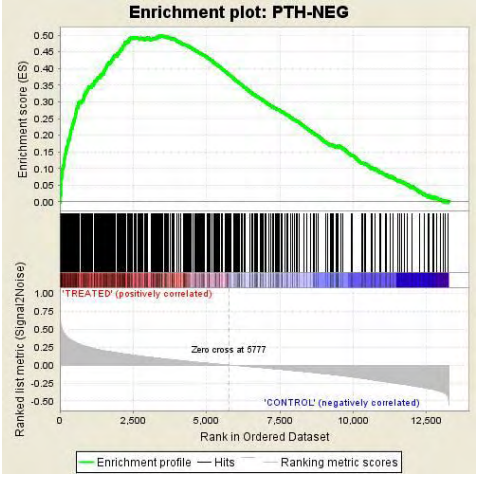
a



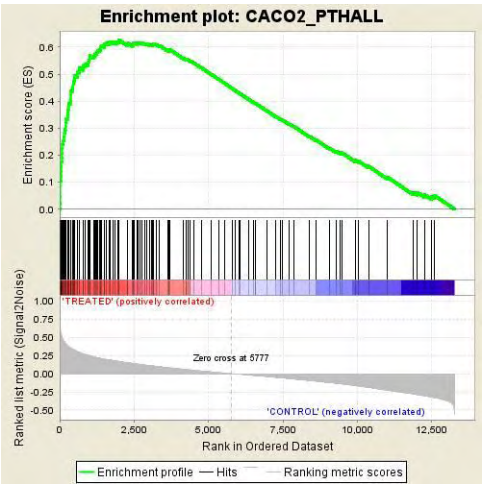
b



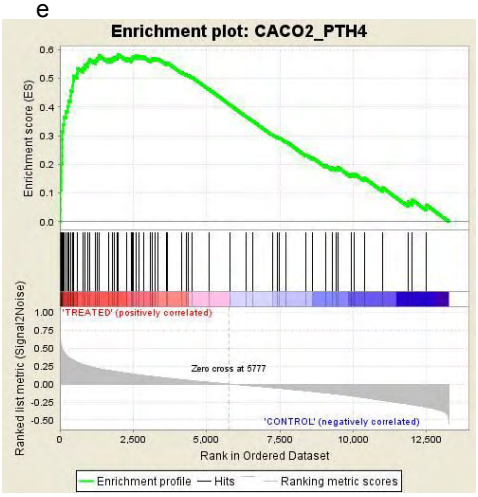
c



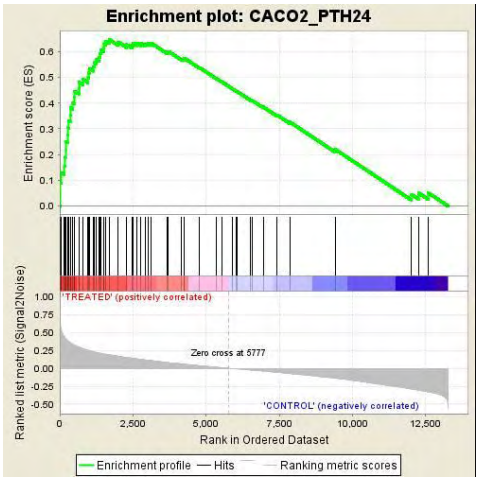
d



e



f



7B.4.1.2Gene sets enriched in control phenotype

There are eleven gene sets enriched in the control phenotype with a FDR<0.05. These represent the gene sets down-regulated in response to calcium and they are shown in Tables 7B.2. Within these eleven gene set there is one cluster of highly similar genes, the remaining gene sets are unrelated. They are shown in Appendix 13, with a brief discussion of their biology. None of these enriched gene sets are derived from colonic tissue.

Table 7B.2 The top 11 gene sets enriched in the control phenotype with an FDR<0.05. The gene sets are ranked by normalised enrichment score (NES). Significance of the enrichment results are represented by p-value and false detection rates (FDR).

Gene set	NES	p-value	FDR
REFRACTORY_GASTRIC_UP	-2.2	0	0
PEPTIDE_GPCRS	-2.1	0	0.003
HSA04080_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION	-2.1	0	0.002
FETAL_LIVER_VS_ADULT_LIVER_GNF2	-1.9	0	0.015
GPCRDB_CLASS_A_RHODOPSIN_LIKE	-1.9	0	0.03
IGF_VS_PDGF_UP	-1.9	0	0.03
MOREAUX_TACI_HI_VS_LOW_UP	-1.8	0	0.04
STEMCELL_COMMON_DN	-1.8	0	0.04
GPCRS_CLASS_A_RHODOPSIN_LIKE	-1.8	0	0.04
HSA03010_RIBOSOME	-1.8	0	0.04
NUCLEAR_RECEPTORS	-1.8	0.003	0.05

7B.4.2 Outcomes for user defined gene sets

The twenty user defined gene sets were employed in this study to address specific hypotheses. The outcomes will be presented separately here and are shown in Table 7B.3. Six of these gene sets were in the top twenty-five gene sets that were enriched in the treated with calcium phenotype and were described above. The two sets of genes representing genes up and down regulated in colorectal cancer (**crc_up** and **crc_down**) were both enriched in the treatment phenotype, these results were statistically significant but these gene sets were found a long way down the list of enriched gene sets, with more than one hundred gene sets showing higher levels of enrichment suggesting the effect is comparatively weak. The results for the sets of genes up and down regulated in adenomas (**adenoma_up** and **adenoma_ down** are contrary to what might have been predicted, with the **adenoma_up** gene set enriched in the treated with calcium phenotype and the **adenoma_down**

gene set none-significantly enriched in the down regulated by calcium phenotype. Again, for both these sets there were a large number of other gene sets more strongly enriched, suggesting that the effect is small.

The **crypt_base** set of genes is not regulated in the opposite direction to the **crypt_top** gene set, also being weakly enriched in the treated phenotype. This may be because a number of the genes up-regulated by calcium are found in the crypt stroma at the crypt base. Similarly the **dn_tcf_dn** gene set was non-significantly weakly enriched in the treated phenotype. Comparison of the genes differentially expressed in response to calcium, with those differentially expressed in the rectal mucosa in response to Celecoxib (a cyclo-oxygenase 2 inhibitor) showed no similarity. In fact, the two gene sets were weakly enriched in the opposite way to that expected, with **celecoxib_up** weakly enriched in the control phenotype and **celecoxib_dn** in the treatment phenotype. Of the user defined gene sets none were significantly enriched in the control phenotype. Two of the sets representing PTH targets in bone and muscle (**pth_bone_up** and **pth_muscle_up**) were weakly enriched as was **cd133_stemcells**. The three other PTH related gene sets (**pth_bone_dn**, **pth_haem_dn**, **pth_haem_up**) showed no significant enrichment in either phenotype.

Table 7B.3 Outcome for the user defined gene sets in the gene set enrichment analysis. They are ranked by normalised enrichment score (NES), with those enriched in the up-regulated by calcium phenotype at the top.

Gene set	NES	p-value	FDR
CRYPT_TOP	+3.2	0	0
CACO2_PTHALL	+3.1	0	0
PTH_NEG	+2.9	0	0
CACO2_PTH24	+2.8	0	0
CACO2_PTH4	+2.6	0	0
DN_TCF4_UP	+2.3	0	0
CELECOXIB_DN	+2.0	0	0.001
CRC_UP	+1.8	0	0.005
ADENOMA_UP	+1.6	0	0.03
CRYPT_BASE	+1.6	0	0.03
DN_TCF_DN	+1.0	0.36	0.47
CRC_DOWN	+1.6	0.009	0.03
CELECOXIB_UP	-1.1	0.3	0.70
ADENOMA_DOWN	-1.2	0.06	0.55
CD133_STEMCELLS	-1.4	0.05	0.27
PTH_BONE_UP	-1.5	0	0.21
PTH_MUSCLE_UP	-1.6	0	0.12

7B.4.3 Comparison of the genes sets correlated with PTH and changed in response to calcium supplementation

To further address the hypothesis that calcium acts on the colonic epithelium by modulating serum PTH, comparison was made between the gene sets changed in response to calcium and those correlated with PTH. It has already been shown that the **pth_neg** set of genes containing the 500 genes most strongly negatively correlated with PTH in-vivo is strongly enriched in the genes up-regulated by calcium. It was predicted that there would be overlap between the other gene sets enriched in the treated with calcium phenotype and the negatively correlated with PTH phenotype. Conversely it was predicted that there would be overlap between the gene sets enriched in the

control phenotype and the positively correlated with PTH phenotype. Comparison was made between all of the gene sets enriched with an FDR<0.05 in the negatively correlated with PTH (104) and treated by calcium phenotypes (204). This showed fifty-nine gene sets which were enriched in both phenotypes; these are shown in Appendix 13. Perhaps more importantly overlap between the top twenty-five gene sets revealed that there were four gene sets in common, these are; **crypt_top**, **caco2_pthall**, **UVC_HIGH_ALL _DN** and **FLECHNER_KIDNEY_ TRANSPLANT_WELL_UP**. The gene set **crypt_top** was the most highly enriched gene set in both the negatively correlated with PTH phenotype and the up-regulated by calcium phenotype.

