DNA methylation within intron 2 of the *Stat1* gene is reduced in colon tissue from interleukin-10 gene-deficient mice compared with healthy control mice

Matthew PG Barnett[1,2], Anna E Russ[1,2], Warren C McNabb[2,3,4], Nicole C Roy[1,2,4,5]

[1] Food Nutrition & Health Team, Food & Bio-based Products Group, AgResearch Limited, Palmerston North, New Zealand

[2] Nutrigenomics New Zealand; www.nutrigenomics.org.nz

[3] AgResearch Limited, Palmerston North, New Zealand

[4] Riddet Institute, Massey University, Palmerston North, New Zealand

[5] Gravida: National Centre for Growth and Development, Private Bag 92019, Auckland 1142, New Zealand

Corresponding Author:

Dr. Matthew Barnett

AgResearch Limited, Food Nutrition & Health Team, Food & Bio-based Products Group, Tennent Drive, Palmerston North 4442, New Zealand

Email address: matthew.barnett@agresearch.co.nz

Abstract

Background. Epigenetic influences have been implicated in the development of autoimmunity. While such mechanisms have been linked to mouse models of intestinal inflammation, and to human inflammatory bowel disease (IBD), the involvement of epigenetic mechanisms in the pathogenesis of intestinal inflammation in the interleukin-10 gene-deficient (*Il10*$^{-/-}$) mouse model of IBD has not yet been reported. This study investigated the hypothesis that changes observed in the expression of *Stat1* and *Ppara* in colon tissue of *Il10*$^{-/-}$ mice are associated with differential methylation of CpG sites within key regulatory regions of these genes.

Methods. Colon tissue was collected from *Il10*$^{-/-}$ and C57BL/6JArc mice at 6 (pre-inflammation) or 12 (established inflammation) weeks of age. Methylation levels of CpG sites within selected regions of the *Stat1* and *Ppara* genes were assessed using MALDI-TOF mass spectrometry in DNA extracted from mouse colon tissue.

Results. Methylation of specific CpG sites within intron 2, but not the proximal promoter, of the *Stat1* gene was reduced in colon tissue from *Il10*$^{-/-}$ mice at 12 weeks of age compared with colon tissue C57BL/6J mice at 12 weeks of age.

Discussion. These data provide preliminary evidence that DNA methylation is altered in the *Il10*$^{-/-}$ mouse model of IBD, and this may be linked with changes in the expression levels of genes that play a key role in inflammation. Further studies are required to confirm these observations, and to establish a causative link between methylation at specific sites (such as intron 2 of *Stat1*) and gene expression.

Introduction

Epigenetic mechanisms, such as DNA methylation, are increasingly recognized as having an important role in the regulation of gene expression in response to environmental influences. They have also been implicated in the development of autoimmune conditions, with a recent genome-wide association study showing that the majority of causal variants linked to autoimmune diseases are non-coding, acting via mechanisms such as histone acetylation and transcription of non-coding RNAs (Farh *et al.*, 2015). There is also evidence that such mechanisms are implicated in the development of inflammatory bowel diseases (IBD) (Stylianou 2013). Genetic variation alone accounts for only approximately 25% of IBD heritability (Franke *et al.*, 2010), consistent with the growing recognition of the importance of epigenetic mechanisms in heredity (Jablonka and Raz 2009). The different incidence of IBD in monozygotic twins is supporting evidence for the role of epigenetic mechanisms in the development of colitis, as are the alterations in global and gene-specific DNA methylation that have been detected in mucosal biopsies of IBD patients (Toyota *et al.*, 2002, Maeda *et al.*, 2006) and in a mouse model of colitis (Hahn *et al.*, 2008).

In a dextran sodium sulphate (DSS)-induced model of colitis, aberrant DNA methylation of three CpG islands was observed in colon epithelial cells, with the methylation levels within these regions being increased from 8 weeks after DSS treatment until the development of colon cancers 7 weeks later (Katsurano *et al.*, 2012). Inflammation triggered by the DSS treatment appeared to be responsible for induction of methylation (Katsurano *et al.*, 2012); this prompted the question of whether inflammation-associated changes in DNA methylation may also occur in other models of colitis.

