EPIGENETIC PROFILING OF BRONCHIAL EPITHELIAL CELLS: DNA METHYLATION

by

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ABSTRACT

Epigenetic regulation of gene expression is critical for normal human development and cellular differentiation. Although each somatic cell in the human body is genetically identical, epigenetic marks including the DNA methylation pattern are tissue-specific and critical for determining the vast array of cellular phenotypes. For studying respiratory disease, the airway epithelium is the ideal target tissue since it is the first point of contact for inhaled particles, viruses and airborne allergens. Cultured airway epithelial cells of asthmatic children show striking phenotypic differences compared to those from non-asthmatic individuals such as poor wound healing and enhanced expression of inflammatory cytokines. The altered state of the asthmatic epithelium could be due to underlying differences in gene expression associated with changes in their DNA methylation profile. Therefore, in this study, the purpose was to determine the extent to which DNA methylation, a reportedly stable epigenetic mark, is altered by standard cell culture conditions over passage for primary and immortalized bronchial epithelial cells. We analyzed four individual primary bronchial epithelial cell lines and a $1HBE_O^-$ immortalized bronchial epithelial cell line in triplicate. CpG DNA methylation was characterized during stages of cell propagation for 1505 CpG sites in promoter regions and/or first exons of 807 genes and compared with its gene expression.

A comparison of $1HBE_O^-$ cells and primary bronchial epithelial cell lines revealed that $1HBE_O^-$ cells have significantly higher levels of overall methylation and more hyper- and heterogeneously-methylated CpG loci. In addition, there were a large number of CpG sites that

had variable DNA methylation over passage in the primary cell lines but not in the immortalized cell lines.

In summary, there were extensive differences in DNA methylation profiles between primary and immortalized bronchial epithelial cell lines during cell propagation, revealing the importance of this epigenetic modification in cell culture. This work is critical because, although little is known about the CpG specific changes that occur in cell culture, passaged cells are still regularly grouped together and phenotyped for major biological studies.

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1 INTRODUCTION

1.1 Asthma

Asthma is a condition that is characterized by airway obstruction which is variable and largely reversible, and chronic inflammation of the respiratory tract that can exacerbate leading to acute episodes known as asthma attacks. It is difficult to identify asthma with certainty since it is a clinical diagnosis made on the basis of a patient's symptoms, medical history, assessment of lung function and exclusion of other alternative diagnoses (1). In addition, due to the variability in the presentation of symptoms, environmental triggers, ages of onset and multiple subtypes it is currently debated whether these constituents are independent or a part of the spectrum of the same disease (2).

Although we are limited in our understanding of the natural history and causes of different subtypes of asthma, it is clear that the prevalence of "asthma" as we know it is on the rise. In 2007, The International Study of Asthma and Allergies in Childhood (ISAAC) reported that the burden of asthma is continuing to increase rapidly in many geographical regions including: Africa, Latin America and parts of Asia (3). In addition, the incidence in most English speaking and Western European countries has only just started to decline after a steep rise in the last two decades. Genetic alterations occur far too slowly to account for such a rapid change in prevalence (1). These epidemiological data suggest that changes in the environment are primarily responsible for the recent increases in incidence (4). However, the increased incidence of allergic disease is primarily experienced by those who have a family history of

asthma/allergy suggesting that it is an interaction between genes and the environmental changes that are responsible for the “epidemic” (5). Epigenetics can bridge the gap between the environment and genetics and provide the basis for new biological models to better understand the pathogenesis of a variety of respiratory diseases including asthma.

1.2 The Airway Epithelium

The airway epithelium is the first point of contact for inhaled particles, viruses and airborne allergens (6). Due to its frontline position, the airway epithelium is an attractive target of study to identify novel mechanisms and new therapeutic strategies that may better treat and someday, prevent asthma.

1.2.1 Types of Airway Epithelial Cells

Briefly, ninety-five percent of the airway epithelium is composed of three main types of cells: basal, ciliated and secretory cells. Ciliated and secretory cells are in contact with the airway lumen while the basal cells are in contact with the basal lamina.

Basal cells are characterized by their large nucleus with highly condensed chromatin and are believed to function as precursor cells for more differentiated cell types (7-9). Due to their potential to differentiate and their capacity to adapt to the environment, it is the basal cells that are believed to primarily attach, divide and grow in primary airway epithelial cell culture systems (10).

There are two main types of secretory cells. Goblet mucous secreting cells are found mainly in the upper airways while Clara cells are found in more distal airways. Goblet cell hyperplasia is

commonly found in chronic asthma and results in excess mucous in the airway (11). Clara cells differ from goblet cells in that their secretory granules are smaller in size and number and they contain an extensive granular endoplasmic reticulum (12).

Ciliated cells are differentiated epithelial cells that arise from either basal or secretory cell types (13). Ciliated cells use their motile cilia to remove mucous entrapped foreign objects from the airways. Dysfunctional ciliated epithelial cells are partially responsible for the mucous plugs that form during asthma attacks due to the inability of the airway to properly clear excess mucous (14).

In all airways, the ciliated cells account for roughly 50% of the epithelial cell population (11, 12). In the larger airways, basal cells are the second most common type of cell found followed by secretory goblet cells (15). As the airway generation number increases, the number of basal cells declines steadily and Clara cells replace the mucous secreting Goblet cells to become the predominant secretory cell type.

1.2.2 The Airway Epithelium and Asthma

Asthma is mainly recognized as an inflammatory disorder involving inflammatory cell infiltration of the conducting airways, but, often overlooked elements of asthma are the structural changes that occur in the airway walls including the airway epithelium. Under normal circumstances, the airway epithelium forms a highly regulated and almost impermeable barrier to the inhaled environment through the formation of tight junctions (16, 17). In asthmatics, the current evidence indicates their airway epithelium is fundamentally abnormal resulting in augmented

susceptibility to environmental injury and flawed repair processes. For instance, the asthmatic epithelium is more susceptible to oxidant induced damage and apoptosis compared to normal epithelium (18, 19). In addition, studies of cultured airway epithelial cells from children who have asthma revealed that they have increased production of the inflammatory cytokines PGE-2 and IL-6. These changes are stable over several passages, and thus support the notion that altered secretory profiles of the epithelium are intrinsic to the origin of asthma rather than occurring secondary to airway inflammation (20).

In addition, current research is starting to paint a compelling portrait of the important role the airway epithelium plays not only as a protective barrier but also in regulating airway inflammation and T helper (Th) lymphocyte differentiation. For example, under normal conditions airway epithelial cells produce anti-inflammatory molecules including IL-10 and TGF-beta in response to pro-inflammatory cytokines suggesting that they may be involved in a negative feedback system to moderate inflammation (21). In addition, recent studies have shown that a novel cytokine, thymic stromal lymphopoietin (TSLP), is produced by airway epithelial cells and is capable of generating TH2-polarized dendritic and mast cell responses (22, 23). Given that i) overexpression of TSLP in airway epithelial cells induces experimental asthma in mice (24) and ii) that TSLP mRNA is overexpressed in the bronchial mucosa of asthmatic patients (22), there is increased support for the hypothesis that asthma is initiated by the epithelial cells themselves via increased production of TSLP (23) and other inflammatory cytokines.

It is important to understand changes occurring at the level of the airway epithelium in order to improve treatments focused on protecting vulnerable airways against environmental injury rather than relying solely on maintenance treatments to reduce airway inflammation and the immune response. Epigenetic mechanisms, such as DNA methylation and/or histone modifications, are potential mechanisms that could mediate the abnormal epithelial cell cytokine expression patterns observed in cultured asthmatic airway epithelial cells and explain the altered *in vivo* condition of the asthmatic airway epithelium.

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1.3 Epigenetics: Overview

Epigenetics, in a broad sense, is one of the bridges between genotype and phenotype—a dynamic phenomenon that mediates the final outcome of a locus or chromosome without changing the underlying stable DNA sequence (25). Specifically, epigenetics refers to "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (26). An epigenome represents these epigenetic events and modifications across the whole genome and importantly each organism is not limited to one epigenome. For example, the vast majority of cells in a multi-cellular organism share an identical genome, yet a diversity of cell types with disparate but stable profiles of gene expression and distinct cellular functions develop. These differences are due in part to tissue-specific epigenomes that regulate gene expression to promote specific pathways of cell differentiation. Thus, cell differentiation may be considered an epigenetic event, governed by changes in the "epigenetic landscape" as initially described by Conrad Waddington (27).

A single human diploid cell contains a total of 6 billion base pairs of DNA packaged into 46 chromosomes. Each base pair is roughly 0.34 nanometers in length and therefore each diploid cell contains approximately 2 metres of DNA (28). Within the nucleus, epigenetic information is organized into chromatin, a compact heterogeneous structure composed of DNA, histone proteins and other components that actively packages DNA within cells and forms chromosomes. Chromatin is responsible for packaging and efficiently organizing this large amount of DNA into the microscopic space of each cell's nucleus so that the 50 trillion cells in our body can function properly. The basic units of chromatin are nucleosomes, nucleoprotein complexes consisting of 146bp of DNA wrapped around a core of four histone proteins: H2A, H2B, H3 and H4 (29, 30). There are 14 contact points between histones and DNA (29) making the nucleosome a stable, yet dynamic, complex suited to one of its roles of packaging DNA in the nucleus (31). These nucleosomes form distinct types of chromatin neighbourhoods that are not uniform across chromosomes such as euchromatin where DNA is loosely wound and kept accessible for transcription and heterochromatin, where DNA is more tightly condensed and is more inaccessible for transcription **(Figure 1.1)**.

Numerous regulators and mediators of the epigenome act dynamically to alter gene expression and change phenotypic characteristics of cells both *in vitro* and *in vivo*. Most epigenetic modifications fall into two main categories: DNA methylation and histone modifications. Briefly, histone modifications such as methylation, acetylation, ubiquitination and phosphorylation that occur post-translationally mainly on the amino acid residues of histone protein tails are one mechanism that alter chromatin structure. These modifications either disrupt or enhance

contact between nucleosomes to unravel or tightly wind chromatin and/or they recruit non-histone proteins that carry out specific enzymatic activities (32). For example, Lysine (K) methylation of H3K9, H3K27 and H4K20 has been strongly associated with gene silencing due to the formation of heterochromatic states (33).

1.4 DNA Methylation

1.4.1 Overview

The focus of my thesis is DNA methylation, one of the best characterized epigenetic modifications of chromatin. DNA methylation is the chemical modification of DNA that occurs via the enzyme-assisted addition of a methyl group to a cytosine. In evolutionary terms, DNA methylation is one of the oldest epigenetic modifications as it is found in bacteriophages, bacteria, fungi, plants, humans and all other mammals (34-38). In mammals, DNA methylation is largely confined to cystosine C5 methylation in the DNA sequence context 5'-CpG-3', also known as Cytosine-Guanine (CpG) dinucleotides, whereas in plants and other species it commonly exists in a variety of other sequence contexts (39). DNA methylation was originally proposed to inhibit gene expression but this is an oversimplification since DNA methylation alters the interactions between many proteins and DNA, leading to changes in chromatin neighbourhoods and either a decrease or increase in gene expression dependent upon a multitude of other factors present in the cell's nucleus and surrounding environment (40). Indeed, a growing body of research has now implicated DNA methylation in a wide array of

processes critical to an organism's development and survival that will be discussed later in this section.

1.4.2 The Process of DNA Methylation

S-adenosyl methionine, formed by the catalyzed reaction of the amino acid methionine with ATP (41-43), is the methyl donor for DNA methylation **(Figure 1.2).** Complex enzymes known as DNA methyltransferases (DNMTs) catalyze the synthesis of methylated cytosine and are responsible for both *de novo* methylation marks and maintaining DNA methylation patterns in normal cells during DNA replication (44). This process is tightly regulated and abnormal hypo- or hyper- methylation is commonly associated with transformed cells (44) .

Unlike other epigenetic mechanisms, there is a clear mechanism for the propagation of cytosine methylation patterns during cell division (45). The enzyme DNMT1 is constitutively expressed in proliferating cells and ubiquitously in somatic tissues throughout mammalian development (46). DNMT1 is commonly referred to as the maintenance DNA methyltransferase, since it is specific for hemi-methylated CpGs and therefore during S phase (47) of DNA replication it methylates CpGs of the nascent DNA strand according to the methylation pattern of the template DNA (reviewed in (48, 49)). Originally, these DNA methylation patterns were thought to be static but recent research is demonstrating the dynamic nature of DNA methylation. Since DNA methylation is currently considered to be a dynamic and possibly even a temporally cyclic epigenetic modification (50, 51) that is capable of responding to environmental change, any

such modifications in DNA methylation could be propagated to the next generation via this mechanism.

The inheritance of these epigenetic changes presumably aids the cells survival by adapting their gene expression and protein profile to match the new environment. In relation to the current study, it follows that the *in vitro* cell culture environment and cell propagation techniques could induce adaptive changes in DNA methylation that would then be passed onto the next lineage of cells via mechanisms propagating existent DNA methylation patterns such as via DNMT1.

On the other hand, the catalytically active DNMT3 family made up of DNMT3a and DMNT3b are highly expressed in embryonic stem cells, early embryos and developing germ cells when *de novo* methylation is known to take place (52). There is evidence that these methyltransferases are involved in the establishment of methylation patterns during gametogenesis and early development that ultimately influence phenotype in adulthood (53). For example, in honeybees, it was shown that siRNA mediated gene expression silencing of DNMT3, induced reprogramming changes that promoted the development of genetically identical larvae into queens with fully developed ovaries rather than into the more common sterile worker (54).

Although it is accepted that DNA methyltransferases are responsible for establishing and maintaining methylation marks in mammals (reviewed by (49)), the debate begins when deliberating about removal mechanisms of DNA methylation. In mammals, genomic methylation patterns are erased and then re-established in primordial germ cells while the cells

are dividing (55). In this case, it is posited that demethylation occurs via a passive mechanism, i.e. methylation marks are not maintained by methyltransferases during DNA replication, rather than active removal of the methyl groups (56). In contrast to mammals, plants, which have an intricate active demethylation system, transmit genomic methylation patterns from one generation to the next with the accrual of additional methylation marks at repeated sequences (57). Therefore, it is necessary for plants to utilize active demethylation to remove or trim the additional methylation in order to prevent interference with expression of nearby genes. There is not the same requirement in mammals due to germ cell erasure of methylation marks (58).