Comparison was made between the gene sets enriched with an FDR<0.05 in the positively correlated with PTH (20) and control (untreated with calcium) phenotypes (11). There were seven overlapping gene sets; these are shown in Appendix 14. There were no gene sets that overlapped in the opposite unpredicted fashion.

7B.5 Discussion

This analysis has demonstrated that dietary calcium supplementation results in the differential expression of a number of sets of genes in the rectal mucosa of the individuals studied. The identification of these gene sets provides evidence on the effect of calcium on the normal rectal mucosa and support for its anti-neoplastic effect. A general observation is the finding that there are a large number of genes enriched in the treated by calcium phenotype and relatively few in the control phenotype. This finding is consistent with the single gene analysis, which identified more genes up-regulated by calcium than down. The large number of enriched gene sets could be seen to make further analysis difficult. However, as a method using ranking, the important gene sets are those found at the top of the list of enriched sets. There is also a lot of overlap in the biological processes represented by the gene sets, hence the high number of enriched gene sets.

7B.5.1 Action of calcium on the colonic epithelium

The aim in analysing this study was to determine the biological processes that are altered in response to calcium by analysing gene sets representing known biological processes. The most obvious finding was the **crypt_top** gene set being the most highly enriched. This observation suggests that calcium results in the increased expression of genes commonly found in differentiated post-mitotic cells at the top of the colonic crypt. This is consistent with the findings of the single gene analysis. In this, the annotation terms enriched in the differentially up-regulated genes represented a number of biological features of differentiated crypt cells (See Appendix 5, 6 and 7). Differentiation of colonic epithelial cells occurs as a consequence of decreased Wnt pathway

signalling, with evidence for this from both in-vitro (Mariadason, Bordonaro et al. 2001) and in-vivo studies (Sansom, Reed et al. 2004). There is also significant overlap between the genes found in differentiated colonic cells and those negatively regulated by wnt signalling (Kosinski, Li et al. 2007). This suggests that calcium induces differentiation by negatively regulating the wnt signalling pathway. Consistent with this is the observation that the set of genes negatively regulated by wnt signalling in vitro **dn_tcf4_up** (van de Wetering, Sancho et al. 2002) is also enriched in the genes up-regulated by calcium. Other evidence that the effect of calcium is mediated by modulation of the Wnt signalling pathway was provided by the single gene analysis. In this, it was observed that calcium supplementation resulted in the down-regulation of a number of genetic markers of entero-endocrine cells. One consequence of the deletion of TCF4 in mice is the absence of entero-endocrine cells, whilst mature enterocytes and goblet cells remain (Korinek, Barker et al. 1998). This suggests that Wnt signalling controls the development of entero-endocrine cells. The effect of calcium in reducing markers of entero-endocrine cells is therefore consistent with a suppressive effect on wnt signalling. Modulation of wnt signalling by calcium in combination with vitamin D has been demonstrated in a micro-array study of gene expression in colonic mucosa of mice (Yang, Kurihara et al. 2008). In this study mice fed a „Western diet' showed changes similar to those found in APC mutant mice. These changes were reversed by calcium and vitamin D. Calcium and vitamin D most notably decreased expression of the wnt effectors beta-catenin and TCF (Yang, Kurihara et al. 2008).

The converse was not seen with calcium having no significant effect on the expression of proliferation associated genes found at the crypt base or those positively regulated by wnt signalling. These results suggest that calcium supplementation increases the numbers of differentiated cells within the crypt.

7B.5.2 Comparison with previous studies of calcium

The induction of genes expressed in differentiated cells by calcium adds to the growing evidence that calcium acts to induce differentiation in the colonic crypt. A study of ninety two patients demonstrated that calcium increased the expression of p21 (CDKN1A), a cyclin-dependent kinase inhibitor only expressed in fully differentiated cells but had no effect on the expression of the proliferation marker MIB-1 (Fedirko, Bostick et al. 2009). Although p21 was not recognised as changed by calcium using either GCOS or RP, it was up-regulated in 2/6 samples using GCOS with a fold change of +1.15. A number of studies have examined the effect of calcium on proliferating cells on the colonic crypt. The common finding in these studies is a reduction in the number of proliferating cells in the upper parts of the crypt, with a redistribution of the proliferation zone to the lower portion of the crypt (Nobre-Leitao, Chaves et al. 1995; Holt, Atillasoy et al. 1998; Holt, Wolper et al. 2001). In the only randomised double blind placebo controlled trial, with 170 patients, no change in overall proliferative index was seen. However, again there was a decrease in the number of proliferating cells in the upper portion of the crypt, resulting in what the authors describe as a normalisation of the distribution of proliferating cells (Bostick, Fosdick et al. 1995). This study has used a different method to those above to investigate the effect of calcium on

colorectal epithelium. In demonstrating an increase in the expression of genes found in differentiated colonic cells the results are consistent with the findings above, showing that calcium results in the earlier differentiation of cells as they migrate up the crypt.

7B.5.1.3 Relationship between gene sets differentially expressed in response to calcium and colorectal neoplasia

There was no clear relationship between calcium supplementation and the gene sets representing the changes found in colorectal neoplasia. The genes both up and down regulated in colorectal cancer (**crc_up** and **crc_down**) were weakly enriched in the calcium treated phenotype. The adenoma gene sets (**adenoma_up** and **adenoma_down**) were also weakly enriched and in the directions opposite of that predicted. This suggests that the effect of calcium is not just to result in the expression of genes in a direction opposite to that seen in colorectal neoplasia. This is not surprising, as the transcriptional changes found in colorectal neoplasia represent the consequences of multiple genetic changes. Further explanation for this is that some of the genes found in the differentiated crypt top are also increased in neoplastic compared to normal colon. Amphiregulin (AREG) is frequently up-regulated in colorectal cancer but is also expressed in differentiated cells at the crypt top (Saeki, Stromberg et al. 1992; Kosinski, Li et al. 2007) and was up-regulated by calcium. Another set of genes commonly down regulated in colorectal neoplasia are genes found in entero-endocrine cells, these were also down regulated in response to calcium. Thus the effect of calcium is not just to alter the expression of genes altered in colorectal neoplasia.

7B.5.1.4 Effect of calcium on normal mucosal markers of the risk of neoplasia

There are features of normal epithelium that have been recognised to indicate an increased risk of colorectal neoplasia. These are of relevance to the action of calcium. Firstly, the presence of proliferating cells in the upper part of the colonic crypt has been demonstrated to predict the risk of current (Ponz de Leon, Roncucci et al. 1988; Risio, Lipkin et al. 1991) or future neoplasia (Scalmati, Roncucci et al. 1990; Anti, Marra et al. 1993). In this study, calcium results in increased expression of markers of differentiation, consistent with a reduction in proliferating cells in the upper portion of the crypt; as has been demonstrated by different methods by others (Bostick, Fosdick et al. 1995; Holt, Atillasoy et al. 1998; Holt, Wolper et al. 2001).

The second feature of normal mucosa that has been shown to predict current (Anti, Armuzzi et al. 2001) and future neoplasia (Keku, Amin et al. 2008) is reduced apoptosis. Apoptosis is a feature of differentiated cells and the **crypt_top** gene set contains genes involved in apoptosis. Apoptosis is also a biological feature of some of the other gene sets enriched in the calcium treated phenotype; **REOVIRUS_HEK293_UP** and **UVC_HIGH_ALL_DN** (DeBiasi, Clarke et al. 2003; Gentile, Latonen et al. 2003). As was discussed in the previous part of this chapter, the differentially expressed genes were also enriched with apoptosis related annotation terms. Calcium has also been shown in other studies to result in increased apoptosis (Miller, Keku et al. 2005; Fedirko, Bostick et al. 2009). These observations indicate that calcium supplementation influences normal mucosa in a manner predicted to reduce the incidence of neoplasia. This is consistent with its

observed chemo-preventive action. It also underlines the importance of understanding the mechanism of action of calcium.