We have consistently observed changes in the expression of several key regulatory genes in inflamed colon tissue of interleukin-10 gene-deficient (*Il10*$^{-/-}$) mice, which has been widely used as a model of human IBD by our group and others (de Buhr *et al.*, 2006, Barnett *et al.*, 2010, Selhub *et al.*, 2013). These genes include signal transducer and activator of transcription 1 (*Stat1*) and peroxisome proliferator-activated receptor alpha (*Ppara*) (Table 1; (Knoch *et al.*, 2009, Barnett *et al.*, 2010)). Although the genetics of IBD is complex, with ninety nine published IBD susceptibility loci (Lees *et al.*, 2011), the *STAT1* gene is, among others, consistently associated with its occurrence in human populations. The *PPARA* gene is not specifically linked with human IBD, however because we have consistently seen this gene differentially expressed in the *Il10*$^{-/-}$ mouse model, we were interested to investigate whether there might also be differential DNA methylation within this gene.

STAT1 is a member of the signal transducer and activator of transcription (STAT) family of transcription factor proteins (Xi *et al.*, 2006). STAT1 can be activated by various ligands, including the inflammation-associated cytokines IFNγ and IL6, and mediates the expression of a variety of genes important for cell viability in response to different cell stimuli and pathogens. STAT1 protein activation and expression is increased in the intestinal mucosa in IBD, although more so in Crohn's disease (CD) than in ulcerative colitis (UC) (Schreiber *et al.*, 2002). Furthermore, a genome-wide association study has shown that *STAT1* is one of several genes differentially expressed in human CD samples compared to those from healthy controls (Wu *et al.*, 2007). Changes in methylation patterns of the *STAT1* gene have been observed in several studies, for example methylation within the *STAT1* gene promoter has been linked to silencing of *STAT1* gene expression in human squamous cell carcinoma of the head and neck (Xi *et al.*, 2006). Furthermore, there is evidence of induction of *Stat1* in response to the DNA demethylating drug 5-aza-2'-deoxycytidine in a mouse model of

pancreatic carcinoma (Shakya *et al.*, 2013). This suggests that DNA methylation is one mechanism by which *Stat1* expression may be influenced.

PPARα is a member of the nuclear hormone receptor group of transcription factors which, through negative regulation of inflammatory cytokine expression, plays an important role in the immune response (Delerive *et al.*, 1999, Zhao *et al.*, 2011). Evidence suggests that expression of the *PPARA* gene is linked to changes in DNA methylation within this gene. For example, methylation at an intergenic CpG island 50 kb upstream of *Ppara* is associated with decreased *Ppara* gene expression in mice (Carone *et al.*, 2010). Furthermore, increased *Ppara* mRNA expression is associated with decreased CpG methylation in the *Ppara* promoter in the liver of mice (Lillycrop *et al.*, 2005). Alterations in methylation state and mRNA levels of *Ppara* may thus be interdependent, and may be modulated by colitis.

The putative role of the *STAT1* gene in the pathogenesis of IBD, our consistent observation of changes of *Stat1* and *Ppara* expression associated with inflammation in *Il10*$^{-/-}$ mice, and the potential for the expression of both genes to be linked to changes in DNA methylation, suggested our hypothesis: that changes observed in the expression of *Stat1* and *Ppara* in colon tissue of *Il10*$^{-/-}$ mice are associated with differential methylation of CpG sites within key regulatory regions of these genes. This hypothesis was investigated by measuring the methylation levels of selected regions of the *Stat1* and *Ppara* genes in C57BL/6J and *Il10*$^{-/-}$ mice at six (prior to inflammation) and twelve (established inflammation, (Knoch *et al.*, 2010)) weeks of age, using matrix-assisted laser deionisation, time of flight (MALDI-TOF) mass spectrometry.

Materials and Methods

Ethics statement. The mouse experiment described in this paper was approved by the AgResearch Limited (Ruakura) Animal Ethics Committee (application 11343), and was carried out in strict accordance with the New Zealand Animal Welfare Act 1999. All efforts were made to minimize suffering, and the animals were observed daily to identify possible adverse effects on their health.

Animals, diet and breeding. Nineteen *Il10*$^{-/-}$ mice (Institute for Laboratory Animal Research designation B6.129P2-Il10<tm1Cgn>/J) on a C57BL/6J genetic background were purchased from the Jackson Laboratory (Maine, USA). Sixteen control C57BL/6JArc mice were obtained from the AgResearch Ruakura Small Animal Colony (Hamilton, New Zealand). Animals were kept under conventional conditions at a temperature of 22 °C, humidity of 60%, air exchange of 12 times/hour and with a 12 hour light/dark cycle. All mice had *ad libitum* access to water, which was refreshed twice a week.

All mice were fed a non-sterile powdered AIN-76A diet (1977) which was made in-house at Plant & Food Research, Palmerston North as previously described (Dommels *et al.*, 2007).