The suggestion that active demethylation may occur in the form of mammalian DNA demethylases has led to the description of several such enzymes, each quite different from the others (56). Candidates proposed for methyl group removal include DNMT3a and 3b, the same enzymes responsible for methyl group addition (59, 60). It is posited that in the absence of S-adenosyl methionine, these enzymes increase the rate of oxidative deamination at the cytosine converting it to thymine(T) or uracil(U) depending on whether it was initially methylated or not (61). In the case of methylated cytosine, the G-T mismatch base pair would then attract glycosylases that would remove the T mismatch base. The demethylation process would be completed by base excision repair mechanisms that insert unmethylated cytosine into the DNA sequence (60). However, the likelihood and efficiency of this reaction has been questioned and whether enzymes exist to actively remove methyl groups in mammals is still being explored. Research in Zebrafish has indicated that demethylation in vertebrates may be more complex than previously thought and involve a coordinated system of specific deaminases, G:T

glycosylases and a Gadd45 family member (62). These findings may explain why it has been so difficult to isolate one enzyme responsible for demethylation in vertebrates.

1.4.3 Frequency, Distribution and Location

In mammals, DNA methylation is largely confined to cytosine C5 methylation of CpG dinucleotides; although, a few studies have detected non-CpG methylation in human cells, and particularly in embryonic stem cells (39, 63, 64). The high rate of mutagenesis of methylated cytosine resulting in a point mutation to thymine has been widely documented in the literature and occurs at a rate 10 to 40 fold higher than at other nucleotides (65, 66). This inherent susceptibility to mutation has resulted in a low frequency of CpG dinucleotides in the genome. In humans, methylated cytosines account for only 0.75-1% of all DNA bases in the genome with at least 70 to 80% of all CpG dinucleotides being methylated (67, 68). However, approximately 5.5 % of all CpGs are contained within CpG Islands (69) that are mainly located near transcription start sites and are predominantly unmethylated in normal tissue samples (67).

CpG islands are broadly defined as “regions of DNA of greater than 500bp with a GC content equal to or greater than 55% and an observed/expected CpG ratio of 0.65” (70). The advantages of this new definition over previous definitions are that these CpG islands are more likely to be associated with the 5’ region of genes and the 500 bp size requirement excludes most Alu-repetitive elements. Approximately 70% of human genes have promoter regions containing CpG islands, whereas the remaining promoters tend to be depleted of CpGs (71). For

the purpose of this study, the definition of a CpG Island has been refined according to Weber et al. in order to define three distinct CpG promoter density categories: High CpG Promoters (HCPs), Intermediate CpG promoters (ICPs) and Low CpG Promoters(LCPs) (72). HCPs contain a 500-bp region with a CpG ratio above 0.75 and GC content above 55%; LCPs do not contain a 500-bp region with a CpG ratio above 0.48; and ICPs are neither HCPs or LCPs and thus contain weak CpG islands with a moderate CpG richness and/or have a GC content below 55%. HCPs are associated with ubiquitous housekeeping genes and highly regulated key development genes (71). LCPs are generally associated with tissue-specific genes (73), while ICPs are reported to demonstrate the strongest correlation between gene expression differences and promoter methylation (74).

Recent studies indicate that only a small subset of human primary cell promoters (<5%) show any DNA methylation (67, 69, 72, 75-77). New studies have established the presence of some tissue-specific methylation marks in normal tissue at these CpG islands. However, a comparison of tissue specific methylation profiles revealed that the majority of differentially methylated regions occur outside of promoters and specifically downstream of promoters within the gene bodies (67). The mechanism that favours protection of most CpG promoter regions from methylation but does not hinder tissue-specific DNA methylation of gene bodies has yet to be resolved. Recent genome-scale DNA methylation maps have indicated that DNA methylation patterns are better correlated with histone methylation patterns than with the genome sequence context in which they occur (73). In the same study, the loss or gain of specific

histone marks in promoter regions of mouse embryonic cells was a strong indicator of inverse changes in associated CpG DNA methylation levels (73).

1.4.4 Proposed Function

Overall, the function of DNA methylation in mammals is not clear but current data points towards its role in gene regulation, global maintenance of the genome and/or control of repetitive elements such as transposons (78, 79). The role of DNA methylation in the inhibition of gene expression is a function that is still debated regularly and depends partially on the location of the DNA methylation in the genome. DNA methylation is thought to only affect the availability of a gene for transcription since other factors need to be present to physically transcribe the gene. In one study of the differentially methylated regions in the 5' un-translated regions of genes on chromosomes 6, 20 and 22, only a small proportion, one-third, was inversely correlated with gene expression (67). Due to the numerous environmental factors capable of altering gene expression in each cell, it is perhaps not surprising that only a small proportion of gene's expression levels are inversely associated with DNA methylation at the promoter region.

Genomic imprinting showcases the important role DNA methylation plays in regulating gene expression. Genomic imprinting is the process by which certain mammalian genes are expressed or silenced in the developing embryo and throughout the life of the organism depending on whether they came from the mother or the father (80-83). In this case, DNA methylation marks certain "imprinted" genes differently in parental germ cells, and inheritance of these epigenetic marks leads to expression from only one of the two parental chromosomes

(84-89). In the developing somatic cells of the child, the imprinted DNA methylation marks that occur mainly in CpG Island-rich genes (90) are maintained, resulting in parent of origin-specific gene expression for certain loci of the genome (As reviewed in (91)). Importantly, abnormalities in genomic imprinting have been associated with various developmental diseases that have long reaching consequences (92, 93).

In addition, there is abundant evidence to support the role of DNA methylation in regulating the genome in other important ways. For example, gene body DNA methylation is postulated to inhibit cryptic transcriptional initiation outside of gene promoter regions (94). In addition, in mammalian genomes, the majority of genomic repeats are methylated (69) and loss of such methylation in plants has been shown to reactivate mobile elements and increase the frequency of transposition thus supporting DNA methylation's role in maintaining genomic integrity (95).

Lastly, DNA methylation in the gene bodies, the region of the gene downstream from the promoter, has long been known to be positively correlated with active transcription (96). Recent evidence has continued to strengthen this contention and there is growing support that this may be a general phenomenon of gene regulation. For example, a recent study of a CD19+ B cell methylome showed that methylation of CpG-rich gene bodies was associated with high transcription levels (97). In addition, the active human X chromosome has been observed to have 2.4 times more gene body methylation than its inactive counterpart (98). It has also been observed in cell lines that highly expressed genes are associated with a pattern of low methylation in the promoter and high methylation in the rest of the gene body whereas weakly

expressed genes are moderately methylated in both promoter and gene body regions (74). For example, hypomethylated CpG sites in the gene body have been associated with low gene expression in cancer cell lines (99).

1.4.5 DNA Methylation and Disease

The best studied relationship between epigenetic alterations and disease are in the context of cancer. Abnormalities in DNA methylation are strongly implicated in the development of certain types of human cancers. Tumour cells have an epigenome that is globally hypomethylated due to loss of methylation at repetitive sequences leading to genome instability (100-102). Simultaneously, promoter CpG islands of tumour suppressor genes such as $p16^{INK4a}$, and Rb undergo hypermethylation and become inactivated allowing uncontrolled growth and cell division (103-106). These initial findings have significantly advanced cancer treatment by being translated into successful clinical diagnostic tests such as those that use highly sensitive methylation assays to detect the probability of early tumour re-occurrence in patients with non-small cell lung cancer who have undergone curative resection (107).

Abnormal DNA methylation patterns have also been linked to several reproductive problems and associated diseases. During normal spermatogenesis, it is known that substantial epigenetic programming occurs in two waves (108-112). Extensive erasure of DNA methylation marks characteristic of somatic cells is followed by the establishment of sex-specific patterns by de novo methylation. In one study, Houshdaran et al. used an extensive panel of DNA methylation assays to characterize the DNA methylation profile of abnormal sperm and

reported elevated methylation at numerous DNA sequences indicating that improper erasure of somatic DNA methylation marks is implicated in poor quality sperm and male infertility (113).

Furthermore, assisted reproductive technologies that involve the manipulation of embryos are associated with an increased risk of children with Angelman syndrome and Beckwith-Wiedemann syndrome (114). Molecular analysis of both syndromes revealed a loss of maternally imprinted methylation at specific gene loci on chromosome 15 and 11 respectively, implicating epigenetic abnormalities in the diseases (114). In addition, epigenetic abnormalities are responsible for a large percentage of embryonic losses and placental hyperplasia observed in animal cloning models (115). However, there is some emerging evidence that underlying infertility problems may play a role rather than the assisted reproductive technologies themselves (116).

Epigenetic dysregulation is also associated with the development of serious neurological and psychiatric disorders. For example, Rett syndrome, a common cause of mental retardation that affects one girl in 10,000 to 15,000 births (117), is associated with the disruption of methyl CpG binding protein 2 (MeCP2) (118) that normally binds methylated DNA. Both over and under expression of this key protein are associated with abnormal brain development suggesting that careful gene regulation is crucial. Due to the complexity of psychiatric disorders such as major depression and schizophrenia and the tissue-specificity of epigenomes, little is known about the role of epigenetic mechanisms in this context for humans. However, there is a growing body of evidence linking abnormal DNA methylation and histone modifications to these psychiatric

disorders using animal models (as reviewed by (119)). In addition, defective epigenetic mechanisms have just begun to be implicated in neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and Huntington's disease (as reviewed by (120)).

1.4.6 DNA Methylation, Aging and the Environment

Due to the dynamic nature of epigenetics, research that disentangles the role of aging, environmental factors, and inter-individual variability of methylation profiles is in its infancy but important information has emerged from the few existing studies. Twin studies are traditionally used to indicate the role of environmental factors in disease. In 2009, Kaminsky et al. demonstrated that dizygotic twins have significantly higher epigenetic discordance in DNA methylation patterns compared to monozygotic twins (121). In another twin study, epigenetic variation was related to differences in disease susceptibility among identical twins. Specifically, identical twins that were older, had spent more time apart and had varying medical histories exhibited more differences in overall content and genomic distribution of DNA methylation marks than their younger counterparts (122).

In general, trends of hypomethylation at repeat elements and hypermethylation at promoter regions found in cancer have also been observed in normal tissues with aging (123). Specifically, recent work has shown that older age is associated with an overall increased promoter methylation in normal prostate and colon tissues for several genes (124, 125). However, Bjornson et al. found that over an 11 to 16 year period there was familial clustering of both increased and decreased methylation levels at specific CpG sites in peripheral blood samples with a majority of the Icelandic cohort exhibiting greater than 5% change in global DNA

methylation (126). An explanation for both increased and decreased methylation associated with aging emerged from the work of Christensen et al. (127). From an analysis of 217 normal human tissues from 10 anatomic sites they showed that the direction and strength of correlation between age and methylation was mainly dependent upon CpG island status irrespective of tissue-type. CpG sites located within CpG Islands were more likely to gain methylation with age while their non-CpG island counterparts were more likely to lose methylation with age. This finding was fairly consistent with the CpG sites found to change the most over time in the Icelandic cohort.

Briefly, environmental factors such as carcinogen exposure, inflammation and diet also contribute to changes in methylation patterns. For example, tobacco, alcohol and asbestos exposure have all been associated with gene-inactivation via alterations in DNA methylation in human cancers of the bladder, head and neck, and lungs (128-133).

Therefore, although more studies are required, it is becoming clear that aging and the environment have the potential to dramatically alter DNA methylation marks both globally and specifically within certain tissues and gene regions to promote the development of disease. This thesis explores the question of whether normal human bronchial epithelial cells undergo changes in DNA methylation due to their cell culture environment as they are propagated and amplified.

1.5 Epigenetics and Asthma

Research into the role that epigenetic mechanisms play in the pathogenesis of asthma is still in its infancy but the initial findings have suggested that they may play an important role. DNA methylation has been shown to initiate the process by which T helper (Th) lymphocytes differentiate to either a Th type 1 (Th1) or proallergic Th type 2 (Th2) pattern of cytokine gene expression (134). Polarization of naive CD4 T cells into Th2 cells is characterized by the expression of interleukin-4 (IL-4), IL-5 and IL-13 and a reduction of interferon-gamma (IFN-γ). Loss of methylation at the proximal promoter and at the conserved intronic regulatory element (CIRE) in the first intron of the IL-4 gene and hypermethylation of the IFN-γ promoter all resulted in greater IL-4 production and Th2 differentiation (135-138). In addition, during TH2 polarization, DNMT3a is recruited to the IFN-γ promoter region and correspondingly the promoter region undergoes progressive *de novo* methylation (138) which in turn decreases the production of IFN-γ. Remarkably, this study demonstrated that methylation of a single CpG site in the promoter region of IFN-γ can abolish its normal gene expression in TH1 cells.

Histone regulation has also been implicated in asthma. Cells obtained by bronchial biopsy and alveolar macrophages from asthmatic patients have increased histone acetyltransferase (HAT) activity and a significant reduction in histone deacetylase-2 (HDAC2) expression and activity (139). These epigenetic changes could promote inflammatory gene expression as has been observed in Chronic Obstructive Pulmonary Disease (COPD) patients (140).

1.6 Thesis Project

The ultimate aim of the broad project that I have been involved in was to test whether epigenetic changes underlie the early phenotypic differences found in bronchial epithelial cells obtained from asthmatic and allergic subjects compared to normal subjects (20). However, before the epigenome of the airway epithelium can be appropriately characterized, fundamental methodological studies need to be conducted. The main objective of this thesis was to determine the extent to which DNA methylation, a reportedly stable epigenetic mark, and gene expression levels are altered by standard cell culture conditions over passage for primary and immortalized bronchial epithelial cell lines. This is important because: 1) there are a limited number and amount of samples that can be obtained, 2) many cells are needed for epigenetic studies and 3) although cultured cells are regularly phenotyped, culture-induced epigenetic changes have not been thoroughly studied.

1.6.1 Previous Research: Observed Phenotypic Changes in Culture

Primary cell culture is used in basic science laboratories around the world as a helpful model system to examine biological processes including signal transduction, cell differentiation, gene regulation and gene-environment interactions. In addition, a plethora of researchers have used passaged cell lines to study disease. The use of primary cultures of human bronchial epithelial cells has been important for understanding the mechanisms underlying respiratory diseases such as asthma (19, 20, 141-145), for characterizing airway viral infections (146-150), and for advancing our knowledge of airway inflammation (146, 151-160). However, it is becoming increasingly recognized that cells *in vitro* behave differently from their *in vivo* counterparts and

that cell culture conditions can influence the differentiation of primary bronchial epithelial cells (as reviewed by Gruenert et al. (15)). For instance, during the development of current bronchial epithelial cell (BEC) culture techniques it has been shown that primary BEC differentiation is influenced by the extracellular matrix (161-167), growth factors (168-176), hormones (177-180), and the presence of an air-liquid interface (161, 166, 167, 181). A limitation of primary culture studies is the small amount of viable cells obtainable. This is overcome by passaging them to produce more of the sample, although this is generally accompanied by the loss or reduction of some differentiated features and eventual cell senescence.