7B.5.5Mechanism of action of calcium

The preceding discussion describes the consequences of calcium supplementation on the rectal epithelium. These are consistent with calcium having an anti-neoplastic effect. An understanding of the mechanism of action of calcium is more pertinent for developing the use of calcium as a chemo-preventive agent. In particular the recognition of individuals who are likely to benefit from calcium and are at risk of colorectal neoplasia would provide a rationale for its use in a chemo-preventive strategy. The hypothesis of this thesis is that that raised PTH acts on the normal colon increasing the risk of neoplasia and that calcium acts by reducing serum PTH. There are a number of results in this chapter that support the hypothesis that calcium influences gene expression in the rectal mucosa by modulating PTH level. Firstly, the influence of calcium on gene expression was predominantly to increase the expression of certain genes and the effect of PTH both in-vitro and in-vivo was predominantly to decrease the expression of certain genes. This raises the possibility that PTH signalling results in transcriptional repression and that calcium, by reducing PTH relieves that repression. This appears to be the case as the gene sets highly enriched in the calcium treated phenotype include the gene sets negatively correlated with serum PTH in-vivo (**pth_neg**) and down-regulated by PTH in-vitro (**caco2_pth_all**). Comparison with the GSEA results in chapter 6B show that **crypt_top** is also the most highly enriched gene set in the negatively correlated with PTH phenotype.

Perhaps the most striking finding in support of this hypothesis was that

the set of genes down-regulated by PTH in-vitro (**caco2_pth_all**) were statistically significantly enriched in the genes up-regulated by calcium. This is striking because it is unusual to find overlapping results from micro-array experiments studying the same biological process (Subramanian, Tamayo et al. 2005). This is particularly when comparing in-vitro and in-vivo studies using different array chips, with two seemingly different interventions. This finding was suggested in the single gene analysis, but was dependent on the finding of only a small number of overlapping genes. This demonstrates the power of GSEA to identify the underlying biological processes where fold changes are relatively small.

As was discussed above the pro-differentiation effect of calcium appears to be mediated by abrogation of wnt signalling. Parathyroid hormone is known to stimulate the wnt signalling pathway in bone (Kulkarni, Halladay et al. 2005) and PTH activation of PTHR1 induces it to interact with the wnt effecter molecule dishevelled resulting in nuclear localisation and activation of beta-catenin independent of wnt (Romero, Sneddon et al. 2010). In the in-vitro studies of Caco2, PTH treatment down-regulated wnt signalling pathway genes suggesting that the wnt signalling pathway was stimulated. Similarly in chapter three the genes negatively correlated with PTH (**pth_neg**) are those found in differentiated colonic cells which are negatively regulated by wnt signalling (Mariadason, Bordonaro et al. 2001). This suggests that PTH up regulates the wnt signalling pathway resulting in down regulation of differentiation related genes.

There is considerable overlap between the gene sets enriched in the calcium treated phenotype and correlated with PTH phenotype. This provides support

for the validity of the results presented in chapter six, indicating that inter-individual variation in gene expression is mediated in part by variation in serum PTH levels. It is of course relevant that for each of the six individuals studied here calcium supplementation resulted in a significant reduction in serum PTH level. This selection ensured that the hypothesised mechanism of action of calcium could be studied and further studies would need to address whether calcium had any effect on gene expression in the absence of a change in PTH level.

An important negative finding is that there was no relationship with the gene sets (**celecoxib_up** and **celecoxib_down**). This indicates that calcium acts by a different mechanism to the COX-2 inhibitor, Celecoxib. The gene sets enriched in the control phenotype provide limited biological information, having no clear unifying biological theme. However, there is also overlap between these gene sets and those positively correlated with PTH. Similarly the two PTH related gene sets (**pth_muscle_up** and **pth_bone_up**), are weakly enriched in the control phenotype, as well as the positively correlated with PTH phenotype. It is also noteworthy that the **cd133_stemcells** gene set is weakly enriched in the control phenotype as well as the positively correlated with PTH phenotype. This raises the possibility that calcium acting via PTH influences colonic stem cells. An influence of calcium, acting via PTH, on colonic stem cells would be consistent with its anti-neoplastic effect, as well as the known functions of PTH. However, this is only hinted at by the data here and further studies would be needed to clarify this. Further studies using different approaches should be undertaken to confirm the changes in

expression of the genes identified in this study. Other approaches could include real-time PCR or immuno-histochemistry.

7B.5.6 Conclusion

This study demonstrates that dietary calcium supplementation results in the increased expression of genes found in differentiated crypt top cells. This suggests that calcium acts by down-regulation of wnt signalling with consequent induction of differentiation. These calcium induced changes in the normal crypt are consistent with a reduced risk of colorectal cancer and contribute further evidence to support an anti-neoplastic action of calcium.

The most important aspect of these results is the overlap between genes regulated by calcium and those regulated by PTH in-vitro and in-vivo. This provides evidence that calcium acts by suppressing serum PTH levels and that it is serum PTH that acts on the rectal epithelium inhibiting the differentiation of colonic cells by stimulating Wnt signalling. It also provides evidence to support the hypothesis that increased serum PTH is a risk factor for the development of colorectal neoplasia as evidenced by its effect on the normal colorectal mucosa.

Chapter 8

CONCLUDING REMARKS

8.1 Calcium acts on the normal colorectal mucosa

The aim of this study was to investigate the effect of calcium on normal rectal epithelium. The main finding was that calcium supplementation results in the increased expression of genes found in differentiated colorectal cells. This suggests that calcium induces differentiation, which occurs as a result of suppression of Wnt signalling. This finding is consistent with previously observed effects of calcium, detected by different methods. It is also indicates that calcium has an anti-neoplastic effect on normal colorectal epithelium.

8.2 Relationship between the effects of dietary calcium and PTH on gene expression

A hypothesis was put forward in this thesis: it proposed that PTH acted directly on colorectal epithelium and that the effects of calcium would have opposing effects by modulating serum PTH. Within the confines of this small study, there is evidence to support this hypothesis. The main support comes from the observed overlap between the genes differentially expressed in response to calcium and those regulated by PTH in-vitro and in-vivo.

Micro-array studies are generally not used to address specific hypotheses but are often used in a more exploratory role. However, the use of GSEA allowed specific hypotheses to be addressed and, I believe, provided significant support for the hypothesis. The results of these studies clearly require further investigation to explore the observations made. The clinical study has provided more samples, which will allow alternative

approaches to investigate further the influence of PTH and calcium on the rectal mucosa.

The evidence linking raised serum PTH to an increased risk of colorectal cancer and the results from this study may have implications for the use of calcium in preventing colorectal neoplasia in routine clinical practice.

There are also other implications of a raised serum PTH being a risk factor for the development of colorectal cancer. The influence of an endocrine factor on tumorigenesis is novel; colorectal neoplasia classically results from events within the cell rather than external hormonal influence. Hyperparathyroidsim is common in the general public, particularly in winter, at high latitudes, it is also potentially reversible. Finally as was discussed in the introduction to this thesis; calcium and vitamin D act together on colorectal neoplasia. These observations have implications for the proposed direct mechanism of action of Vitamin D.