Experimental design. Following a 7 day stand-down period to allow the *Il10*$^{-/-}$ mice to acclimatize following transportation, mice were randomly assigned to one of the following sampling groups: pre-inflammation (6 weeks; *Il10*$^{-/-}$ mice n=9, C57 mice n=8) or post-inflammation (12 weeks; *Il10*$^{-/-}$ mice n=10, C5L/6JArc mice n=8). These times were based on previous data from this mouse model (Knoch *et al.*, 2010) which showed little evidence of inflammation prior to 7 weeks of age, and peak inflammation occurring between 10 and 12 weeks of age. Once mice had been assigned to groups and transferred to individual cages, all animals received a single oral inoculation of intestinal bacteria (*Enterococcus* species (10^6)

and complex intestinal flora derived from normal C57BL/6JArc mice, total volume 200 µL) as has been previously applied in this mouse model (Knoch *et al.*, 2009, Barnett *et al.*, 2010).

Sample collection. At 6 or 12 weeks of age, samples were collected from mice as previously described (Russ *et al.*, 2013). Briefly, mice were euthanized using CO_2 asphyxiation followed by cervical dislocation. The intestine was quickly removed, cut open lengthwise and flushed with 0.9% sodium chloride to remove any traces of digesta. Sections of each intestinal region (duodenum, jejunum, ileum and colon) were then taken and frozen in liquid nitrogen before storage at -85 °C for gene profiling (which has already been reported (Russ *et al.*, 2013)) and DNA methylation analysis.

DNA extraction. Genomic DNA was extracted from whole colon tissue using an AllPrep® DNA/RNA/Protein mini kit (Qiagen; Cat. Number: 80004). The extracted DNA was quantified and its purity assessed using a NanoDrop ND1000 (Thermo Fisher Scientific, Wilmington, Delaware, USA). Samples were only used in subsequent analyses if they were of high purity (A260/A280 > 1.8; A260/A230 > 1.6). Where there was evidence of impurities such as proteins, chaotropic salts or phenol being present (as indicated by a low A260/A230 ratio), samples were further purified by ammonium acetate precipitation of the DNA using glycogen (Ambion, Inc.; Cat. Number: AM9510) as a carrier (according to the manufacturer's instructions) to remove these impurities. DNA was stored at −20 °C until required for further analysis.

Selection of targets for methylation analysis. Although methylation changes in the promoter region are often associated with regulation of gene expression (Lillycrop *et al.*, 2005, Xi *et al.*, 2006, Manning *et al.*, 2008), several studies have shown that CpG methylation within introns is also associated with changes in gene expression (Godler *et al.*, 2011, Mostovich *et al.*, 2011, Xue *et al.*, 2011). We therefore used Sequenom EpiDesigner software to design primers to target sequences within either the promoter region, or within introns, in the *Stat1* and *Ppara* genes. For both sequences within the *Ppara* gene, and for the intronic sequence within *Stat1*, we also targeted regions containing CpG islands.

Table 2 shows information on the chromosomal location and primer sequences for the amplicons targeted for Sequenom analysis. Two regions of interest were selected for each gene.

Sequenom methylation analysis. DNA samples were analyzed using Sequenom MassARRAY technology as previously described (Couldrey and Lee 2010, Couldrey *et al.*, 2011). Briefly, the EZ-DNA methylation kit (Zymo Research; Cat. Number: D5001) was used to produce methylation-dependent sequence variations of C to T and regions of interest were amplified using T7 tagged PCR primers. *In vitro* amplification and transcription was performed on the reverse strand with simultaneous U specific cleavage by RNAseA. Samples were subject to mass spectrometry to provide high-resolution DNA methylation analysis, quantitative to 5% methylation for informative CpG dinucleotides (Coolen *et al.*, 2007). For fragments containing a single CpG site, DNA methylation state was calculated by the ratio of methylated to un-methylated fragments. For cleavage products containing multiple CpG sites the average methylation status of the fragment is reported.

Statistical analysis. Statistical analyses were performed using GenStat (VSN International, Hemel Hempstead, UK; 9th edition, 2006 or 10th edition, 2007). Differences due to strain or sampling age were analyzed using residual maximum likelihood (REML).

A probability value of less than 0.05 was considered significant. Unless otherwise stated, data are presented as the mean ± standard deviation.

Results

Table 3 summarizes the quality of data obtained by Sequenom analysis. Of the two regions targeted in *Ppara*, only the *Ppara*_pp37 region yielded data of sufficient quality for statistical analysis, whereas both regions analysed within the *Stat1* gene yielded high quality data.