Aberrant epigenetic regulation under normal cell culture conditions has raised concern over the validity of *in vitro* model systems that involve persistent proliferation because of the abnormal CpG island methylation that occurs (182-184). For example, preliminary observations of the Myo D1 gene revealed de novo methylation at CCGG sites that was not apparent *in vivo* and therefore must have occurred *in vitro* (183). Initial studies showed that primary and immortalized cell lines respond differently to cell culture. During propagation, primary cultures demonstrate decreasing DNMT1 activity and decreasing 5-methylcytosine content, while immortalized cultures do not (185, 186).

In addition, instability of DNA methylation in cell culture has been observed in embryonic stem cell lines with additional epigenetic disparity occurring when cells were adapted to a serum-free culture system. Remarkably, most of the epigenetic changes induced *in vitro* were not transient but stably inherited by the cultures, even after differentiation (187). Furthermore, a genome-wide study of ten primary mouse tissues studied the relationship between passage number and

hypermethylation of high-CpG-density promoter regions. In one example, neural precursor cells displayed no hypermethylation in these regions initially but as the passage number increased a small set of HCPs started to acquire hypermethylation (73). The methylation patterns acquired by the cultured cells during later passages were similar to malignant cancerous cells that are known to harbour hypermethylated CpG islands (188, 189). Possible explanations for these changes are that during culture *de novo* methylation could silence genes that are non-essential in the artificial culture environment (190) while demethylation could enhance expression of genes that promote cell survival and proliferation (191).

In a recent study by Eckhardt et al., a variety of primary cell types were passaged for three passages to minimize the risk of introducing aberrant methylation. As a side study, they compared the methylation levels of selected CpG regions before and after culturing and did not detect any difference in average methylation (67). However, it is important to recognize the limitations of this methodology. In contrast to this thesis, they did not study changes at CpG nucleotide resolution for a wide variety of genes but instead reported an averaged DNA methylation value from approximately 16 CpG loci per amplicon region for a few selected regions.

1.6.2 Hypotheses and Aims

It has been shown that cultured airway epithelial cells of asthmatic children show striking phenotypic changes compared to the cells of non-asthmatic individuals. It is plausible that cell culture could differentially alter normal and asthmatic airway epithelial cells due to underlying differences in bronchial cell subtypes cultured or other unknown factors. This *in vitro*

phenomenon could be a confounding factor that impedes future research. Therefore, it was the intent of this thesis to explore the hypothesis **that epigenetic changes occur in cell culture to alter the development and differentiation of primary and immortalized bronchial epithelial cells.** Besides examining "global" patterns of DNA methylation and gene expression during propagation in cell culture, we examined in-depth, 4 candidate genes that are important regulators of epithelial cell phenotype and undergo epigenetic changes as a function of passage. The specific aims of the study were to:

1. a. Characterize CpG-site specific DNA methylation in normal and immortalized bronchial epithelial cells during propagation in cell culture for 1505 CpG sites located in promoter regions and/or first exons of 807 genes.

   b. Compare and contrast DNA methylation of normal bronchial epithelial cells and immortalized airway epithelial cells.

2. Study the associations between DNA methylation and gene expression and determine which CpG sites are the most highly correlated with gene expression for both primary and immortalized cell lines.

3. Select 4 biologically relevant candidate genes that show changes in DNA methylation and/or gene expression over passage for in-depth studies and to confirm the validity of the gene expression data.

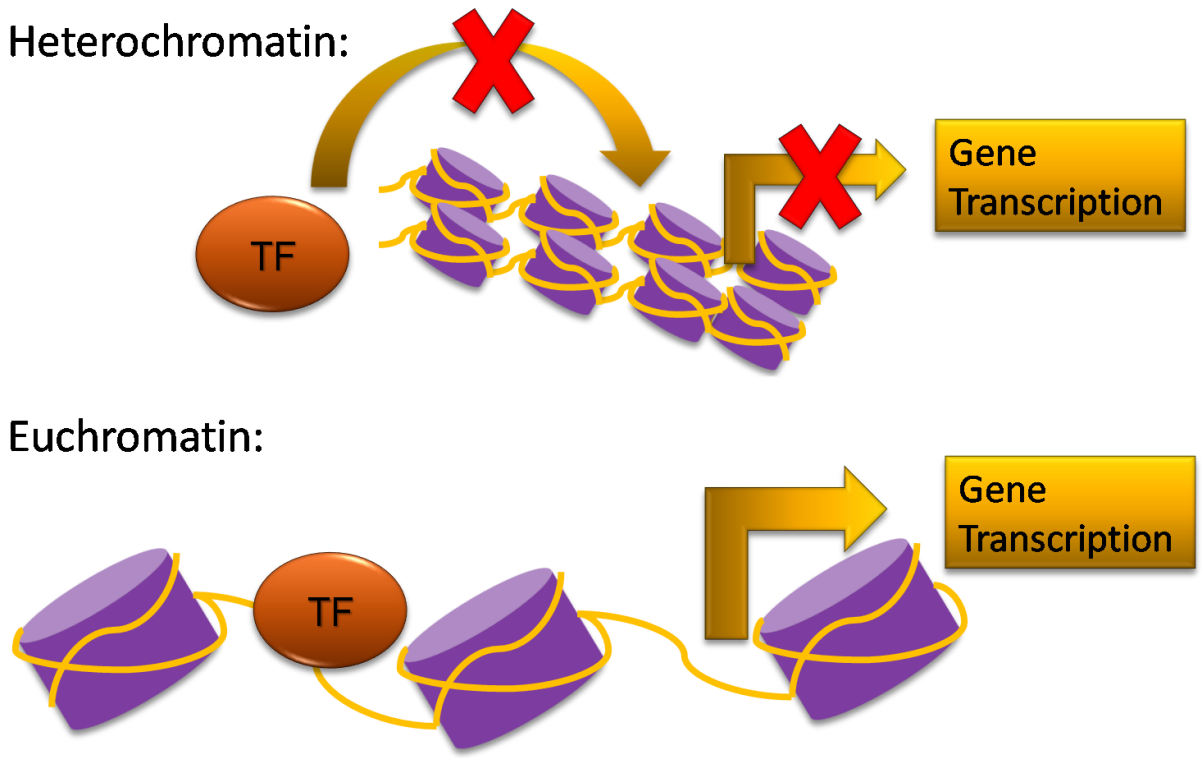

Figure 1.1 Chromatin Neighbourhoods. Simplified theoretical model of how chromatin structure influences gene transcription. In a heterochromatic state, transcription factors (TFs) cannot properly access the DNA (yellow lines) to initiate transcription because the DNA is tightly coiled around the nucleosomes (purple cylinders). However, in a euchromatic state, the DNA is easily accessible and thus gene transcription is able to occur.

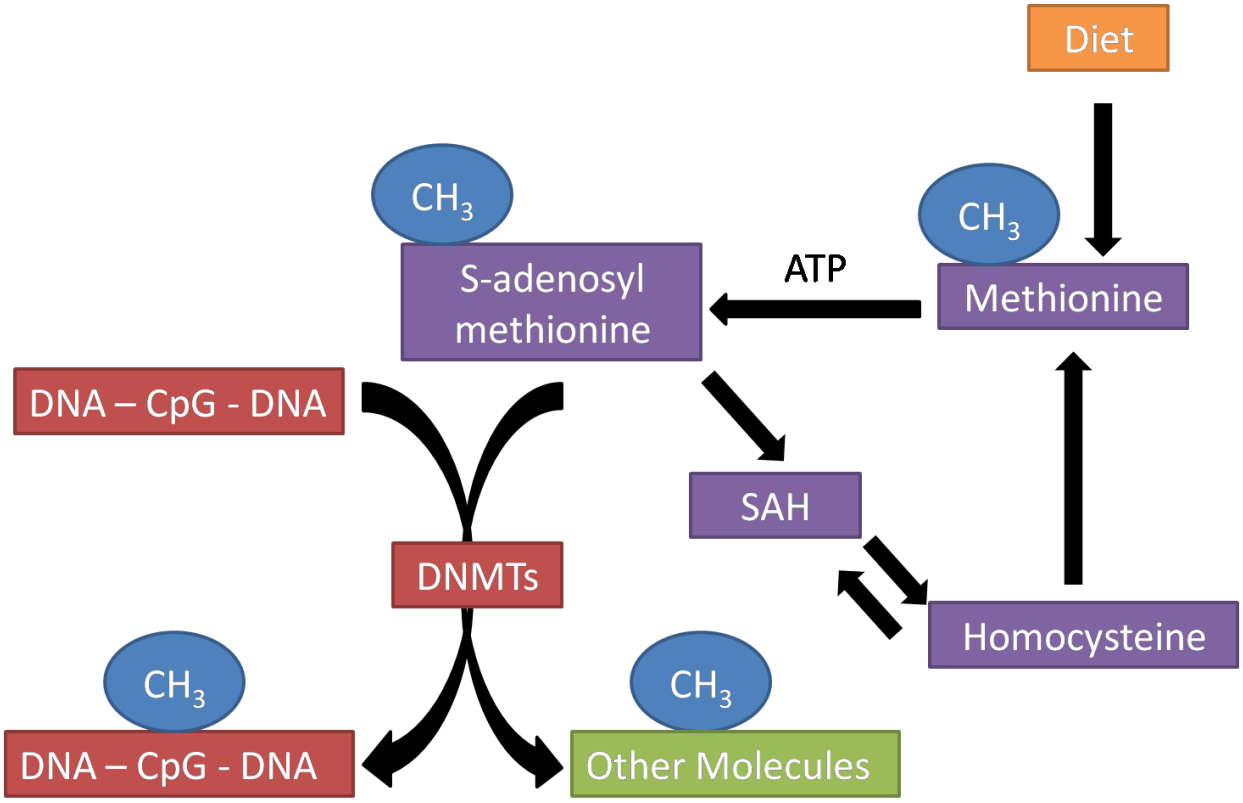

Figure 1.2 Simplified Methyl Group Donor Pathway. S-adenosyl methionine is the methyl donor for DNA methylation. Initially, methionine comes from dietary sources and is subsequently regenerated from homocysteine (purple pathway). In the presence of the enzyme methionine adenosyl transferase, methionine reacts with ATP to form S-adenosyl methionine. DNA Methyltransferases (DNMTs) transfer the methyl group (CH_3) from S-adenosyl methionine to a genomic CpG site (red pathway) or to other molecules that are regulated by methylation (green pathway).

2 METHODS

2.1 Experimental Design

As shown in **Figure 2.1**, primary bronchial epithelial cells from four individuals and an immortalized bronchial epithelial cell line in triplicate were passaged under normal cell culture conditions. Cell passaging or splitting is a technique that enables the cells to continue to grow and propagate under cultured conditions for extended periods of time **(Figure 2.2)**. At each passage, DNA and RNA were collected. In total, DNA and RNA was successfully collected at passages 1, 2 and 4 for primary bronchial epithelial cell lines and at passages 1,2,3,4 for each immortalized bronchial epithelial cell line. The DNA was bisulfite-converted and hybridized to the Illumina GoldenGate Methylation Array that interrogates 1505 CpG sites across 807 genes. The RNA was hybridized to the Illumina Humanref-8 BeadChip that interrogates 24,500 transcripts across the transcriptome. From the initial data analysis of the Illumina Methylation arrays and Illumina BeadChips, four genes were selected for validation studies and more in-depth analysis. Quantitative Real-Time PCR was used to confirm relative gene expression levels over passage for these selected genes. This study was found to be acceptable on ethical grounds for research involving human subjects. The reference number is PHC REB H07- 01116 (Appendix A).

2.2 Primary Cell Culture

Human transplant donor lungs, age 20 to 25, deemed unsuitable for transplantation and donated for medical research were obtained through the International Institute for the

Advancement of Medicine(192). Primary human bronchial epithelial cells (BECs) were isolated by protease digestion of human airways as previously described with minor modifications (193). Briefly, after surgical removal specimens were washed in Custodiol® HTK (Histidine-Tryptophan-Ketoglutarate) solution and packed on ice for transportation. The trachea and bronchi to the 3rd generations were dissected and rinsed 3 times in cold Phosphate-Buffered Saline (PBS) without Ca^{2+} and Mg^{2+} to completely remove blood and mucus plugs. The tight junctions connecting the epithelium on intact segments of trachea and bronchi (2-4cm long) were then dissociated with 1.4 mg/ml Pronase and 0.1 mg/ml of DNase (Roche Diagnostics, Indianapolis, USA) per 100ml of MEM (Fisher Scientific, Philadelphia, USA) at 4°C for 16 hours. During the dissociation process, some damaged cells release their DNA which would normally cause a massive inflammatory response. The addition of 0.1 mg/ml of DNase removes the DNA and prevents massive apoptosis during cell preparation. After digestion the tracheal/bronchial tube was rinsed with Eagle's Minimal Essential Medium (MEM) and loosened cell clumps were strained through a 70 µm nylon mesh (Becton-Dickinson, Franklin Lakes NJ). To neutralize Pronase, cells were re-suspended and incubated in MEM with 10% Fetal Bovine Serum (FBS) for 10 min and washed twice with MEM by centrifugation at 4°C. Approximately 20 x 10^6 cells in total were recovered from both the trachea and bronchial tissues of each individual. Adherent cells, normally basal epithelial cells, were suspended in tissue culture flasks and incubated at 37°C for 30 min in BEGM (Cambrex).

BECs were grown to confluence on six-well tissue culture plates (Corning Costar, Cambridge, MA) maintained in 1ml of Bronchial Epithelial Growth Medium (BEGM, Cambrex) containing

100U/ml penicillin and 100ug/ml streptomycin, at 37°C in a humidified 5% CO_2 atmosphere **(Figure 2.3)**. The first time these cells were placed on the tissue culture plates, they were at Passage 0. These cells were then amplified and passaged under normal cell culture conditions. Twenty-percent of the cells from each passage were used to seed the next passage and eighty-percent of the cells were collected for DNA/RNA extraction and analysis once the epithelial cells had reached 90-100% confluence. Passage number refers to the number of times the cell line has been trypsinized, re-plated and allowed to grow back to confluence. The higher the passage number the further away the epithelial cells are from the primary source i.e the lung epithelium.

2.3 Immortalized Cell Culture

Immortalized cell lines can grow and divide indefinitely under optimal cell culture conditions (194). Immortalization of cells can occur naturally such as when cells from tumour origin are cultured; however, considerable progress has been made to transform primary cells into non-cancerous immortalized cells that more accurately represent the primary tissue (195). Immortalized $1HBE_O^-$ cells are human bronchial epithelial cells that were immortalized by overexpression of Simian Virus-40 (SV40) large-T antigen (a viral oncogene) and have previously been characterized in detail (15, 196). Overexpression of SV40 allows the human bronchial epithelial cells to overcome senescence presumably via interacting with the tumour suppressor proteins Rb and p53 (197, 198). $1HBE_O^-$ cells were grown on six-well culture plates in low glucose Dulbecco's Modified Eagle's Media (DMEM) containing 10% Fetal Bovine Serum (FBS) and incubated at 37 C in 5% CO2 **(Figure 2.4)**. Cells were passaged when approximately 90%

confluent. Due to their proliferation and high survival rate, the $1HBE_O^-$ cell line potentially augments the types of analyses that can be performed if there epigenetic and gene expression profiles are similar to the primary bronchial cell lines. An advantage of using SV40 transformed bronchial epithelial cells instead of other types of immortalized cells derived from lung carcinomas is that the original primary cell origin is known whereas this is difficult to determine in naturally transformed cells.