Appendices
Appendix 1

The one hundred and fifty six genes identified as differentially up regulated by calcium using the GCOS>3 approach. The genes are listed by the number of subjects in which an increase was seen and then by fold change. I= Number of subject showing increased expression. FC=fold change

Gene symbol	Gene title	I	FC
TMPRSS2	transmembrane protease, serine 2	6	1.52
EZR	ezrin (villin 2)	6	1.31
CLCA4	chloride channel, calcium activated, family member 4	5	1.73
PDE9A	phosphodiesterase 9A	5	1.49
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	5	1.34
IER3	immediate early response 3	5	1.32
LDLR	low density lipoprotein receptor	5	1.28
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	4	1.71
ACTG2	actin, gamma 2, smooth muscle, enteric	4	1.64
FHL1	four and a half LIM domains 1	4	1.63
ISG20	interferon stimulated exonuclease gene 20kDa	4	1.57
CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1	4	1.56
RHOF	ras homolog gene family, member F (in filopodia)	4	1.45
DHRS9	dehydrogenase/reductase (SDR family) member 9	4	1.45
TRIM31	tripartite motif-containing 31	4	1.43
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	4	1.39
PLA2G2A	phospholipase A2, group IIA (platelets, synovial fluid)	4	1.37
ARL14	ADP-ribosylation factor-like 14	4	1.34
CYP51A1	cytochrome P450, family 51, subfamily A, polypeptide 1	4	1.32
IDI1	isopentenyl-diphosphate delta isomerase 1	4	1.25
GCNT3	glucosaminyl (N-acetyl) transferase 3, mucin type	4	1.25
TWF1	twinfilin, actin-binding protein, homolog 1 (Drosophila)	4	1.24
LAMA3	laminin, alpha 3	4	1.23
CFTR	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	4	1.19
RNF19B	ring finger protein 19B	4	1.19
FOS	v-fos FBJ murine osteosarcoma viral	4	-1.08

	oncogene homolog		
SLC6A14	solute carrier family 6 (amino acid transporter), member 14	3	2.13
HBEGF	heparin-binding EGF-like growth factor	3	1.89
FKBP5	FK506 binding protein 5	3	1.82
SPINK4	serine peptidase inhibitor, Kazal type 4	3	1.8
TM4SF1	transmembrane 4 L six family member 1	3	1.77
DUOX2	dual oxidase 2	3	1.63
AREG	amphiregulin	3	1.56
RARRES3	retinoic acid receptor responder3	3	1.53
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	3	1.45
PLN	phospholamban	3	1.45
COL1A2	collagen, type I, alpha 2	3	1.45
GNA13	guanine nucleotide binding protein (G protein), alpha 13	3	1.43
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	3	1.41
CNN1	calponin 1, basic, smooth muscle	3	1.4
GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	3	1.39
S100P	S100 calcium binding protein P	3	1.39
DUSP6	dual specificity phosphatase 6	3	1.36
OASL	2'-5'-oligoadenylate synthetase-like	3	1.34
PDLIM5	PDZ and LIM domain 5	3	1.34
GREM1	gremlin 1, cysteine knot superfamily, homolog (Xenopus laevis)	3	1.34
KCNK1	potassium channel, subfamily K, member 1	3	1.33
F2RL1	coagulation factor II (thrombin) receptor-like 1	3	1.33
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6	3	1.33
TXNIP	thioredoxin interacting protein	3	1.32
IGFBP5	insulin-like growth factor binding protein 5	3	1.31
DPT	dermatopontin	3	1.3
SFN	stratifin	3	1.3
OLFM4	olfactomedin 4	3	1.3
HRASLS2	HRAS-like suppressor 2	3	1.29
SEPT2	septin 2	3	1.29
CALD1	caldesmon 1	3	1.29
SC4MOL	sterol-C4-methyl oxidase-like	3	1.29
DSC2	desmocollin 2	3	1.28
INPP1	inositol polyphosphate-1-phosphatase	3	1.28
MMP12	matrix metallopeptidase 12	3	1.28
FHL2	four and a half LIM domains 2	3	1.27
ETNK1	ethanolamine kinase 1	3	1.27
S100A14	S100 calcium binding protein A14	3	1.27
TRIM15	tripartite motif-containing 15	3	1.27
ELOVL6	ELOVL family member 6, elongation of long chain fatty acids	3	1.27

KLF6	Kruppel-like factor 6	3	1.27
TRIB1	tribbles homolog 1 (Drosophila)	3	1.27
MGP	matrix Gla protein	3	1.27
SNRK	SNF related kinase	3	1.26
COL6A3	collagen, type VI, alpha 3	3	1.26
COL1A1	collagen, type I, alpha 1	3	1.26
SLC26A3	Solute carrier family 26, member 3	3	1.25
SFRS3	splicing factor, arginine/serine-rich 3	3	1.25
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	3	1.25
ATP6V1A	ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A	3	1.25
PTP4A1	protein tyrosine phosphatase type IVA, member 1	3	1.24
CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	3	1.24
LMNA	lamin A/C	3	1.24
PAFAH1B1	platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	3	1.24
DES	desmin	3	1.24
AA156240	CDNA clone IMAGE:6025865	3	1.24
MAPK6	mitogen-activated protein kinase 6	3	1.23
GLS	glutaminase	3	1.22
YWHAZ	tyrosine 3-monooxygenase	3	1.22
GLUL	glutamate-ammonia ligase (glutamine synthetase)	3	1.22
HSPD1	heat shock 60kDa protein 1 (chaperonin)	3	1.22
DNAJA1	DnaJ (Hsp40) homolog, subfamily A, member 1	3	1.22
ID1	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	3	1.22
MYL9	myosin, light chain 9, regulatory	3	1.22
CD59	CD59 molecule, complement regulatory protein	3	1.21
FEM1C	fem-1 homolog c (C. elegans)	3	1.21
AMD1	adenosylmethionine decarboxylase 1	3	1.21
TNC	tenascin C (hexabrachion)	3	1.21
MAR6	membrane-associated ring finger (C3HC4) 6	3	1.21
MUC4	mucin 4, cell surface associated	3	1.2
RRM2	ribonucleotide reductase M2 polypeptide	3	1.2
BMP2	bone morphogenetic protein 2	3	1.2
CD97	CD97 molecule	3	1.2
ANXA1	annexin A1	3	1.2
TAGLN	transgelin	3	1.2
RFK	riboflavin kinase	3	1.2
KLF4	Kruppel-like factor 4 (gut)	3	1.19
RRAGC	Ras-related GTP binding C	3	1.19
SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4	3	1.19

MYH11	myosin, heavy chain 11, smooth muscle	3	1.19
MGC14376	hypothetical protein MGC14376	3	1.19
CLIC5	chloride intracellular channel 5	3	1.19
CEACAM7	carcinoembryonic antigen-related cell adhesion molecule 7	3	1.18
MUC13	mucin 13, cell surface associated	3	1.18
TNFRSF21	tumor necrosis factor receptor superfamily, member 21	3	1.18
IFI27	interferon, alpha-inducible protein 27	3	1.18
TMEM70	transmembrane protein 70	3	1.17
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	3	1.17
PLEKHC1	pleckstrin homology domain containing, family C (with FERM domain) member 1	3	1.17
GUCA2B	guanylate cyclase activator 2B (uroguanylin)	3	1.17
SPRED2	sprouty-related, EVH1 domain containing 2	3	1.16
IFNGR1	interferon gamma receptor 1	3	1.16
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase	3	1.16
FDFT1	farnesyl-diphosphate farnesyltransferase 1	3	1.15
PALLD	palladin, cytoskeletal associated protein	3	1.15
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	3	1.15
CAV1	caveolin 1, caveolae protein, 22kDa	3	1.15
CRIP1	cysteine-rich protein 1 (intestinal)	3	1.14
TNS1	tensin 1	3	1.14
SPARC	secreted protein, acidic, cysteine-rich (osteonectin)	3	1.14
CRK	v-crk sarcoma virus CT10 oncogene homolog (avian)	3	1.13
ANKRD12	ankyrin repeat domain 12	3	1.12
UBE2A	ubiquitin-conjugating enzyme E2A (RAD6 homolog)	3	1.11
SLC30A1	solute carrier family 30 (zinc transporter), member 1	3	1.11
TAGLN2	transgelin 2	3	1.11
SPINK5	serine peptidase inhibitor, Kazal type 5	3	1.11
MAP1LC3B	microtubule-associated protein 1 light chain 3 beta	3	1.1
hCG_1776259	hypothetical protein FLJ23556	3	1.1
FAS	Fas (TNF receptor superfamily, member 6)	3	1.09
SLC44A4	solute carrier family 44, member 4	3	1.09
KIAA0101	KIAA0101	3	1.09
FLNA	filamin A, alpha (actin binding protein 280)	3	1.09
BCL10	B-cell CLL/lymphoma 10	3	1.08
CYP3A5	cytochrome P450, family 3, subfamily A, polypeptide 5	3	1.08
RBM25	RNA binding motif protein 25	3	1.07
RBM5	RNA binding motif protein 5	3	1.07