As shown in Figure 1, no significant differences were observed (either with respect to time or genotype) in the methylation of CpG sites within the analysed regions of *Ppara*. As shown in Figure 2, no significant differences were observed (either with respect to time or genotype) in the methylation of CpG sites within the proximal promoter region of *Stat1*. However, the targeted region within intron 2 showed an overall reduction of methylation in *Il10*$^{-/-}$ mice at 12 weeks of age compared to C57 mice at 12 weeks of age ($P < 0.001$). Specifically, a significant reduction in percentage methylation was observed at CpGs 1 (*Il10*$^{-/-}$: 10 ± 5% *vs.* C57: 30 ± 5%, $P < 0.001$), 4-5 (*Il10*$^{-/-}$: 30 ± 9% *vs.* C57: 44 ± 4%, $P < 0.01$) and 12 (*Il10*$^{-/-}$: 18 ± 13% *vs.* C57: 37 ± 6%, $P < 0.01$) within this region. The apparent reduction in methylation when comparing 12 week old *Il10*$^{-/-}$ mice with 6 week old mice of the same genotype was not significant ($P = 0.21$).

Discussion

In this study, we observed a reduction in methylation levels in three CpG sites within intron 2 of the *Stat1* gene. As shown in Table 1, we have consistently observed increased *Stat1* mRNA levels in 12 week old *Il10*$^{-/-}$ mice when compared to C57 mice of the same age, or when compared with 6 week old *Il10*$^{-/-}$ mice. These observations represent preliminary evidence that alteration of the methylation status of the *Stat1* gene may have a role in regulating the expression of this gene in this mouse model, and potentially in human IBD.

While there was no change in the methylation levels of the *Stat1* gene within the sites assessed in the promoter, there was a change at a CpG island located within intron 2. This is consistent with observations that changes in methylation status within the intron regions of genes can be associated with changes in gene expression (Godler *et al.*, 2011, Mostovich *et al.*, 2011, Xue *et al.*, 2011), and this may be specifically the case at a CpG island within an intron (Fujiwara *et al.*, 2015). Methylation is not the only mechanism regulating gene expression of *STAT1*; for example, interferon gamma signalling and transcription factors are also involved. More research, such as using *in vitro* methylation assays, is therefore required to determine whether there is a causal relationship between a change in CpG methylation within this intron region and increased gene expression level of *STAT1* in IBD. Understanding such mechanisms could inform the development of novel therapies to reduce inflammation by reducing STAT1 gene or protein expression.

Consistent with the result presented here, other studies have demonstrated that genetic and epigenetic aberrances in particular genes interact, and that these interactions are associated with altered gene expression in IBD (Cooke *et al.*, 2012, Kim *et al.*, 2012). A recent study of mucosal genome-wide methylation changes in rectal biopsies from UC and CD patients found consistent differences in DNA methylation between IBD cases and controls at regulatory sites within a number of genes, including genes which have been implicated in IBD susceptibility through genome-wide association analyses (Cooke *et al.*, 2012). The mRNA levels of a subset of these genes were analysed by quantitative real-time PCR (qPCR) and their expression difference between cases and controls was correlated with DNA

methylation (Cooke *et al.*, 2012). Similarly, another recent study found that reduced methylation of the *STAT4* gene was correlated with increased *STAT4* gene expression, and in addition, there was a correlation between risk alleles and methylation status in the promoter region (Kim *et al.*, 2012).

Dysregulation of STAT signaling has been implicated in tumor formation and progression (Yu and Jove 2004). The observation of induction of *Stat1* in response to the DNA demethylating drug 5-aza-2'-deoxycytidine in a mouse model of pancreatic carcinoma (Shakya *et al.*, 2013) is evidence supporting the potential role of methylation in controlling STAT1 expression. Although much of the research into the STAT family has been in the context of cancer, there is also clearly a role for STAT proteins, and particularly STAT1, in inflammation. Inhibition of T cell proliferation by airway epithelial cells, mediated by activation of the IFNγ/STAT1 pathway, may be a mechanism by which an immune threshold is set, preventing unwanted chronic inflammation (Deppong *et al.*, 2012). STAT1 signal transduction also appears to be necessary for activation of anti-inflammatory genes such as indoleamine 2,3-dioxygenase (*IDO1*) (Diegelmann *et al.*, 2012), and to play a role in suppression of the chronic inflammatory reaction in a guinea pig model of asthma (Cai *et al.*, 2012). Finally, hypomethylation of *STAT1* has been observed in naive CD4+ T cells from patients with primary Sjögren's Syndrome which, like IBD, is an autoimmune disease (Altorok *et al.*, 2014). This is further evidence that altered DNA methylation within the *STAT1* gene may have a role in inflammation, and potentially supports the suggestion that our observed increase of *Stat1* gene expression in the *Il10*$^{-/-}$ mouse model may reflect the involvement of this gene in the anti-inflammatory response. However, it must be noted that the observed *STAT1* hypomethylation in Sjogren's syndrome was on the promoter, not in intron 2 as we have observed here.