2.4 Genomic DNA and RNA Extraction

Primary and immortalized bronchial epithelial cells were collected from cell plates, centrifuged at 1000RPM for 5 minutes to form pellets and stored at -80 degrees celsius. DNA and RNA were then extracted simultaneously from the primary and immortalized bronchial epithelial cell pellets using the AllPrep DNA/RNA kit (Qiagen). In separate purification reactions, genomic DNA was purified using the AllPrep DNA spin column, and total RNA was purified using an RNeasy Mini spin column. Afterwards, DNA concentration was determined by measuring the intensities at 260 and 280nm using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies; Rockland, DE). DNA samples with low concentration or 260/280 ratios outside of the range for normal DNA (1.80 to 1.90) were cleaned using the DNA Clean & Concentrator-5 kit from Zymo Research (Orange, CA). Prior to the gene expression array and RT-PCR experiments, RNA quality was measured on the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and all RNA samples had an RNA integrity number (RIN) greater than 9.5 which means the RNA was intact and of high quality (199).

2.5 Sodium Bisulfite Conversion

The process of bisulfite conversion of DNA exploits the different sensitivities of cytosine and 5-methylcytosine (5-MeC) to de-amination by bisulphite under acidic conditions—in which cytosine undergoes conversion to uracil, whereas methylated cytosine is non-reactive (200). The efficiency of the reaction is well documented with approximately 99% of unmethylated CpGs undergoing conversion and 99% of methylated CpGs being protected from conversion (201). Bisulfite treatment of 500ng to 1µg DNA per sample was done using the EZ DNA Methylation-Gold Kit (Zymo Research) according to the manufacturer's instructions prior to the Illumina GoldenGate DNA Methylation array.

2.6 Illumina Array: DNA Methylation

DNA methylation profiling of bronchial epithelial cells was done using the Illumina Goldengate Methylation platform array which utilizes the same Illumina SNPgenotyping platform that is used for SNP detection (202). The technique is based on the fact that nonmethylated cytosines(C) are converted to uracil (U) when treated with bisulfite, while methylated cytosines remain unchanged (203). Because the hybridization behaviour of uracil is similar to that of thymine (T), the detection of the methylation status of a particular cytosine can be carried out following bisulfite treatment by using a genotyping assay for a C/T polymorphism. The assay procedure is similar to standard SNP genotyping, except that four oligonucleotides, two allele-specific oligonucleotides (ASOs), and two locus-specific oligonucleotides (LSOs) are required for each assay site rather than three. The methylation status of an interrogated CpG site is determined by the ratio of the fluorescent signal from the methylated allele over the sum of

the fluorescent signals of both methylated and unmethylated alleles. This provides a continuous measure of the levels of DNA methylation of a specific CpG site, ranging from 0 in the case of completely unmethylated sites to 1 for completely methylated sites. Multiple readings for each CpG site serve as internal controls to validate the microarray findings. On average, the methylation state of two CpG sites per gene was determined. In total, 1505 CpG sites in 807 genes on the Illumina CancerPanel were interrogated with the majority of the CpG loci located in HCPs **(Figure 2.5)**. This panel includes important genes involved in cell cycle regulation, cell differentiation and apoptosis that are prime candidates to undergo changes under cell culture conditions. Bisulfite conversion of DNA, hybridization and scanning were done at the Centre for Molecular Medicine and Therapeutics (CMMT) by experienced technicians.

2.7 Illumina BeadChip: Gene Expression

Gene expression microarray experiments were performed using three Illumina HumanRef-8 Expression BeadChips (Illumina, San Diego, USA) that contains 8 arrays per chip. The array set contains 24,000 different bead types representing NCBI Reference Sequence gene transcripts with some of the transcripts measured in duplicate by two different probe sets. Each Illumina bead-type carries thousands of 50-mer transcript-specific probe sequence oligonucleotides and each bead-type is represented approximately 30 times in each array. Multiple copies of each probe set provide built in redundancy that improves the robustness and precision of gene expression array measurements (204). Each of the 24 RNA samples was hybridized to a single array. Target cDNA for the microarrays was prepared following the manufacturer's protocol.

Briefly, 1 ug of total RNA from each bronchial epithelial cell sample was reverse transcribed into cDNA and subsequently in vitro transcribed and biotinylated using the Illumina Total Prep RNA Amplification Kit (Illumina, San Diego, USA). After hybridization and stringent washing, the bound cDNA was stained using Streptavidin – Cy3. Gene signals on the microarray were scanned using the Bead Array Reader and the Sentrix Scan Software (Illumina, San Diego, USA). Hybridization and scanning of BeadChips was performed at the McGill University and Genome Québec Innovation Centre by their qualified technicians.

2.9 Real-Time Quantitative PCR

Reverse transcription was performed with 2 µg of total RNA, a mix of random and oligo(dT) primers and Omniscript reverse transcriptase (Qiagen). Real-time PCR reactions used 10ng of cDNA, 200nM of forward and reverse primers and the FastStart Universal SYBR Green Master Mix (Roche Diagnostics) in a final 10 µl reaction. PCR cycling conditions were as follows: 10 min at 95°C; 50 PCR cycles (95°C for 15 sec; 60°C for 30 sec; 72°C for 31 sec); a melting cycle consisting of 95°C for 15 sec, 60°C for 15 sec, and a ramping cycle up to 95°C in 20 min with a fluorescence reading at each °C followed by 15 sec at 95°C. PCR primers in these assays are designed to span introns to minimize potential problems from genomic DNA contamination. All measurements were made in triplicate. A melting curve was performed after each run to confirm that there is a single amplified product. Initially, expression levels were computed for each primer pair across several samples from cycle threshold values (Cts) obtained from the instrument. Then normalized relative expression values and quality control evaluations were implemented using the qBase method developed by Hellemans et al. (205). Relative expression

levels are normalized with respect to a set of three reference primer pairs (GAPDH, PUM1 and RPL13a). **Table 2.1** contains the specific forward and primer sequences for all reference and candidate genes interrogated. The normalization of relative quantities with reference genes relies on the assumption that the reference genes are stably expressed across all tested samples. In the qBase software, the stability of the reference genes is tested by calculating the coefficient of variation. In addition, inter-run calibration is used to correct for run-to-run variations from a selected calibrator sample repeated across all runs. Real-time quantitative PCR and normalisation of the data was done by experienced technicians at the Centre de développement des biotechnologies (CDB) de Sherbrooke.

2.10 Statistics

2.10.1 Data Organisation

These data were organised with BeadStudio methylation and gene expression software from Illumina (San Diego, CA). All methylation array data points are represented by fluorescent signals, Cy5 and Cy3, from both methylated and unmethylated alleles respectively. The methylation level is given by the formula:

$$\beta = (\max(Cy5,0)) / (|Cy3| + |Cy5| + 100)$$

The average methylation beta value (β) is derived from the approximately 30 bead replicate methylation measurements that exist for each of the 1505 CpG loci. Raw average beta values were analyzed without normalization as recommended by the software providers. All CpG loci on the X chromosome were excluded from analysis to remove any bias from differences in

gender. For the remaining CpG loci, the detection P-value was used to determine quality and consistency of the average β value. CpG loci with a mean detection P-value >0.05 across all samples were eliminated from analysis (n=50, 3.5%). The final methylation data set contained 24 samples (12 primary and 12 immortalized bronchial epithelial cell derived) and 1372 CpG loci. For many subsequent analyses these 1372 CpG loci were distributed into three categories depending on the region they were located in: High, Intermediate and Low density CpG Promoters. This categorization is based on the CpG content analysis of each Illumina CpG probe by Weber et al.(72).

Analysis of gene expression microarray data was carried out using the Illumina Gene Expression Software (San Diego, CA). The arrays were normalized by the average normalization algorithm so that the means of all gene expression signals for the different arrays became equal. This method was chosen since the HumanRef-8 BeadChip arrays compare large numbers of genes, and the expression levels can be assumed to be roughly similarly distributed. To control for background noise, gene expression microarray data was subjected to background subtraction. This is done by using the average value of control beads conjugated with nonspecific oligonucleotides as a baseline expression signal to which 18,631 transcript gene expression signals were calibrated for each sample. Although this potentially results in the exclusion of a large number of probes with negative values, it has been shown that significant data compression results when no background subtraction is performed (206). In this case, 2,367 probes were removed because of negative values after background subtraction and a further 5,822 transcript probes with a mean detection P-value >0.05 across all samples were eliminated

from further analysis. The final gene expression data set contained 10,442 transcripts for each of the primary and immortalized bronchial samples. After normalization, each bead-type signal was calculated by averaging corresponding bead signals after removing the outliers using median absolute deviation.

Subsequent analyses of methylation and gene expression data were carried out using Significance Analysis of Microarray (SAM) software, Excel 2007 and SPSS v 15.0.

2.10.4 Aim 1 – CpG Methylation Characterization

Average DNA Methylation:

Un-paired Student's t-tests were used to evaluate differences in average methylation between immortalized and bronchial epithelial cell lines. A repeated measures ANOVA test was used to test the differences in average DNA methylation over passage. This approach was used instead of a standard ANOVA because it models the correlation between repeated measures and takes into consideration that the average DNA methylation is being measured for each individual's cell line at different time points in cell culture. Prior to analysis, the alpha level, i.e. the acceptable level of error, was set at 0.05. P-values lower than 0.05 were considered significant and resulted in rejection of the null hypothesis.

Inter-passage DNA Methylation Correlation:

Pearson's correlation coefficient (r) reflects the degree of linear relationship between CpG-specific DNA methylation average beta values from two different passages of the same cell line

for all of the interrogated CpG loci. A correlation value of +1 means that there is a perfect positive linear correlation between passages, -1 means a perfect inverse correlation, and 0 means there is no correlation between the two selected passages.

CpG-specific changes in methylation from Passage 1 to Passage 4:

For the top 10 CpG sites that had the largest changes in DNA Methylation from passage 1 to 4, paired, two-tailed Student's t-tests were used to determine whether it was a significant change in DNA methylation for immortalized and primary bronchial epithelial cells respectively. P-values were conservatively corrected for multiple testing by multiplying the p-value by the number of tests (in this case 10). Therefore, an uncorrected p-value would need to be <0.005 to be significant after Bonferroni correction.

2.10.3 Aim 2 - DNA Methylation and Gene Expression Correlation

The 1372 CpG loci were divided into three sub-categories: unmethylated (<20% methylation), hypermethylated (>80% methylation) and heterogeneously methylated (20 – 80% methylation). For each category, Spearman's rank correlation coefficient (Rs) gives an estimate of the association between average beta (DNA Methylation) and average signal (Gene Expression) for the genes that are represented on both microarrays and provides a measure of how closely the two sets of rankings agree with each other. The significance of a Spearman's rank correlation can be determined by looking up the correlation value and degrees of freedom in a significance table **(Figure 2.6)**. This coefficient was used in lieu of Pearson's correlation coefficient because of the non-normal distribution of the data for both data sets. The correlation test was

conducted with Illumina's BeadStudio analysis software separately on primary bronchial epithelial samples and immortalized airway epithelial samples.

2.10.2 Aim 3 – Candidate Gene In-Depth Analysis

Significance of Analysis of Microarrrays:

The significance of analysis of microarrays (SAM) platform is an open source statistical program designed for finding significant genes in microarray experiments (207). The software was written by Balasubramanian Narasimhan and Robert Tibshirani. SAM uses repeated permutations of the data to determine if the expression or methylation of any genes are significantly related to the dependent variable, in this case: cell culture passaging. Adjustment of a delta-tuning parameter can alter the number of false-positives and was set at 0.20 for this study. The cutoff for significance is determined by the q-value which for this study was based on the false positive rate of 0%. The q-value is the lowest false dicovery rate at which the gene is considered to be significant (208). For this study, the one class timecourse slope and one class time course signed area were the experimental setups used to determine whether a CpG site's methylation or a gene's expression had changed significantly over passage number in primary and immortalized cells. The slope option discovers significant genes that linearly increase or decrease in methylation/gene expression over passage whereas the signed area mode pulls out significant genes that vary significantly over pasage number. Specifically for the SAM analysis, we eliminated additional CpG loci that have beta values <0.10 or >0.95 across all samples and individuals. This led to the exclusion of 688 consistently invariant CpG sites in addition to the

133 CpG loci previously removed for high p-detection values and location on the X-chromosome. Therefore, for the SAM analysis, 684 CpG loci were studied for linear or dynamic changes in DNA methylation over passage.

Array Validation: Real-time quantitative PCR

Fold change values between passages of the same individual or replicate were used to compare the microarray gene expression data and the normalized real-time qPCR results. Significance was determined by an un-paired, two-tailed, t-test.

2.11 Candidate Gene Selection Process

Candidate genes were selected based on their meeting the following criteria:

1) Significant linear changes (q-value =0%) in DNA methylation over passage in either primary or immortalized cell line, as determined by time course SAM analysis.

   OR

2) Significant non-linear changes (q-value<10%) in DNA methylation over passage and high correlation (Rs Spearman > 0.55) with gene expression changes in primary or immortalized cell line.

   AND

3) Biological relevance to the development and differentiation of the airway epithelial cell as indicated by an extensive search of the literature and consultation with airway epithelial cell experts.