STX3	syntaxin 3	3	1.07
ACSL1	acyl-CoA synthetase long-chain family member 1	3	1.06
CXADR	coxsackie virus and adenovirus receptor	3	1.06
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3	1.06
APPL2	adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 2	3	1.05
CD177	CD177 molecule	3	1.05
PTPRC	protein tyrosine phosphatase, receptor type, C	3	1.05
COL6A2	collagen, type VI, alpha 2	3	1.04
COL15A1	collagen, type XV, alpha 1	3	1.02
LIN7C	lin-7 homolog C (C. elegans)	3	1.01
SLC1A1	solute carrier family 1 member 1	3	1.01
RDH11	retinol dehydrogenase 11 (all-trans/9-cis/11-cis)	3	1
ZKSCAN1	zinc finger with KRAB and SCAN domains 1	3	-1.07
EFNA1	ephrin-A1	3	-1.08

Appendix2

The seventy seven genes identified as differentially down regulated by calcium using the GCOS>3 approach. The genes are listed by the number of subjects in which a decrease was seen and then by fold change. D= Number of subject showing decreased expression. FC= Fold change

Gene symbol	Gene title	D	FC
HBB	hemoglobin, beta	4	-1.35
CXCL14	chemokine (C-X-C motif) ligand 14	4	-1.22
HBA2	hemoglobin, alpha 2	4	-1.22
IGLV2-14	immunoglobulin lambda variable 2-14	4	-1.21
HBA1	hemoglobin, alpha 1	4	-1.2
SORL1	sortilin-related receptor, L(DLR class) A repeats-containing	4	-1.14
IGL@	Immunoglobulin lambda locus	4	-1.05
RIMBP2	RIMS binding protein 2	3	-1.62
SLC22A4	solute carrier family 22 (organic cation transporter), member 4	3	-1.53
CHGB	chromogranin B (secretogranin 1)	3	-1.48
TNFRSF17	tumor necrosis factor receptor superfamily, member 17	3	-1.44
IGHA1	Immunoglobulin heavy constant alpha 1	3	-1.41
IGKC	Immunoglobulin kappa constant	3	-1.41
STAP1	signal transducing adaptor family member 1	3	-1.39
MSTP9	macrophage stimulating, pseudogene 9	3	-1.39
SST	somatostatin	3	-1.35
PILRB	paired immunoglobin-like type 2 receptor beta	3	-1.35

AMPD1	adenosine monophosphate deaminase 1 (isoform M)	3	-1.34
C10orf116	chromosome 10 open reading frame 116	3	-1.33
ALOX5	arachidonate 5-lipoxygenase	3	-1.33
SLC6A8	solute carrier family 6 (neurotransmitter transporter, creatine), member 8	3	-1.32
HAPLN1	hyaluronan and proteoglycan link protein 1	3	-1.31
MT1M	metallothionein 1M	3	-1.3
IGLJ3	immunoglobulin lambda joining 3	3	-1.29
IGKV1D-13	immunoglobulin kappa variable 1D-13	3	-1.26
RNASE6	ribonuclease, RNase A family, k6	3	-1.24
ADAMDEC1	ADAM-like, decysin 1	3	-1.24
ABCA8	ATP-binding cassette, sub-family A (ABC1), member 8	3	-1.23
TYROBP	TYRO protein tyrosine kinase binding protein	3	-1.23
PYY	peptide YY	3	-1.23
TRPM6	transient receptor potential cation channel, subfamily M, member 6	3	-1.23
NDRG1	N-myc downstream regulated gene 1	3	-1.22
GCG	glucagon	3	-1.21
CHGA	chromogranin A (parathyroid secretory protein 1)	3	-1.21
GOLGA8B	golgi autoantigen, golgin subfamily a, 8B	3	-1.21
AMACR	alpha-methylacyl-CoA racemase	3	-1.2
TPH1	tryptophan hydroxylase 1 (tryptophan 5-monooxygenase)	3	-1.2
HLA-DPA1	major histocompatibility complex, class II, DP alpha 1	3	-1.2
CKB	creatine kinase, brain	3	-1.19
LIPA	lipase A, lysosomal acid, cholesterol esterase	3	-1.18
IGSF6	immunoglobulin superfamily, member 6	3	-1.18
INSM1	insulinoma-associated 1	3	-1.17
GALNT12	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 12 (GalNAc-T12)	3	-1.17
DNASE1L3	deoxyribonuclease I-like 3	3	-1.17
SLC26A2	solute carrier family 26 (sulfate transporter), member 2	3	-1.17
RHOBTB3	Rho-related BTB domain containing 3	3	-1.17
FXYD3	FXYD domain containing ion transport regulator 3	3	-1.17
IL1R2	interleukin 1 receptor, type II	3	-1.16
MT1H	metallothionein 1H	3	-1.15
HLA-DQA1	major histocompatibility complex, class II, DQ alpha 1	3	-1.15
SCG5	secretogranin V (7B2 protein)	3	-1.14
GREM2	gremlin 2, cysteine knot superfamily,	3	-1.14

	homolog (Xenopus laevis)		
HLA-DRB1	major histocompatibility complex, class II, DR beta 1	3	-1.13
IL8	Interleukin 8	3	-1.13
CCL21	chemokine (C-C motif) ligand 21	3	-1.13
MT1F	metallothionein 1F	3	-1.12
MT1G	metallothionein 1G	3	-1.11
TGOLN2	trans-golgi network protein 2	3	-1.11
TFF3	trefoil factor 3 (intestinal)	3	-1.11
TTRAP	TRAF and TNF receptor associated protein	3	-1.1
BAMBI	BMP and activin membrane-bound inhibitor homolog	3	-1.09
PLOD2	procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	3	-1.09
PTTG1	pituitary tumor-transforming 1	3	-1.09
INSL5	insulin-like 5	3	-1.09
MT2A	metallothionein 2A	3	-1.08
SLC3A1	solute carrier family 3, member 1	3	-1.08
FTH1	ferritin, heavy polypeptide 1	3	-1.07
WASL	Wiskott-Aldrich syndrome-like	3	-1.03
IGKC	immunoglobulin kappa constant	3	-1.03
PCK1	phosphoenolpyruvate carboxykinase 1 (soluble)	3	-1.02
PIGR	polymeric immunoglobulin receptor	3	-1.02
CCNA2	cyclin A2	3	-1.01
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	3	-1
IGHG1	immunoglobulin heavy constant gamma 1 (G1m marker)	3	1.01
MUC2	mucin 2, oligomeric mucus/gel-forming	3	1.01
WWTR1	WW domain containing transcription regulator 1	3	1.05
LOC339047	hypothetical protein LOC339047	3	1.07
RSAD2	radical S-adenosyl methionine domain containing 2	3	1.56

Appendix 3

The sixty eight genes identified as differentially up regulated in response to calcium using Rank Product.The genes are ranked by percentage false positive (PFP) and then fold change. PFP is equivalent to the false detection rate (FDR). FC= Fold change

Gene Symbol	Description	FC	PFP
TM4SF1	transmembrane 4 L six family member 1	+2.12	0
CLCA4	chloride channel regulator 4	+1.81	0
CCL20	chemokine (C-C motif) ligand 20	+1.78	0
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	+1.66	0
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	+1.43	0
TRIM31	tripartite motif-containing 31	+1.88	0.001
GNA13	guanine nucleotide binding protein (G protein), alpha 13	+1.54	0.002
EREG	epiregulin	+1.50	0.003
LARP4	La ribonucleoprotein domain family, member 4	+1.62	0.003
TMPRSS 2	transmembrane protease, serine 2	+1.59	0.003
SPINK4	serine peptidase inhibitor, Kazal type 4	+1.53	0.003
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	+1.72	0.009
HBEGF	heparin-binding EGF-like growth factor	+1.62	0.009
DPT	dermatopontin	+1.44	0.01
CXCL9	chemokine (C-X-C motif) ligand 9	+1.55	0.012
ELOVL6	ELOVL family member 6, elongation of long chain fatty acids	+1.71	0.013
OLFM4	olfactomedin 4	+1.73	0.013
EZR	ezrin	+1.49	0.014
DUOX2	dual oxidase 2	+1.87	0.017
IL6ST	interleukin 6 signal transducer (gp130, oncostatin M receptor)	+1.44	0.02
MXD1	MAX dimerization protein 1	+1.52	0.024
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	+1.40	0.024
NCOA2	nuclear receptor coactivator 2	+1.43	0.025
CALD1	caldesmon 1	+1.41	0.026
SLC6A14	solute carrier family 6 (amino acid transporter), member 14	+1.58	0.028
NEAT1	nuclear paraspeckle assembly transcript 1	+1.29	0.029
PLN	phospholamban	+1.43	0.032
ATF3	activating transcription factor 3	+1.59	0.033
HIPK3	homeodomain interacting protein kinase 3	+1.39	0.034