Although methylation changes were found within the intron 2 region of *Stat1* in this study, no change in methylation levels was found for the promoter region of *Stat1*. Nor was there any evidence of altered DNA methylation in the *Ppara* gene. Unfortunately it was beyond the scope of this exploratory study to assess methylation at all CpG sites within these genes. Other methods of assessing DNA methylation, such as bisulfite conversion or methyl-DNA immunoprecipitation followed by high throughput sequencing (Pomraning *et al.*, 2009) would enable more comprehensive measurement of methylation changes within the *Ppara* and *Stat1* genes. Wider assessment of the methylome using approaches such as methylation specific amplification microarray (Kellermayer *et al.*, 2010) would enable more comprehensive measurement of methylation changes in this model, both within within the *Ppara* and *Stat1* genes and in other genes of interest.

This study does have a number of limitations. Perhaps most importantly, the *Il10*$^{-/-}$ mice were obtained from the Jackson laboratory, whereas the C57BL/6JArc controls were obtained from AgResearch Ruakura small animal facility (although these mice were originally derived from the Jackson laboratory). It is well known that there can be wide variances in the different C57BL/6 lines (Bryant *et al.*, 2008, Zurita *et al.*, 2011, Simon *et al.*, 2013). We do not have any data to estimate the degree of such variation in this instance, and although both strains are originally from the Jackson laboratory (Zurita *et al.*, 2011), we cannot exclude the possibility that the methylation differences are due to genetic variation between the mouse strains, rather than to either the *Il10*$^{-/-}$ mutation, or inflammation, *per se*. Using wild-type littermates as controls for the *Il10*$^{-/-}$ mice in a future study would enable this point to be clarified.

Because DNA was obtained from intact colon tissue in this study, it is possible that the composition of different cell types within the samples analysed differed between the groups. If this were the case, differences in DNA methylation could simply be due to these differences in cell type. While we are unable to categorically rule this possibility out, we have also undertaken genome-wide gene expression analysis in colon epithelial cells obtained from the mice used in this study (Russ *et al.*, 2013). These results have shown differences in expression of both *Stat1* and *Ppara* mRNA in epithelial cells which are consistent with those observed in intact colon tissue, and we therefore suggest that the differences in methylation are also likely to be consistent. Measuring methylation of the target genes in epithelial cells was beyond the scope of the current study, but is of interest for future studies.

In summary, to the best of our knowledge we have shown for the first time changes in DNA methylation within the *Stat1* gene in colon tissue from the *Il10*$^{-/-}$ mouse model of IBD, which may be linked to the inflammation observed in this model. Epigenetic mechanisms act upstream of transcription and identifying their involvement in the inflammatory process and its regulation is an important step in gaining further insight into the pathogenesis of autoimmune diseases such as IBD. Many studies have described gene expression profiles in IBD-affected tissues and mouse models of IBD. Further investigation of the epigenetic changes in these tissues, particularly those which enable the identification of causal links between these changes and the underlying phenotype, will improve understanding of how these pathways are involved in the development of IBD, as well as providing insights into potential therapeutic approaches to its treatment or prevention.

Supplementary Material

The level of methylation at each CpG island for individual mice is provided as Supplementary File 1 (Microsoft Excel).

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Tables

Table 1. Summary of previously published data showing consistent changes in expression of *Ppara* and *Stat1* genes in the *Il10*$^{-/-}$ mouse model of IBD. Table shows fold change (FC) and P values (for qPCR data) or false discovery rates (FDR; for microarray data) for the comparisons described.