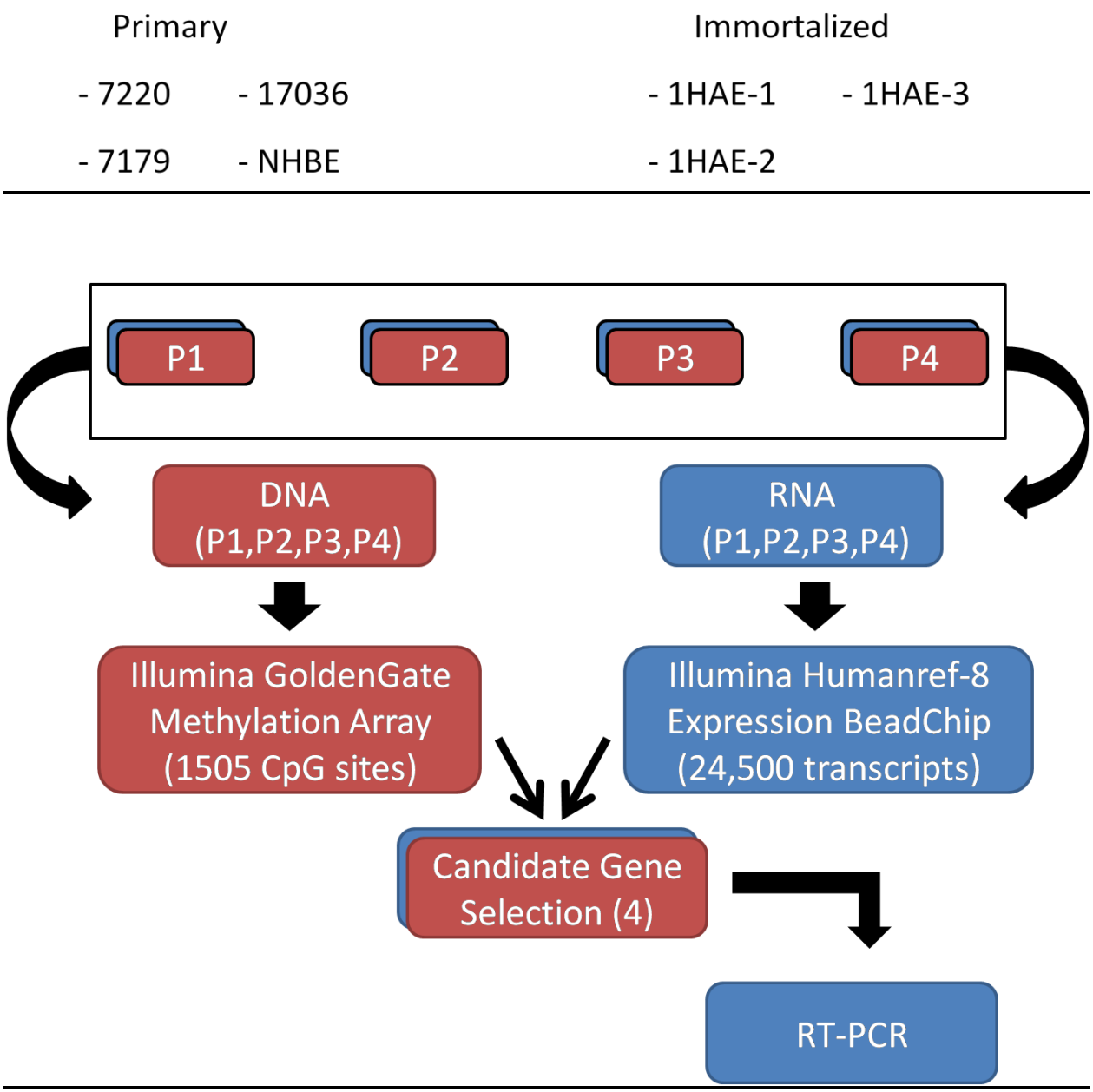

Figure 2.1 Experimental Design. Primary bronchial epithelial cells from four individuals and an immortalized bronchial epithelial cell line in triplicate were passaged under normal cell culture conditions. At each passage, DNA and RNA were collected. In the DNA stream (Red Pathway) DNA was bisulfite-converted and hybridized to the GoldenGate platform. In the RNA stream (Blue Pathway), RNA was hybridized to the Illumina Humanref-8 BeadChip that interrogates 24,500 transcripts across the transcriptome. From the data analysis of both RNA and DNA methylation arrays, 4 genes were selected for validation studies and more in-depth analysis.

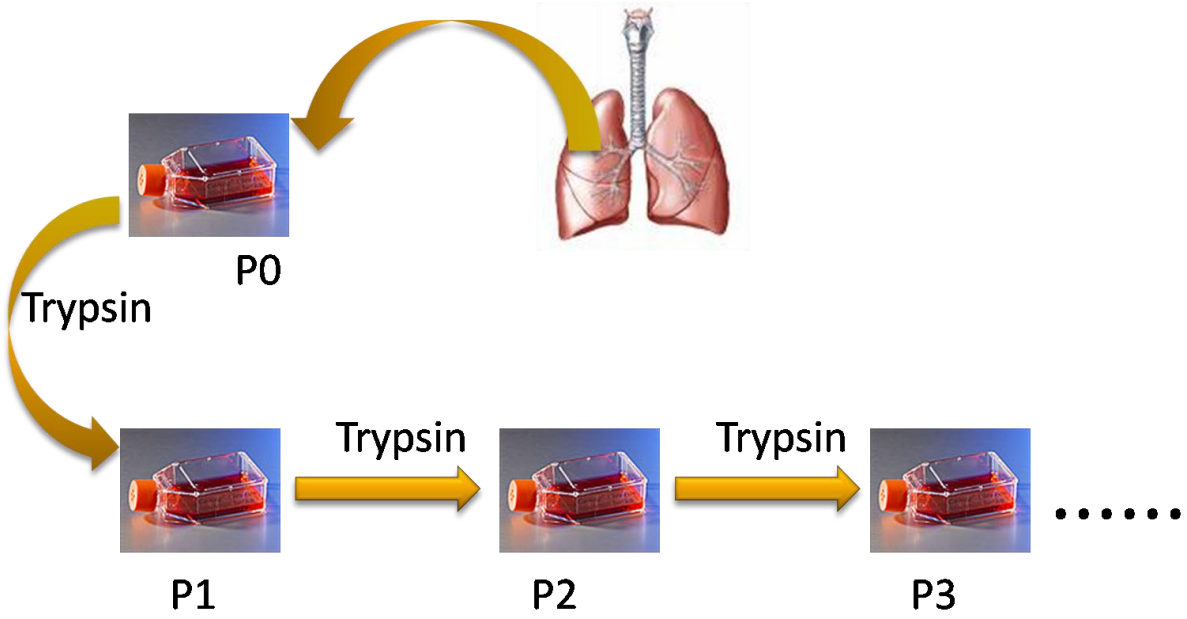

Figure 2.2 Cell Culture Passaging. Primary bronchial epithelial cells were removed from donor lungs by placing the dissected airways into a solution of pronase that acts on the tight junctions to release the epithelial cells. These cells were then placed on a tissue flask. This equals Passage0. Cell passaging or splitting is a technique that enables the cells to stay alive and keep growing under cultured conditions for extended periods of time. Passage number refers to the number of times the cell line has been trypsinized, re-plated and allowed to grow back to confluence. The higher the passage number the further away from the primary source the sample was derived from. At each passage, 20% of the cells were passaged to the next flask and 80% of the cells were collected for DNA/RNA analysis once the cells had reached 90-100% confluence.

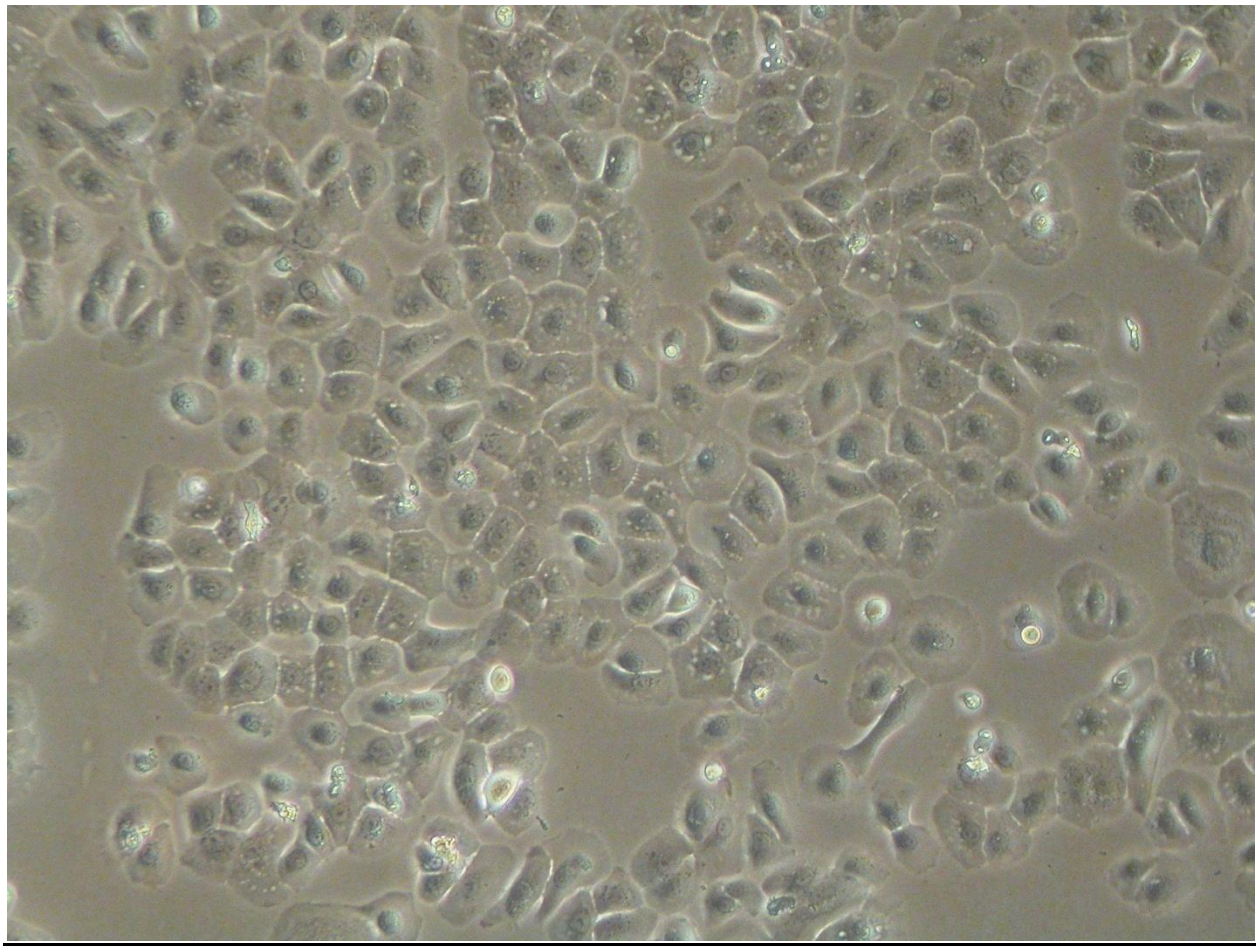

Figure 2.3 Image of Primary Bronchial Epithelial Cells in Cell Culture. 20x image of primary bronchial epithelial cells at passage 1. Primary bronchial epithelial cells were grown in BEGM culture media containing 100U/ml penicillin and 100ug/ml streptomycin, at 37°C in a humidified 5% CO_2 atmosphere.

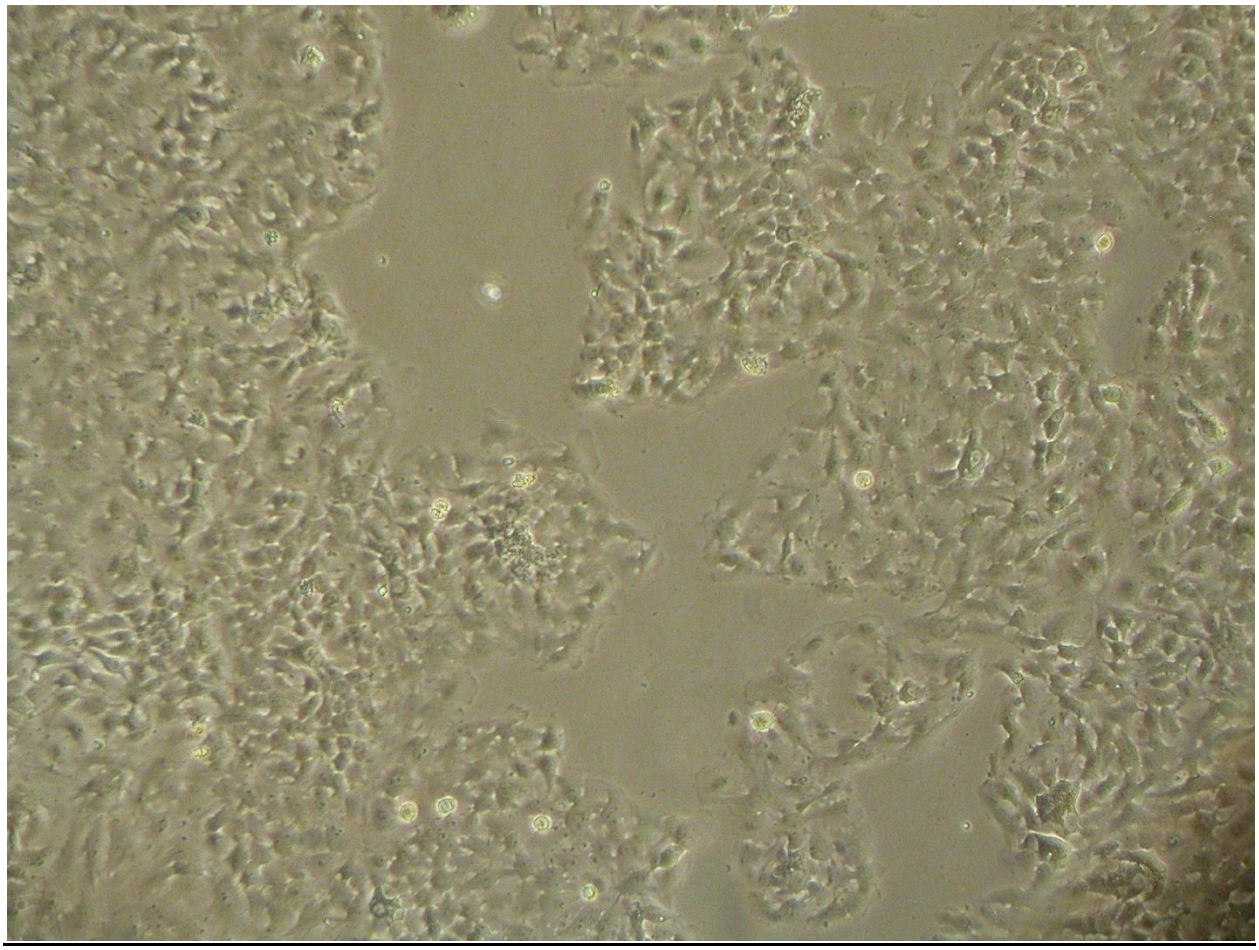

Figure 2.4 Immortalized Bronchial Epithelial Cells. 10x image of immortalized bronchial epithelial cells at passage 1. Immortalized bronchial epithelial cells were grown in DMEM culture media containing 10% FBS and incubated at 37 C in 5% CO2, at 37°C in a humidified 5% CO_2 atmosphere.