CMAH	cytidine monophosphate-N-acetylneuraminic acid hydroxylase	+1.36	0.035
ACTG2	actin, gamma 2, smooth muscle, enteric	+1.38	0.04
ABCG2	ATP-binding cassette, sub-family G (WHITE), member 2	+1.41	0.042
MFAP5	microfibrillar associated protein 5	+1.44	0.043
FHL1	four and a half LIM domains 1	+1.35	0.045
SCYL2	SCY1-like 2 (S. cerevisiae)	+1.43	0.045
CLIC5	chloride intracellular channel 5	+1.47	0.047
ETNK1	ethanolamine kinase 1	+1.49	0.05
EPHA2	EPH receptor A2	+1.44	0.051
DHRS9	dehydrogenase/reductase (SDR family) member 9	+1.54	0.054
CEACAM 1	carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	+1.56	0.058
CFTR	cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)	+1.34	0.059
LDLR	low density lipoprotein receptor	+1.34	0.06
KPNA4	karyopherin alpha 4 (importin alpha 3)	+1.50	0.06
PAFAH1 B1	platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	+1.44	0.06
AREG	amphiregulin	+1.41	0.06
FKBP5	FK506 binding protein 5	+1.22	0.06
KIF2A	kinesin heavy chain member 2A	+1.55	0.061
TWF1	twinfilin, actin-binding protein, homolog 1 (Drosophila)	+1.41	0.061
TXNIP	thioredoxin interacting protein	+1.23	0.062
IER3	immediate early response 3	+1.35	0.062
SGK1	serum/glucocorticoid regulated kinase 1	+1.51	0.064
GREM1	gremlin 1, cysteine knot superfamily	+1.31	0.066
ARL14	ADP-ribosylation factor-like 14	+1.52	0.068
IGFBP5	insulin-like growth factor binding protein 5	+1.31	0.073
SCAMP1	secretory carrier membrane protein 1	+1.42	0.074
MMP12	matrix metallopeptidase 12 (macrophage elastase)	+1.15	0.082
GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	+1.32	0.083
PDE9A	phosphodiesterase 9A	+1.39	0.084
KNTC1	kinetochore associated 1	+1.35	0.087
THBS1	thrombospondin 1	+1.29	0.087
COL1A2	collagen, type I, alpha 2	+1.37	0.087
ZBTB16	zinc finger and BTB domain containing 16	+1.13	0.088
SLC4A4	solute carrier family 4, sodium bicarbonate cotransporter, member 4	+1.37	0.088

SYNM	synemin, intermediate filament protein	+1.48	0.088
ZNF165	zinc finger protein 165	+1.42	0.089
CLIC4	chloride intracellular channel 4	+1.43	0.089
GUCA2B	guanylate cyclase activator 2B (uroguanylin)	+1.39	0.094
MGP	matrix Gla protein	+1.09	0.097

Appendix 4

Shows the eight genes identified as differentially down regulated in response to calcium using Rank Product. The genes are ranked by percentage false positive (PFP) and then fold change.

Gene Symbol	Description	FC	PFP
SST	somatostatin	-1.75	0.003
HBA1	hemoglobin, alpha 1	-1.24	0.005
MT1M	metallothionein 1M	-1.35	0.005
MS4A1	membrane-spanning 4-domains, subfamily A, member 1	-1.01	0.048
IGHG1	Immunoglobulin lambda heavy chain	-1.34	0.052
RNASE6	ribonuclease, RNase A family, k6	-1.18	0.056
CXCL13	chemokine (C-X-C motif) ligand 13	-1.14	0.066
HBB	hemoglobin, beta	-1.31	0.095

Appendix 5

The 117 GO biological process annotation terms enriched in the 179 genes differentially up-regulated by calcium. The p-value and false detection rate (FDR) for each term is shown. The notation E indicates to the power of 10 (x10).

Biological process (BP)	*P* value	FDR
epidermal cell differentiation	6.7E-05	1.1E-01
negative regulation of protein kinase activity	1.3E-04	1.0E-01
muscle organ development	1.5E-04	7.7E-02
sterol biosynthetic process	1.5E-04	6.2E-02
negative regulation of kinase activity	1.7E-04	5.5E-02
regulation of cell migration	1.7E-04	4.6E-02
negative regulation of transferase activity	2.5E-04	5.8E-02
regulation of locomotion	4.0E-04	8.1E-02
regulation of cell motion	4.6E-04	8.2E-02
epithelium development	4.8E-04	7.7E-02
cholesterol biosynthetic process	5.2E-04	7.6E-02
response to organic substance	5.3E-04	7.1E-02
regulation of cell proliferation	6.7E-04	8.2E-02
regulation of MAP kinase activity	7.0E-04	7.9E-02
negative regulation of MAP kinase activity	1.2E-03	1.3E-01
response to mechanical stimulus	1.3E-03	1.2E-01
epithelial cell differentiation	1.3E-03	1.2E-01
epidermis development	1.7E-03	1.5E-01
negative regulation of cell proliferation	1.8E-03	1.4E-01
isoprenoid metabolic process	2.4E-03	1.8E-01
isoprenoid biosynthetic process	2.4E-03	1.8E-01
ectoderm development	3.1E-03	2.1E-01
keratinocyte differentiation	3.2E-03	2.1E-01
response to progesterone stimulus	3.3E-03	2.1E-01
sterol metabolic process	3.7E-03	2.2E-01
negative regulation of transcription factor activity	3.8E-03	2.2E-01
enzyme linked receptor protein signaling pathway	3.9E-03	2.2E-01
positive regulation of defense response	4.1E-03	2.1E-01
regulation of smooth muscle cell proliferation	5.3E-03	2.6E-01
positive regulation of response to stimulus	5.7E-03	2.7E-01
negative regulation of DNA binding	5.7E-03	2.6E-01
negative regulation of cytokine-mediated signaling pathway	6.2E-03	2.7E-01
negative regulation of molecular function	6.6E-03	2.8E-01
steroid biosynthetic process	8.0E-03	3.3E-01
negative regulation of cell migration	8.7E-03	3.4E-01
negative regulation of binding	9.3E-03	3.5E-01
steroid metabolic process	9.4E-03	3.5E-01
positive regulation of phosphorylation	9.8E-03	3.5E-01
regulation of phosphorylation	1.0E-02	3.6E-01
negative regulation of locomotion	1.1E-02	3.6E-01
positive regulation of phosphorus metabolic process	1.1E-02	3.7E-01
positive regulation of phosphate metabolic process	1.1E-02	3.7E-01
regulation of leukocyte chemotaxis	1.2E-02	3.7E-01
cholesterol metabolic process	1.2E-02	3.7E-01