			qPCR		Microarray		Reference
	Gene	RefSeq ID	FC	P value	FC	FDR	
***Il10*$^{-/-}$ mice, 12 wk *vs.* *Il10*$^{-/-}$ mice, 6 wk**	*Ppara*	NM_011144	-2.6	<0.001	-3.0	<0.001	(Russ *et al.*, 2013)
	Stat1	NM_009283	2.0	<0.001	2.1	0.033	(Russ *et al.*, 2013)
***Il10*$^{-/-}$ mice, 12 wk *vs.* C57BL/6J mice, 12 wk**	*Ppara*	NM_011144	-2.6	<0.001	-2.4	0.002	(Russ *et al.*, 2013)
			NA	NA	-2.3	<0.01	(Barnett *et al.*, 2010)
			-5.0	<0.01	-2.5	<0.01	(Knoch *et al.*, 2010)
	Stat1	NM_009283	3.1	<0.001	3.6	<0.001	(Russ *et al.*, 2013)
			NA	NA	2.5	<0.001	(Barnett *et al.*, 2010)
			NA	NA	5.1	<0.001	(Knoch *et al.*, 2009)

453 **Table 2. Primers for Sequenom analysis of CpG methylation**

Gene	***Ppara***		***Stat1***	
Amplicon ID	mus_*Ppara*_pp12	mus_*Ppara*_pp37	mus_*Stat1*_int2_2	mus_*Stat1*_pp3
Genomic co-ordinates	chr15: 85564996-85565378	chr15: 85565771-85566091	chr1: 52177118-52177592	chr1: 52175244-52175553
Strand	+	–	+	-
Length	383	321	475	310
Left primer length	25	26	25	27
Left primer sequence	aggaagagag GTGAGGA TAGATAG GAGGGTT GGTA	aggaagagag TTAGTTTT TGGGTAT TTGAGGT TGTA	aggaagagag AGGTTTA TTATGGG GTTTGTTT TTT	aggaagagag TGTTTTTG TAATTTTT ATGTTTTG GAG
Right primer length	25	22	25	25
Right primer sequence	cagtaatacgac tcactatagga gaaggctCAA CAAATAA CCAAAAT ACCAAAA T	cagtaatacgac tcactatagga gaaggctCAA CAACCAA TCAAACA CTACC	cagtaatacgac tcactatagga gaaggctAAC TCCAACC CACCACT ATAACAT A	cagtaatacgac tcactatagga gaaggctATC AAACAAT CACCAAA AAAACCA C

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Table 3. Summary of data available following Sequenom analysis of amplicons within the *Stat1* and *Ppara* genes

Gene	***Ppara***		***Stat1***	
Amplicon	**pp_12**	**pp_37**	**pp_3**	**int2_2**
No. of samples[a]	28	28	28	28
No. samples with usable spectra[b]	14	28	28	26
Usable spectra (%)[c]	50	100	100	92.9
CpGs within amplicon[d]	8	13	5	4
Total CpGs analysed[e]	224	364	140	112
CpGs with data[f]	98	364	128	91
Usable data (%)[g]	43.8[h]	100	91.4	81.3

[a] The total number of individual mice from which DNA was available for Sequenom EpiTyper analysis.

[b] Samples for which at least one CpG site within the amplicon in question returned valid data.

[c] The number of samples with usable spectra divided by the total number of samples (n = 28) multiplied by 100.

[d] Those CpG sites for which any data were generated. Note that not all CpGs within each amplicon could be assessed, due to limitations of the Sequenom method.

[e] The number of CpGs within the amplicon multiplied by the total number of samples analysed.

[f] The total number of CpGs that returned a valid reading within that amplicon across all samples analyses.

[g] Valid data shown as a percentage of the total CpGs analysed, within the amplicon in question across all samples analysed.