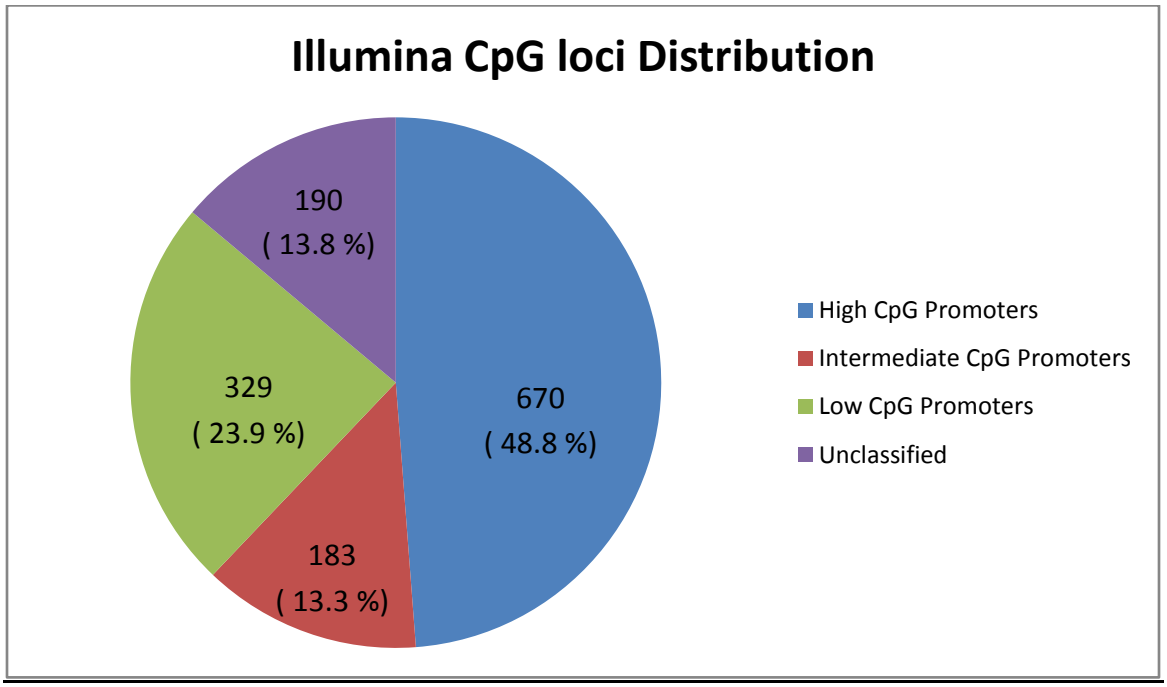

Figure 2.5 Distribution of Illumina CpG Loci Interrogated in this Study. Distribution of the CpG loci was done by using the definition by Weber et al that classified promoter regions into three CpG density categories: High CpG Promoters, Intermediate CpG promoters and Low CpG Promoters(72). The vast majority of CpG loci are located in High density CpG promoter regions (48.8%), followed by Low density CpG promoters (23.9%) and intermediate CpG promoters (13.3%). Some of the CpG loci were not classified at the time of this study (13.8%).

Gene	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	GTGAAGGTCGGAGTCAACGGATTT	TGCCATGGGTGGAATCATATTGGA
PUM1	TGAGGTGTGCACCATGAAC	CAGAATGTGCTTGCCATAGG
RPL13A	CCTGGAGGAGAAGAGGAAAGAGA	TTGAGGACCTCTGTGTATTTGTCAA
CLDN4	GTGCCTTTGAAGTCTTTGTCTTCCTTCCT	GGTGCTTCTCTCCTCTCCGCTC
IFNGR2	ATTTTCGTTGCTGTCGGTGCTGG	CTCTAAGATGGGCTGAGTTGGGTCTTTTAAA
KRT13	ACCAACACTGCCATGATTCAGACCAG	GCACTCCGTCTCTGCCACCG
TGFBI	AGCCCTGCCACCAAGAGAACG	GGAGAGACTTTAGCCGCACCAGG

Table 2.1 Quantitative Relative Expression RT-PCR Primer Sequences. Each primer pair entry is listed by its gene, forward, and reverse primer sequences. A gene name surrounded by stars ('*') identifies reference primer pairs used in normalization (i.e. a housekeeping gene).

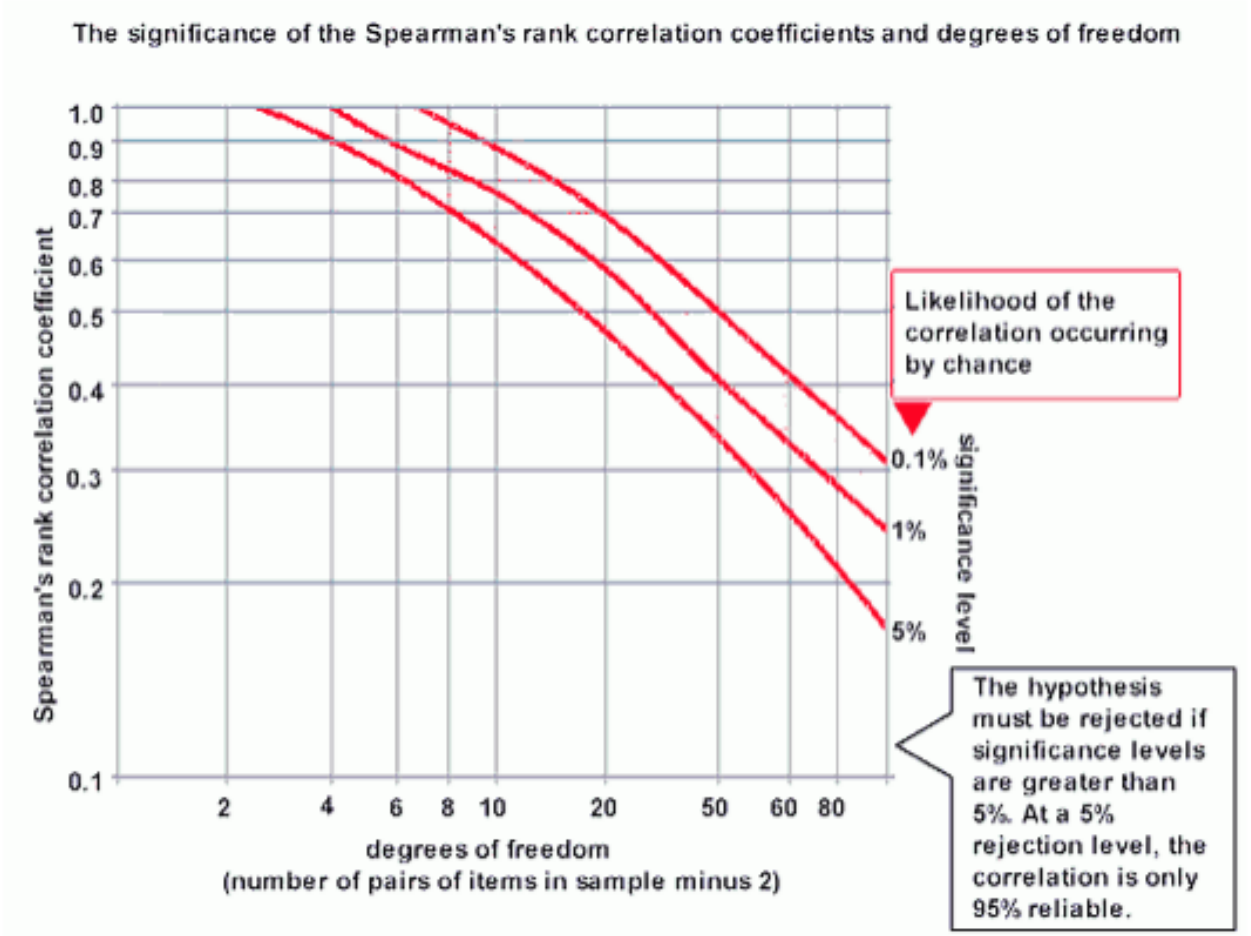

Figure 2.6 Spearman's Rank Correlation Coefficient Significance Table. In the calculation of gene expression and DNA methylation correlation, 12 pairs of items were used = 10 degrees of freedom. Therefore, a spearman rank correlation coefficient for this comparison must be greater than 0.65 in order to be significant at a 5% ($p<0.05$) significance level. Taken with permission from Barcelona Field Studies Centre (http://geographyfieldwork.com)

3 RESULTS

3.1 Aim 1 – Characterization of CpG-Site Specific DNA Methylation

CpG-site specific DNA methylation was assessed at passages 1,2 and 4 in four normal bronchial epithelial cell lines and passages 1,2,3 and 4 in three immortalized bronchial epithelial cell lines for 1505 CpG sites located in promoter regions and/or first exons of 807 genes. The following analysis is based on the 1372 CpG loci that met the inclusion criteria.

Clustering of primary and immortalized bronchial epithelial cell lines by their DNA Methylation Profile. Unsupervised hierarchical clustering based on Manhattan distance of CpG DNA methylation separated the samples into two distinct groups: immortalized bronchial epithelial cells and primary bronchial epithelial cells. **Figure 3.1** displays in a heatmap the autosomal CpG loci that distinguish the primary bronchial epithelial cells from the immortalized airway epithelial cells. CpG loci not displayed in the heatmap were either hypermethylated (>0.80) or hypomethylated (<0.20) consistently for all samples. Two-hundred and eight CpG sites were hypermethylated in the immortalized cell samples compared to primary while only thirty-five CpG sites were hypermethylated in primary cell samples compared to immortalized. However, there were no significant differences in gene expression levels of transcripts associated with these differentially methylated sites (**Figure 3.2**). **Figure 3.3** illustrates the difference in average methylation calculated from 1372 CpG sites between immortalized and primary cell lines. The average DNA methylation of immortalized cells was significantly greater ($p<0.0001$) than primary bronchial cell lines. There were no significant inter passage differences

(F=1.19, p=0.367) in average DNA methylation between passages 1,2 and 4 of the primary bronchial epithelial cells or between passages 1,2,3,4 of the immortalized bronchial epithelial cells.

Categorization of CpG loci according to their methylation status. For primary bronchial epithelial cells, the majority of CpG loci overall show a bimodal distribution of methylation: 23.3 % of the loci were hypermethylated (>80% methylation), 61.7 % were unmethylated (<20% methylation) and 15.0 % were heterogeneously methylated (20 – 80% methylation). The large percentage of unmethylated CpG loci is in agreement with previous studies that show low methylation in strong CpG Islands and within regions 1000 base pairs from the transcription start site for other normal tissues (67, 72).

For immortalized bronchial epithelial cells, the CpG loci overall show a similar distribution with the notable presence of significantly more hyper and heterogeneously methylated CpG sites than primary cell lines ($p<0.0001$). Of the 1372 CpG loci, 32.4 % of the loci were hypermethylated (>80% methylation), 45.7 % were unmethylated (<20% methylation) and 21.9 % were heterogeneously methylated (20 – 80% methylation). The presence of significantly more CpG island hypermethylation ($p<0.05$) in immortalized cell lines compared to primary cell lines has been previously shown in human cancer cell lines (209) but this is the first study to indicate the presence of enhanced CpG island hypermethylation in cell lines immortalized by the over-expression of SV-40 large- T antigen.

Location of DNA Methylation. There was also considerable distributional variation in the type of CpG Promoters (low, intermediate, high density) in which hypermethylated, heterogeneously and unmethylated CpG sites were located. For primary cell lines **(Figure 3.4)**, the majority of methylated CpG loci were in LCPs (58.0%) and secondly found in ICPs (27.9%). Heterogeneously methylated CpG loci were mainly in HCPs (44.1%) and unmethylated CpG loci were also mainly located in HCPs (63.0%). Similarly, in immortalized cell lines (**Figure 3.5)**, methylated CpG loci were primarily in LCPs (45.2%) but secondly were found in HCPs (23.9%). Heterogeneously methylated CpG loci were in HCPs (42.8%) and the vast majority of unmethylated CpGs were in HCPs (69.2%). Although, immortalized cell lines are significantly more hyper and heterogeneously methylated than primary cells, there was no significant difference between the cell lines with respect to the percentage of methylated CpGs distributed into weak, intermediate and strong CpG Islands (LCP,ICP and HCPs) ($p>0.05$).

3.1.2 Inter-Passage Comparisons

As shown in **Figure 3.6**, a comparison of DNA methylation profiles between passages of the same cell line revealed overall high correlations. Inter-passage Pearson correlations of primary bronchial epithelial cell lines ranged from 0.85 to 0.98 with a trend towards higher correlations occurring between the two latest passages (Passage 2 and 4) with the exception of one primary cell line that had higher inter-passage correlations in general. Overall, immortalized bronchial epithelial cell lines showed a trend towards higher correlation than primary cell lines with Pearson R values ranging from 0.88 to 0.98 between passages with the majority above 0.90. The CpG sites that showed the greatest absolute change in DNA methylation from passage 1 to

passage 4 in primary bronchial epithelial cells were located in the following genes: *IPF1, KCNK4, MSH2 and RUNX1T1* that are involved in regulating apoptosis and cell cycle regulation **(Table 3.1)**. However, none of these reached significance when the p-value was adjusted for multiple testing. The CpG sites that showed the greatest absolute change in DNA methylation from passage 1 to 4 in immortalized bronchial epithelial cells were located in the following genes: *NEFL, ETS1, and MCF2* and also did not reach significance after correcting for multiple testing (**Table 3.2)**. Three CpG sites of the ten sites to change the most were shared between primary and immortalized cell lines: *RUNX1T1, NEFL, and MMP3*. It follows that these sites are unable to maintain their normal DNA methylation marks due to cell culture propagation rather than cell culture conditions themselves since immortalized and primary cells were grown in different media.

3.1.4 Inter-Individual and Inter-Replicate Passage Comparisons

DNA methylation profiles of primary bronchial epithelial cells from different individuals showed a trend towards being more highly correlated at later passages. As shown in **Figure 3.7**, a comparison of the 1372 CpG loci for between individual cell lines showed a relatively low correlation for passage 1 samples in the primary bronchial epithelial cell lines with a range of R Pearson correlations between 0.78 and 0.84. However, these individual differences in DNA methylation profiles decreased as indicated by the higher correlation between individual samples at the higher passage numbers with R Pearson correlations ranging from 0.84 to 0.93 and 0.81 to 0.91 for passage 2 and passage 4 inter-individual comparisons respectively.

A comparison of the CpG methylation profiles of the three replicates of immortalized bronchial epithelial cells tended to be more highly correlated than inter-individual passage comparisons of primary bronchial epithelial cell lines. However, even though these cells were derived from the same source and as previously shown had very high inter-passage correlations there still exists distinct CpG methylation profiles in the immortalized cell line replicates that are maintained in culture **(Figure 3.7)**. Overall, inter-replicate passage comparisons of the immortalized bronchial epithelial cell lines had R Pearson correlations that ranged from 0.84 to 0.95 with no evident trends towards higher or lower correlation dependent upon passage number.