negative regulation of cell motion	1.2E-02	3.6E-01
regulation of transcription factor activity	1.3E-02	3.8E-01
response to wounding	1.3E-02	3.8E-01
regulation of smooth muscle cell migration	1.4E-02	3.9E-01
regulation of phosphate metabolic process	1.4E-02	3.9E-01
regulation of phosphorus metabolic process	1.4E-02	3.9E-01
extracellular matrix organization	1.5E-02	4.1E-01
toll-like receptor signaling pathway	1.6E-02	4.2E-01
response to protein stimulus	1.7E-02	4.2E-01
response to nutrient	1.8E-02	4.3E-01
negative regulation of smooth muscle cell proliferation	1.9E-02	4.5E-01
regulation of binding	1.9E-02	4.5E-01
regulation of protein kinase activity	2.1E-02	4.7E-01
response to steroid hormone stimulus	2.1E-02	4.6E-01
regulation of cytokine-mediated signaling pathway	2.2E-02	4.7E-01
inactivation of MAPK activity	2.2E-02	4.7E-01
maintenance of protein location in cell	2.2E-02	4.7E-01
response to glucose stimulus	2.2E-02	4.7E-01
osteoblast differentiation	2.2E-02	4.7E-01
ossification	2.2E-02	4.6E-01
regulation of leukocyte activation	2.3E-02	4.7E-01
extracellular structure organization	2.3E-02	4.8E-01
negative regulation of immune system process	2.3E-02	4.7E-01
lipid biosynthetic process	2.4E-02	4.7E-01
pattern recognition receptor signaling pathway	2.4E-02	4.7E-01
response to hexose stimulus	2.5E-02	4.7E-01
response to monosaccharide stimulus	2.5E-02	4.7E-01
regulation of DNA binding	2.5E-02	4.7E-01
blood vessel development	2.5E-02	4.7E-01
regulation of kinase activity	2.5E-02	4.7E-01
anti-apoptosis	2.6E-02	4.7E-01
striated muscle tissue development	2.7E-02	4.8E-01
protein kinase cascade	2.7E-02	4.8E-01
cytoskeleton organization	2.7E-02	4.7E-01
bone development	2.8E-02	4.7E-01
positive regulation of innate immune response	2.8E-02	4.7E-01
apoptosis	2.8E-02	4.7E-01
cell motion	2.8E-02	4.7E-01
vasculature development	2.9E-02	4.7E-01
actin cytoskeleton organization	2.9E-02	4.7E-01
regulation of cell activation	2.9E-02	4.7E-01
maintenance of protein location	3.0E-02	4.7E-01
innate immune response-activating signal transduction	3.0E-02	4.7E-01
activation of innate immune response	3.0E-02	4.7E-01
negative regulation of signal transduction	3.0E-02	4.7E-01
regulation of transferase activity	3.1E-02	4.7E-01
tissue morphogenesis	3.1E-02	4.7E-01
programmed cell death	3.2E-02	4.7E-01
muscle tissue development	3.3E-02	4.8E-01
maintenance of location in cell	3.3E-02	4.8E-01
epidermis morphogenesis	3.4E-02	4.8E-01
response to extracellular stimulus	3.4E-02	4.8E-01
positive regulation of cytokine production	3.4E-02	4.8E-01

biological adhesion	3.5E-02	4.8E-01
cell adhesion	3.5E-02	4.8E-01
immune response-activating signal transduction	3.5E-02	4.8E-01
MAPKKK cascade	3.6E-02	4.8E-01
leukocyte mediated immunity	3.6E-02	4.7E-01
terpenoid metabolic process	3.7E-02	4.8E-01
regulation of leukocyte migration	3.7E-02	4.8E-01
response to drug	3.8E-02	4.8E-01
anion transport	3.8E-02	4.9E-01
negative regulation of catalytic activity	3.9E-02	4.9E-01
immune response-regulating signal transduction	3.9E-02	4.9E-01
immune effector process	4.1E-02	5.0E-01
actin filament-based process	4.1E-02	5.0E-01
regulation of innate immune response	4.3E-02	5.1E-01
response to calcium ion	4.3E-02	5.1E-01
positive regulation of cell migration	4.5E-02	5.2E-01
positive regulation of macrophage activation	4.5E-02	5.2E-01
positive regulation of DNA metabolic process	4.7E-02	5.4E-01
regulation of lymphocyte activation	5.0E-02	5.5E-01
positive regulation of immune response	5.0E-02	5.5E-01
response to carbohydrate stimulus	5.0E-02	5.5E-01

Appendix 6

The 34 GO cellular component annotation terms enriched in the 179 genes differentially up-regulated by calcium. The p-value and false detection rate (FDR) for each term is shown.

Cellular component (CC)	*P* value	FDR
extracellular region part	4.9E-05	1.4E-02
plasma membrane	1.5E-04	2.1E-02
extracellular region	1.7E-04	1.6E-02
extracellular matrix	4.3E-04	3.0E-02
proteinaceous extracellular matrix	6.6E-04	3.7E-02
vesicle	7.6E-04	3.6E-02
extracellular matrix part	8.7E-04	3.5E-02
actin cytoskeleton	4.7E-03	1.5E-01
extracellular space	5.5E-03	1.6E-01
contractile fiber part	5.9E-03	1.5E-01
cytoplasmic vesicle	6.2E-03	1.5E-01
contractile fiber	8.1E-03	1.7E-01
insoluble fraction	8.6E-03	1.7E-01
anchoring junction	9.1E-03	1.7E-01
cell cortex	1.2E-02	2.0E-01
cell fraction	1.3E-02	2.0E-01
anchored to membrane	1.4E-02	2.0E-01
collagen	1.4E-02	1.9E-01
plasma membrane part	1.6E-02	2.2E-01
organelle membrane	1.6E-02	2.1E-01
adherens junction	1.8E-02	2.2E-01
membrane-bounded vesicle	2.4E-02	2.6E-01

endoplasmic reticulum membrane	2.8E-02	2.9E-01
collagen type I	3.1E-02	3.1E-01
apical plasma membrane	3.5E-02	3.3E-01
cytoplasmic membrane-bounded vesicle	3.7E-02	3.4E-01
nuclear envelope-endoplasmic reticulum network	3.7E-02	3.3E-01
secretory granule	4.0E-02	3.4E-01
endomembrane system	4.0E-02	3.3E-01
membrane fraction	4.1E-02	3.3E-01
endosomal part	4.2E-02	3.2E-01
endosome membrane	4.2E-02	3.2E-01
cytoskeleton	4.3E-02	3.2E-01
cell surface	4.9E-02	3.5E-01

Appendix 7

The 12 GO molecular function annotation terms enriched in the 179 genes differentially up-regulated by calcium. The p-value and false detection rate (FDR) for each term is shown.

Molecular function (MF)	*P* value	FDR %
extracellular matrix structural constituent	1.0E-03	3.7E-01
identical protein binding	5.3E-03	6.9E-01
anion transmembrane transporter activity	9.2E-03	7.5E-01
structural molecule activity	1.3E-02	7.7E-01
chloride ion binding	1.4E-02	7.1E-01
actin binding	2.2E-02	8.1E-01
anion binding	2.5E-02	8.0E-01
protein dimerization activity	3.4E-02	8.5E-01
enzyme inhibitor activity	3.8E-02	8.5E-01
kinase binding	3.8E-02	8.2E-01
double-stranded DNA binding	4.7E-02	8.6E-01
chloride channel activity	4.9E-02	8.4E-01

Appendix 8

The eight GO biological process annotation terms enriched in the 80 genes differentially down-regulated by calcium. The p-value and false detection rate (FDR) for each term is shown. The notation E indicates to the power of 10 (x10).

Biological process (BP)	*P* value	FDR
immune response	3.1E-08	1.5E-05
antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	2.6E-03	4.8E-01
defense response	6.9E-03	6.8E-01
digestion	9.0E-03	6.7E-01
cell surface receptor linked signal transduction	2.1E-02	8.8E-01
antigen processing and presentation	3.3E-02	9.4E-01
chemotaxis	3.8E-02	9.4E-01
taxis	3.8E-02	9.4E-01

Appendix 9

The six GO cellular component annotation terms enriched in the 80 genes differentially up-regulated by calcium. The p-value and false detection rate (FDR) for each term is shown.

Cellular component (CC)	*P* value	FDR
extracellular region	3.2E-05	3.8E-03
MHC class II protein complex	1.4E-03	8.4E-02
integral to plasma membrane	6.8E-03	2.4E-01
MHC protein complex	1.3E-02	2.7E-01
extracellular region part	2.2E-02	3.6E-01

Appendix 10

The nine GO molecular function annotation terms enriched in the 80 genes differentially up-regulated by calcium. The p-value and false detection rate (FDR) for each term is shown.

Molecular function (MF)	*P* value	FDR %
cadmium ion binding	5.8E-06	1.1E-03
antigen binding	9.1E-04	8.4E-02
chemokine activity	1.1E-03	7.0E-02
MHC class II receptor activity	1.2E-03	5.6E-02
chemokine receptor binding	1.4E-03	5.1E-02
hormone activity	1.4E-03	4.5E-02
iron ion binding	3.0E-03	7.9E-02
copper ion binding	3.9E-03	8.9E-02
cytokine activity	1.2E-02	2.3E-01

Appendix 11

The top twenty five enriched gene sets in the treated with calcium phenotype. The gene sets are grouped into clusters with similar biology or from the same study. A brief description of the biology is given along with references for the source publication The gene sets are from the C2 gene sets found on the MSIGDb website, CGP=chemical and genetic perturbations.