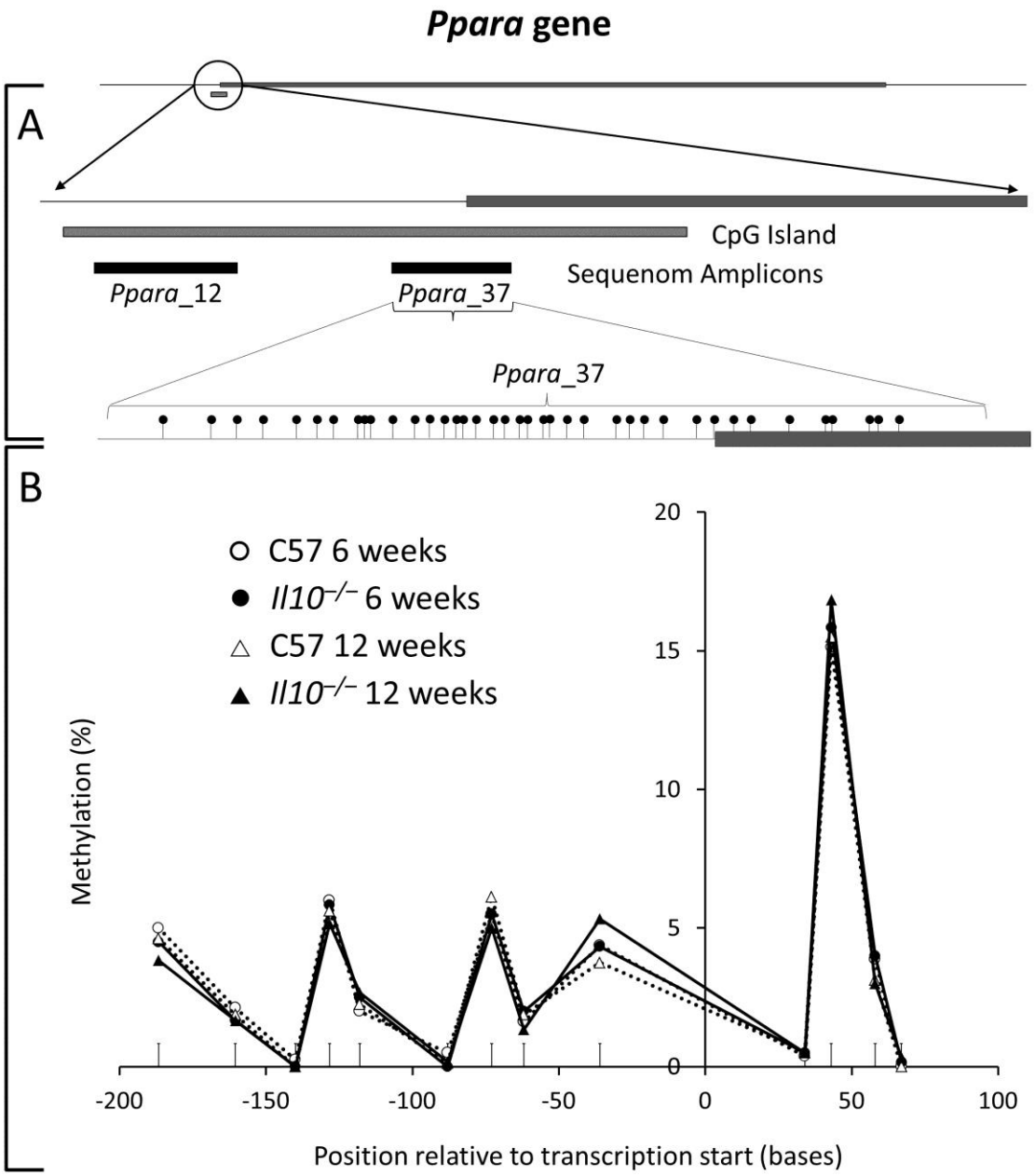
[h] This amplicon did not provide data of sufficient quality to warrant further analysis.

Figure Legends

Figure 1. Methylation of CpG sites at the transcription start site of the *Ppara* gene. The promoter region (*Ppara*_12 amplicon) and transcription start site (*Ppara*_37 amplicon) of the *Ppara* gene were targeted to establish if differential DNA methylation was occurring at specific CpG sites within these regions. Panel A represents the position of CpG islands, target amplicons, and CpG sites within the target region, while the graph in Panel B represents mean values for the percentage methylation at each site within each treatment group, as measured using Sequenom EpiTYPER. No significant differences were observed either with respect to age (6 weeks *vs.* 12 weeks) or genotype ($Il10^{-/-}$ mice *vs.* C57 mice) for any of the CpG sites measured. Error bars along the x-axis represent the least significant difference for comparisons between groups. Insufficient data was obtained from the *Ppara*_12 amplicon to assess methylation within this region, as is shown in Table 3. The data from which this figure is derived are included as Supplementary File 1.