3.2 Aim 2 – CpG-Site Specific DNA Methylation and Gene Expression

In primary bronchial epithelial cells, the average normalized gene expression signal from the BeadChip was 680.4, 897.7, and 1111.6 for hypermethylated, heterogeneous and unmethylated CpG loci respectively **(Table 3.3).** However, even though there is a trend towards less methylated sites having higher gene expression signals, these differences were not significant in the ANOVA analysis ($F=2.35, p=0.095$). Similarly, in immortalized bronchial epithelial cell lines there was no significant association between degree of methylation for the 1372 interrogated CpG sites and level of gene expression signal ($F=2.030$, $p=0.130$). Although, the average gene expression signal of unmethylated CpG loci was higher than the other two degrees of CpG methylation.

3.2.1 Primary Bronchial Epithelial Cells

The top 15 positive and negative gene expression and CpG site correlations as determined by Rs Spearman co-efficient of correlation and sorted by methylation status of the CpG sites are shown in **tables 3.4 and 3.5** respectively. As mentioned in the methods section, Spearman correlations greater than 0.65 are significant with a 5% likelihood of occurring by chance for ten degrees of freedom. The level of correlation is one indicator of the role that DNA methylation plays in regulating gene expression for these selected genes. Of note is the similar strength of positive correlation (range 0.64 to 0.85) and negative correlation (-0.62 to -0.84) between DNA methylation and gene expression indicating that DNA Methylation of hypermethylated, heterogeneously and unmethylated CpG sites is significantly associated with both low and high gene expression levels dependent upon the specific CpG loci analyzed.

3.2.2 Immortalized Bronchial Epithelial Cells

Similarly, the top 15 positive and negative correlations between gene expression and CpG site of immortalized bronchial epithelial cell lines showed strong direct and inverse correlations. Positive Rs Spearman correlations ranged from 0.58 to 0.81 and negative correlations ranged from -0.60 to -0.86 regardless of the methylation status of the CpG loci **(Table 3.6 and 3.7**).

3.3 Aim 3 – In-Depth Study of Selected Candidate Genes

Four candidate genes were selected based on two distinct sets of criteria as discussed in the methods section.

3.3.1 Criteria 1

Of all variant CpG sites analyzed using the SAM software by a one-class time course slope analysis, only DNA methylation of four genes (KRT 13, CLDN4, TEK and PDGFRA) significantly changed over passage (q-value = 0%) in primary cell culture consistently in a linear manner. As shown in **Figure 3.8**, these significant genes were selected based on their deviation from the standard observed/expected curve generated by SAM permutation testing. In this particular case, all significant genes were increasing in methylation which is consistent with previous research that has shown that increased methylation at CpG islands occurs in primary culture over passage (73). A similar analysis of the immortalized cell culture lines revealed no CpG sites that significantly changed in a linear manner over passage and only one CpG site that deviated over passage. These findings are consistent with other studies that show that primary and immortalized cell lines respond differently to cell culture (185, 186). For instance, although primary cell culture induces de novo methylation, immortalized cells do not gain methylation during cell culture propagation, potentially because they are already highly methylated compared to their primary cell counterparts (183). On the basis of these observations and a review of the literature, KRT13 and CLDN4 were the first two candidate genes selected due to their change in DNA methylation and biological relevance to the functioning of the airway epithelium. For both the CpG sites interrogated for these two genes, the CpG loci was initially heterogeneously methylated but gained methylation over passage under primary cell culture conditions to become hypermethylated.

The Keratin 13 (KRT13) gene is responsible for encoding an intermediate filament protein that is responsible for the structural integrity of epithelial cells. In primary bronchial epithelial cell lines, KRT13 becomes hypermethylated over passage ending at a level similar to that seen in the immortalized bronchial epithelial cell lines (**Figure 3.9)**.

Claudin-4 (CLDN4) is a gene that encodes an integral membrane protein which belongs to the claudin family and is a component of tight junction strands. Similar to KRT13, CLDN4 becomes hypermethylated over passage ending at a level similar to its status in the immortalized cell lines (**Figure 3.10)**.

3.3.2 Criteria 2

For primary bronchial epithelial cell lines, numerous CpG sites dynamically changed over passage by increasing or decreasing in methylation non-linearly from passage to passage. From these initial results, a study of DNA methylation/gene expression correlation and a review of the literature, Interferon gamma receptor 2 (INFGR2) and Transforming Growth Factor, Beta-Induced (TGFBI) were the next two candidate genes selected for follow-up analysis.

The INFGR2 gene encodes the non-ligand-binding beta chain of the gamma interferon receptor. In primary bronchial epithelial cell lines, IFNGR2 undergoes dynamic changes in DNA methylation (q-value= 0%), with a peak in the amount of DNA methylation occurring at passage 2 and then a loss of DNA methylation by passage 4 (**Figure 3.11)**. In addition, IFNGR2 had a significant positive Rs Spearman correlation co-efficient of 0.73 when comparing DNA methylation and gene expression levels at each passage.

TGFBI is a gene responsible for encoding the RGD-containing protein that binds to type I, II and IV collagens. Therefore, it is an especially important protein involved in cell-collagen interactions and can also serve to inhibit cell adhesion. As shown in **Figure 3.12**, TGFBI has a high Rs Spearman correlation value for DNA methylation/gene expression of 0.57 but there was not much consistency in the overall nature of DNA methylation/gene expression changes between individual primary bronchial epithelial cell lines. Another reason why TGFBI was selected as the final candidate gene is because of the strong body of literature implicating that dense methylation of the TGFBI promoter region is associated with inhibition of gene expression and has been linked to invasiveness in human tumours. The prior knowledge that TGFBI is strongly regulated by DNA methylation is important since only a small proportion of genes seem to be regulated largely by DNA methylation

3.3.3 Quantitative RT-PCR Validation

In order to validate the Illumina microarray results, the fold-change in gene expression between passages from the microarray data for CLDN4, IFNGR2, TGFBI and KRT13 was compared with the corresponding quantitative real-time PCR fold-change values. Both the micro-array data and the qPCR data revealed that KRT13 is not expressed in primary or immortalized bronchial epithelial cells and therefore it is not displayed in the fold-change table. The direction and strength of the expression change was reproduced with quantitative RT-PCR for the three other genes in primary and immortalized cell culture as shown in **Table 3.8** and **Table 3.9**, respectively.

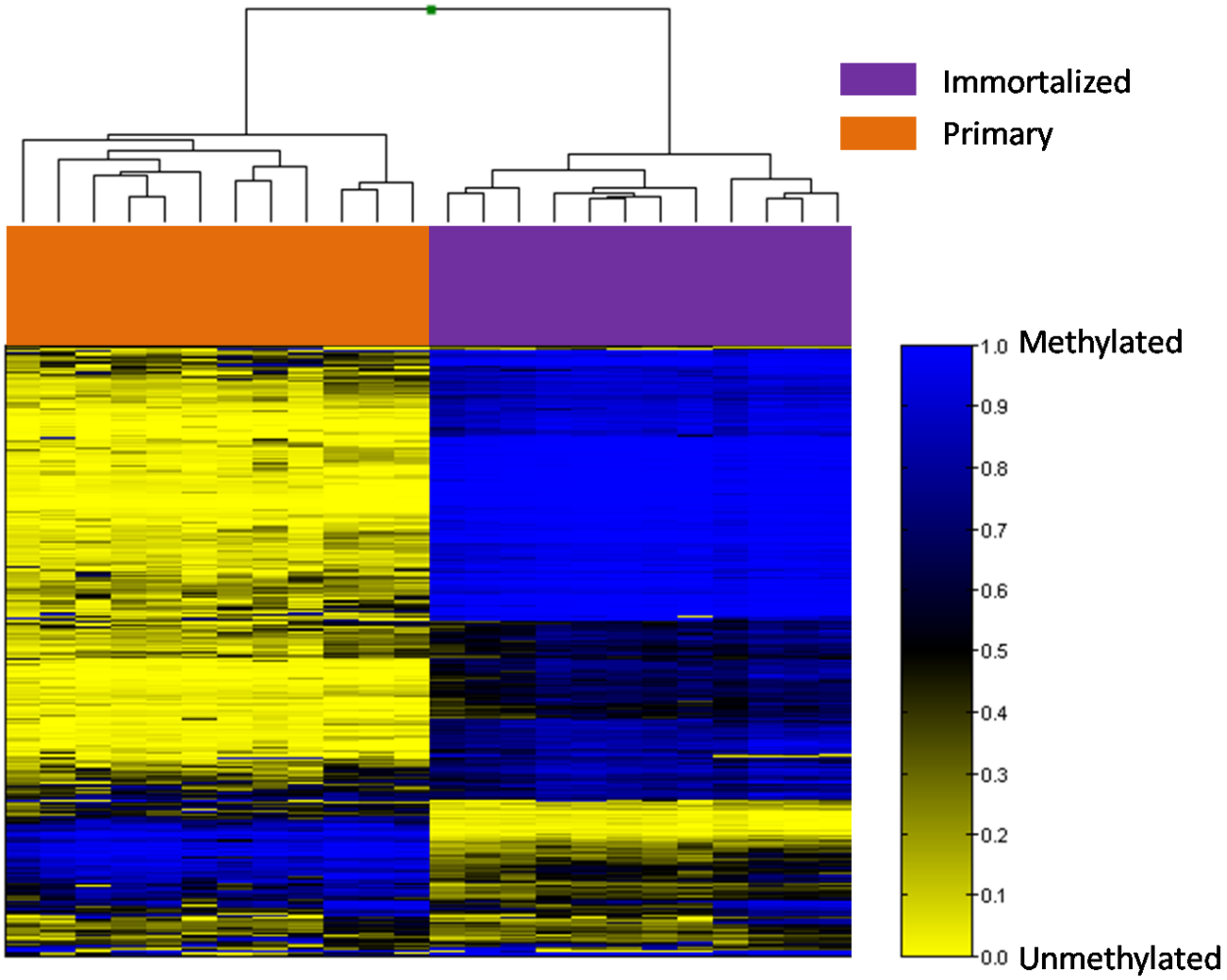

Figure 3.1 Manhattan Clustering of Average Beta Values in Primary and Immortalized Cell Lines. All passage samples were subjected to unsupervised hierarchical sample and CpG methylation clustering based on Manhattan distance. Each column represents a sample and each row represents a CpG locus (300 most variable autosomal loci are shown here). Above the heatmap, purple indicates samples from the immortalized cell cultures and orange indicates samples from the primary cell cultures. In the heat map, blue=methylated and yellow=unmethylated.

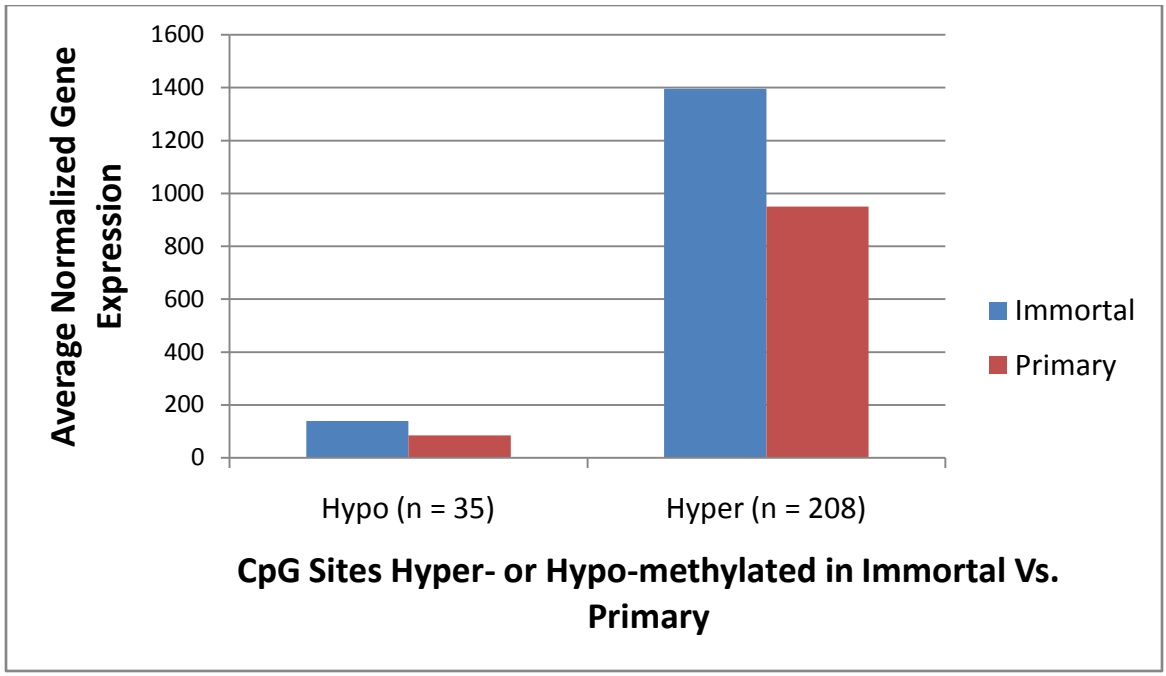

Figure 3.2 Cell Line Comparison of Average Gene Expression for Differentially Methylated CpG Sites. Average gene expression signals for the CpG sites that were hypomethylated and hypermethylated for immortalized bronchial epithelial cells compared to primary. The blue bars represent the immortalized cell samples while the red bars represent the primary cell samples. Average gene expression in immortalized cell samples was overall higher for both hyper and hypo-methylated sites compared to primary cell lines. However, there were no significant differences in gene expression between immortalized or primary bronchial epithelial cell lines or between the two groups of sites within each cell line for these sites due to a large standard deviation in gene expression (P values were >0.05).