Gene Set	Collection	Tissue origin	Involved biology and source publication
CRYPT_TOP	User defined	Human colon	Genes found in differentiated crypt top cells (Kosinski, Li et al. 2007)
DN_TCF4_UP	User defined	Human colon	Genes negatively regulated by wnt signalling (van de Wetering, Sancho et al. 2002)
CACO2_PTHALL CACO2_PTH24 CACO2_PTH4	User defined	Human colon	Genes down regulated in the Caco2 cell line in response to PTH treatment (Chapter 2).
PTH_NEG	User defined	Human colon	Genes for which expression was negatively correlated with serum PTH levels (Chapter 4).
ET743_SARCOMA_24HRS_DN ET743_SARCOMA_48HRS_DN ET743_SARCOMA_DN ET743_SARCOMA_72HRS_DN	CGP	Sarcoma cell line	Genes down-regulated in soft-tissue sarcoma cell lines in-vitro at different time points following treatment with novel chemotherapy agent (ET743) (Martinez, Sanchez-Beato et al. 2005)
UVB_NHEK3_C1 UVB_NHEK3_ALL UVB_NHEK3_C0	CGP	Primary human keratinocytes	Genes regulated by UVB exposure of primary human keratinocytes (Gentile, Latonen et al. 2003)
UVC_HIGH_ALL_DN UVC_XPCS_ALL_DN	CGP	Human fibroblasts	Gene expression changes due to exposure to UVC radiation. Increased expression of cell cycle arrest and apoptosis associated genes was the main finding(Gentile, Latonen et al. 2003).
IFNA_HCMV_6HRS_UP CMV_HCMV_TIMECOURSE_ALL_UP	CGP	Human fibroblasts	The transcriptional response of fibroblasts to exposure to CMV
REOVIRUS_HEK293_UP	CGP	HEK293	The transcriptional response to exposure to reovirus of human embryonic kidney cells
DSRNA_UP	CGP	Neuronal cell	Transcriptional response of neuronal cell line to the viral component

		line	double-stranded RNA
DER_IFNB_UP	CGP	Fibrosarcoma cell line	Genes up-regulated in fibrosarcoma cells in response to interferon
CHEN_HOXA5_TARGETS_UP	CGP	Breast cancer cell line	Genes up-regulated in response to HOXA5 induction in a breast cancer cell line
FSH_OVARY_MCV152_DN	CGP	Ovarian cell line MCV152	Genes down-regulated in response to FSH stimulation of the ovarian cell line MCV152
FLECHNER_KIDNEY_TRANSPLANT_WELL_UP	CGP	Human kidney transplant	Genes differentially expressed between well functioning and rejected kidneys (Flechner, Kurian et al. 2004)
CANCER_UNDIFFERENTIATED_META _UP	CGP	Various human cancers	Represents a meta-signature of genes enriched in various poorly differentiated cancers, but not derived from colorectal cancer. (Rhodes, Yu et al. 2004)
BRCA_PROGNOSIS_NEG	CGP	Human breast cancers	Genes enriched in poor prognosis breast cancers from patients with BRCA1 mutations.
STEMCELL_ COMMON_UP	CGP	Murine stem cells	Genes commonly enriched in embryonic, neural and haematopoietic stem cells compared to differentiated tissue.

Appendix 12

The top twenty five enriched gene sets in the treated with calcium phenotype. The gene sets are grouped into clusters with similar biology or from the same study. A brief description of the biology is given along with references for the source publication. All the gene sets are from the C2 gene sets found on the MSIGDb website, CGP=chemical and genetic perturbations.

Gene Set	Collection	Tissue origin	Involved biology and source publication
HSA04080_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION GPCRDB_CLASS_A_RHODOPSIN_LIKE GPCRS_CLASS_A_RHODOPSIN_LIKE PEPTIDE_GPCRS	CP	Human	G-protein coupled receptors
REFRACTORY_GASTRIC_UP	CGP	Human gastric cancer	Genes enriched in gastric cancers resistant to treatment with 5FU(Kim, Choi et al. 2004)
FETAL_LIVER_VS_ADULT_LIVER_ GNF2	CP		
IGF_VS_PDGF_UP	CGP	Human myoblasts	Genes differentially expressed between IGF versus PDGF treated myoblasts (Kuninger, Kuzmickas et al. 2004)
MOREAUX_TACI_HI_VS_LOW_UP	CGP	Human multiple myeloma cells (MMC)	Gene expression profiles comparing MMC's with low expression of TACI (Moreaux, Cremer et al. 2005)
STEMCELL_COMMON_DN	CGP	Murine stem cells	Genes commonly down-regulated in embryonic, neural and haematopoietic stem cells compared to differentiated tissue.
HSA03010_RIBOSOME	CP	Generic	Kegg
NUCLEAR_RECEPTORS	CP	Generic	Kegg

Appendix13

The 59 gene sets enriched in both the negatively correlated with PTH phenotype (chapter 6B) and treated by calcium phenotype in the GSEA analyses with a FDR of<0.05. The gene sets of particular interest are highlighted.

Gene sets negatively correlated with PTH and up-regulated by calcium
CRYPT_TOP, HEARTFAILURE_ATRIA_DN, RNA_TRANSCRIPTION_REACTOME, **CRC_DOWN**, UVC_HIGH_ALL_DN, HSC_LATEPROGENITORS_ADULT, HCC_SURVIVAL_GOOD_VS_POOR_DN, ROME_INSULIN_2F_UP, BRCA1_OVEREXP_UP,**CACO2_PTHALL**, HSC_LATEPROGENITORS_SHARED, HSC_LATEPROGENITORS_FETAL, GOLDRATH_HP, FLECHNER_KIDNEY_TRANSPLANT_WELL_UP, **CACO2_PTH24**, STEMCELL_COMMON_UP, SERUM_FIBROBLAST_CORE_UP, ET743_SARCOMA_48HRS_DN, WANG_MLL_CBP_VS_GMP_DN, UVB_NHEK3_C0, UVB_NHEK3_ALL, UVC_HIGH_D7_DN, UVC_HIGH_D3_DN, KUROKAWA_5FU_IFN_SENSITIVE_VS_RESISTANT_DN, CMV_HCMV_TIMECOURSE_16HRS_UP, SMITH_HTERT_UP, ET743_SARCOMA_DN, HSA05131_PATHOGENIC_ESCHERICHIA_COLI_INFECTION_EPEC, REOVIRUS_HEK293_UP, ET743_SARCOMA_24HRS_DN, HSA05130_PATHOGENIC_ESCHERICHIA_COLI_INFECTION_EHEC, UVB_NHEK3_C1, CHANG_SERUM_RESPONSE_UP, UVB_NHEK2_DN, FSH_OVARY_MCV152_DN, HYPOXIA_RCC_NOVHL_UP, HSC_MATURE_ADULT, HDACI_COLON_SUL16HRS_UP, RCC_NL_UP, IGLESIAS_E2FMINUS_UP, ET743_SARCOMA_72HRS_DN, JECHLINGER_EMT_DN, HDACI_COLON_CURSUL_UP, UVB_SCC_UP, GENOTOXINS_ALL_24HRS_REG, HDACI_COLON_SUL48HRS_UP, BAF57_BT549_DN, CMV_HCMV_TIMECOURSE_18HRS_UP, HSC_MATURE_SHARED, PASSERINI_EM, CHEN_HOXA5_TARGETS_UP, EMT_DN, PENG_RAPAMYCIN_DN, LEE_E2F1_UP, HDACI_COLON_SUL_UP, ELONGINA_KO_DN, **CACO2_PTH4**, UVB_NHEK1_DN, MRNA_PROCESSING

Appendix 14

This table lists all the gene sets enriched in both the positively correlated with PTH phenotype (chapter 4) and control phenotype in the GSEA analyses with a FDR of<0.05.

Gene sets positively correlated with PTH and down-regulated by calcium
REFRACTORY_GASTRIC_UP, PEPTIDE_GPCRS, HSA04080_NEUROACTIVE_LIGAND_RECEPTOR_INTERACTION, GPCRDB_CLASS_A_RHODOPSIN_LIKE, IGF_VS_PDGF_UP, MOREAUX_TACI_HI_VS_LOW_UP, GPCRS_CLASS_A_RHODOPSIN_LIKE,

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