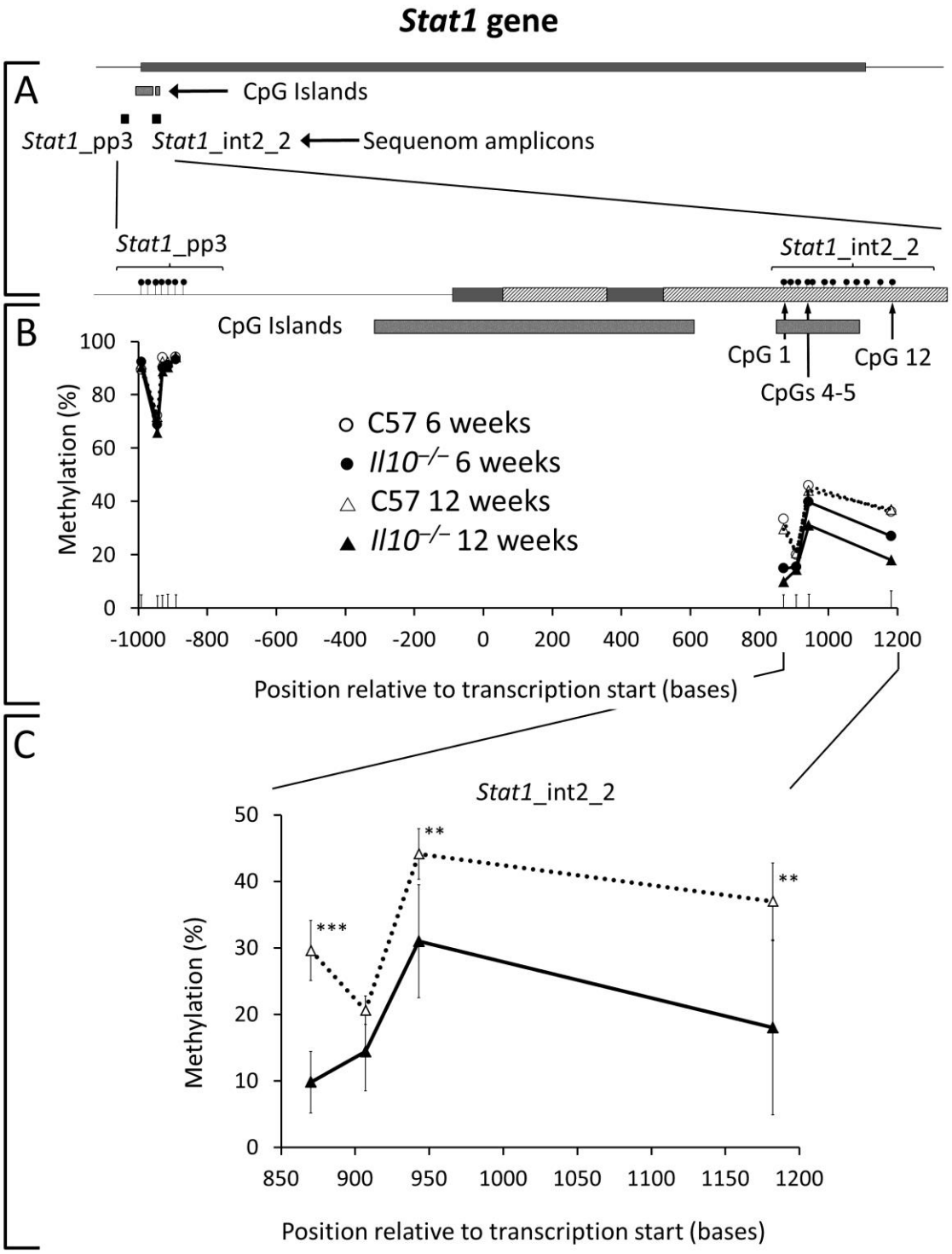
Figure 2. Methylation of CpG sites in the *Stat1* proximal promoter and within intron 2. The proximal promoter region (*Stat1*_pp3 amplicon) and a region within intron 2 (*Stat1*_int2_2 amplicon) of the *Stat1* gene were targeted to establish if differential DNA methylation was occurring at specific CpG sites within these regions. Panel A represents the position of CpG islands, target amplicons, and CpG sites within the target regions, while Panel B represents mean values for the percentage methylation at each site within each treatment group, as measured using Sequenom EpiTYPER. No significant differences were observed either with respect to age (6 weeks vs. 12 weeks) or genotype ($Il10^{-/-}$ mice *vs.* C57 mice) for the CpG sites with the *Stat1*_pp3 amplicon, however the targeted region within intron 2 (highlighted in Panel C) showed an overall reduction of methylation in $Il10^{-/-}$ mice at 12 weeks of age compared to C57 mice at 12 weeks of age ($P < 0.001$). A significant reduction in methylation was observed at CpGs 1 ($P < 0.001$, ***), 4-5 ($P < 0.01$, **) and 12 ($P < 0.01$, **) within this region, as indicated by the arrows in Panel B, and as shown in Panel C. Error bars along the x-axis in panel B represent the least significant difference for comparisons between groups, while those in panel C indicate the standard deviation. The intron regions are indicated by the stripes within panel B. The data from which this figure is derived are included as Supplementary File 1.

504 **Figure 1**



505
506

507 **Figure 2**



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