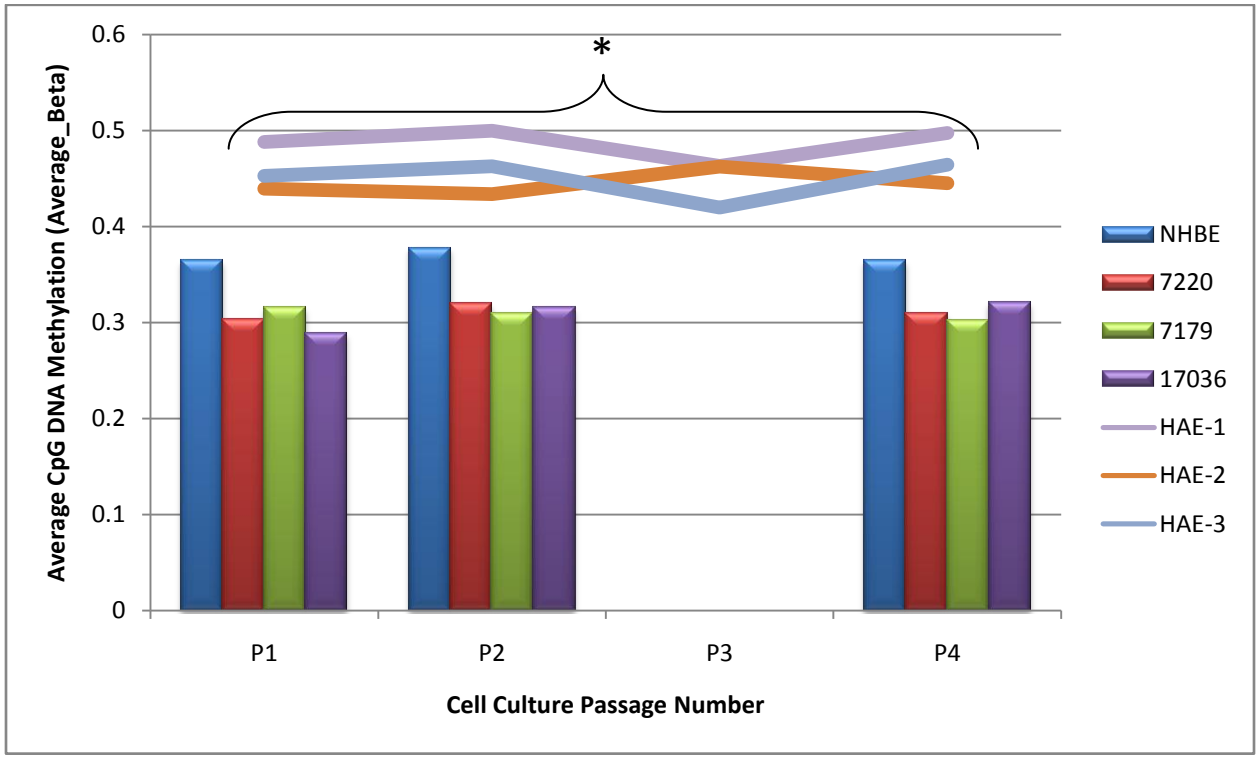

Figure 3.3 Average DNA Methylation Beta Values over Passage in Primary and Immortalized Cell Lines. DNA methylation beta values for all valid CpG probes (n=1372) of the 1505 initially interrogated were averaged for all samples at passage 1,2,3,4 and passage 1,2 and 4 for immortalized and primary cell lines respectively. The primary cell lines NHBE, 7220, 7179 and 17036 are represented in the bottom bar graph portion. The three replicates of the 1HAE Immortalized cell line are represented in the upper line graph. An average beta value of 1=methylated and 0=unmethylated. The 1HAE immortalized cell line is significantly more methylated than primary bronchial epithelial cell lines ($p<0.05$). There are no significant differences over passage for immortalized or primary bronchial epithelial cell lines ($P<0.05$).

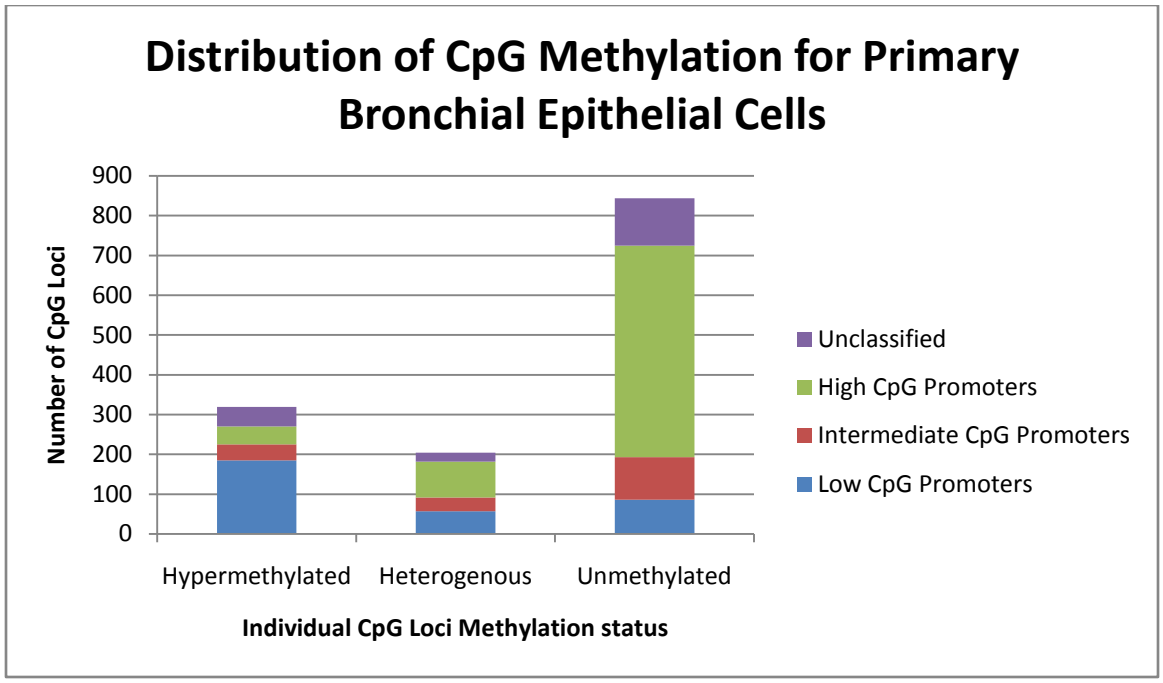

Figure 3.4 Distribution of CpG Methylation for Primary Bronchial Epithelial Cells. CpG loci were distributed into three distinct categories based on their methylation level: Methylated (>80% methylation), heterogenously methylated (Between 20-80% methylation) and unmethylated) (<20% methylation). Methylated CpG loci (n=319) were mainly located in Low CpG density promoters (58.0%) and secondly found in heterogenous CpG density promoters (27.9%). Heterogenously methylated CpG loci (n=204) were mainly in High CpG Promoters (44.1%) and unmethylated CpG loci (n=844) were mainly located in High CpG promoters (63.0%).

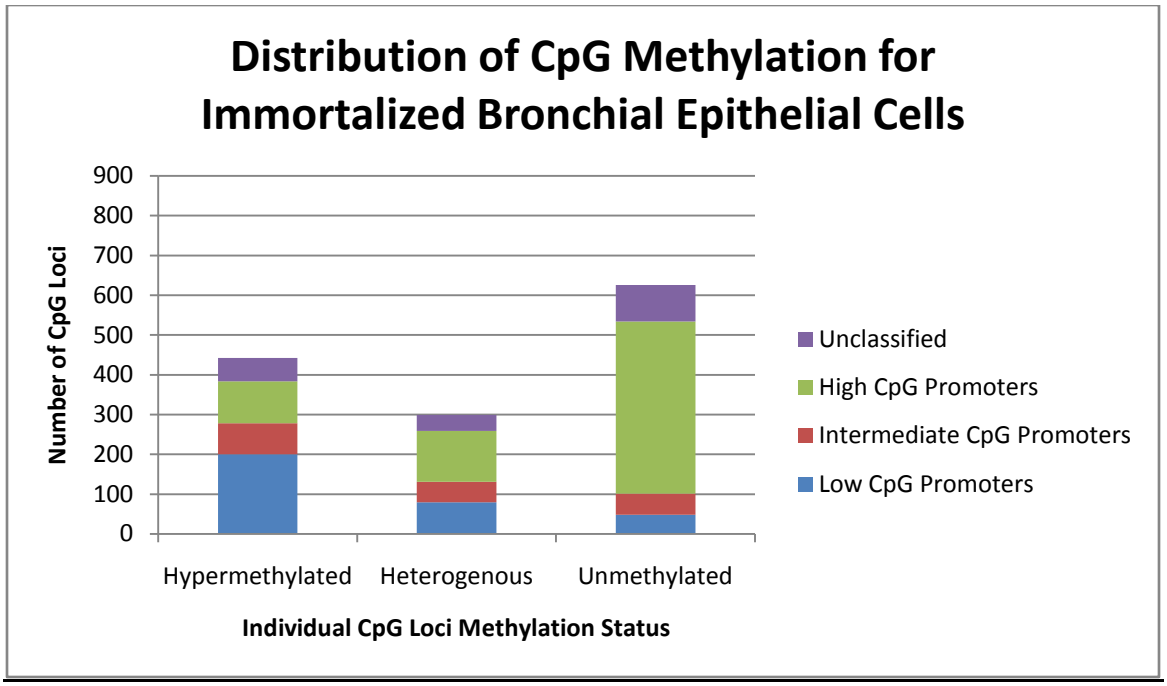

Figure 3.5 Distribution of CpG Methylation for Immortalized Bronchial Epithelial Cells. CpG loci were distributed into three distinct categories based on their methylation level: Methylated (>80% methylation), heterogenously methylated (Between 20-80% methylation) and unmethylated) (<20% methylation). Methylated CpG loci (n=442) were mainly located in Low CpG density promoters (45.2%) and secondly found in High CpG density promoters (23.9%). Heterogenously methylated CpG loci (n=299) were mainly in High CpG Promoters (42.8%) and unmethylated CpG loci (n=626) were mainly located in High CpG promoters(69.2%).

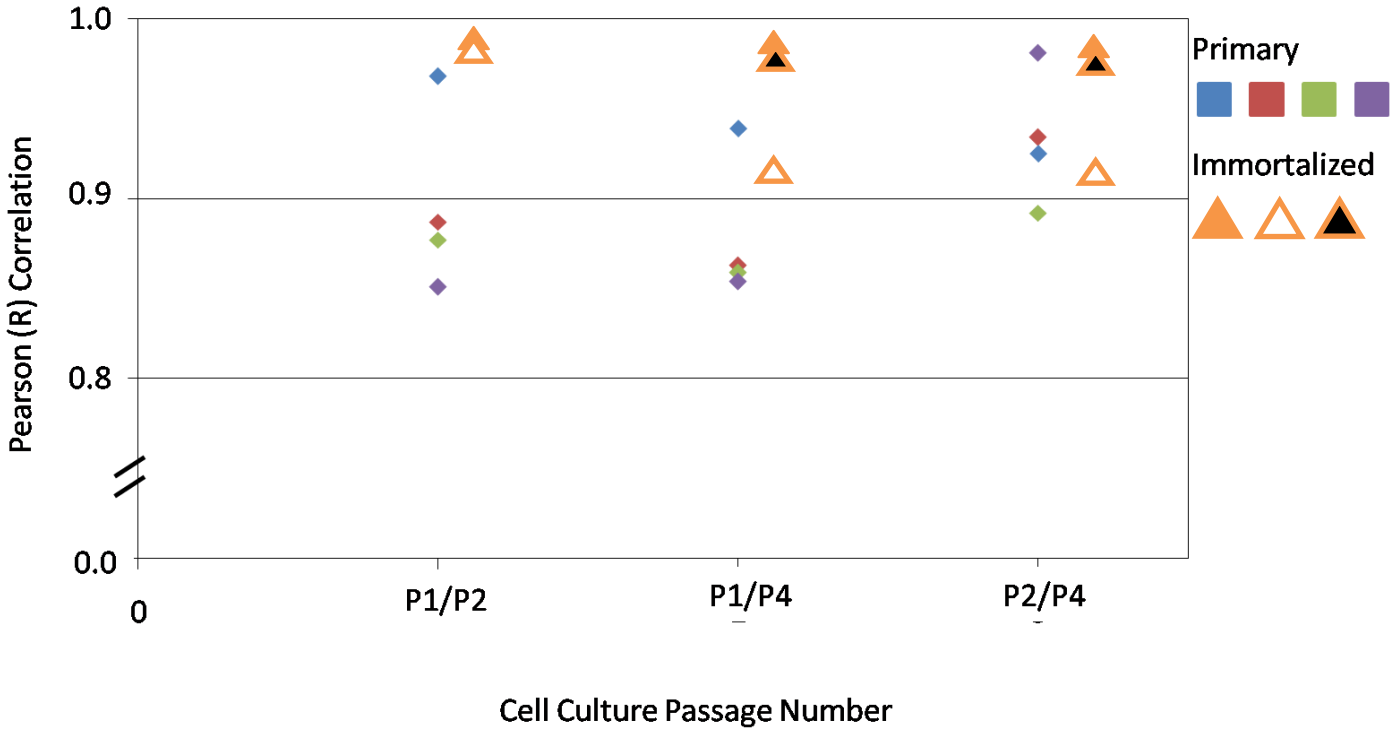

Figure 3.6 Inter-Passage Pearson (R) Correlations in Primary and Immortalized Bronchial Epithelial Cell Lines. The 1372 CpG loci were evaluated for there R Pearson correlations between passage 1,2,3,4 and passage 1,2 and 4 for immortalized and primary cell lines respectively. The four primary cell lines 7220, 7179, 17036 and NHBE are represented by red, green, purple and blue diamonds, respectively. The orange triangles represent the three immortalized cell lines for each inter-passage comparison. The closer the R Pearson value to 1 the higher the correlation between passages of the same individual.

CpG Loci	CpG Promoter Density	Methylation Difference	p-value	Adjusted p-value
IPF1_P750	HCP	0.60	0.05	0.50
KCNK4_P171	HCP	0.37	0.11	1
MSH2_P1008	HCP	0.32	0.12	1
RUNX1T1_P103	Unknown	-0.30	0.08	0.80
NEFL_E23	HCP	-0.30	0.33	1
KRT13_P341	LCP	0.27	0.01	0.09
MMP3_P16	LCP	-0.27	0.15	1
TEK_E75	LCP	0.27	0.12	1
PDGFRA_E125	ICP	0.25	0.08	0.80
IL12B_P392	LCP	0.25	0.42	1

Table 3.1 The 10 CpG Sites Showing the Greatest Change over Passage in Primary Bronchial Epithelial Cells. The Methylation difference (passage 4 minus passage 1, with 0 to 1 representing no methylation to complete CpG methylation at each time point; a positive value indicates a gain of methylation over passage). P-values were corrected by multiplying the unadjusted p-value by 10 to account for multiple testing. Highlighted CpG loci are those that consistently show large changes in DNA methylation over passage for primary and immortalized bronchial epithelial cell lines.

Gene Symbol	CpG Promoter Density	Methylation Difference	p-value	Adjusted p-value
NEFL_E23	HCP	-0.49	0.18	1
ETS1_E253	HCP	-0.38	0.33	1
FMR1_P484	HCP	-0.38	0.17	1
MCF2_P1024	Unknown	0.36	0.37	1
CDKN1A_P242	HCP	-0.36	0.33	1
RUNX1T1_P103	Unknown	-0.34	0.24	1
WNT8B_P216	LCP	0.33	0.43	1
HPN_P374	ICP	0.33	0.43	1
MMP3_P16	LCP	0.32	0.41	1
INS_P804	LCP	0.32	0.44	1

Table 3.2 The 10 CpG Sites Showing the Greatest Change over Passage in Immortalized Bronchial Epithelial Cells. The Methylation difference (passage 4 minus passage 1, with 0 to 1 representing no methylation to complete CpG methylation at each time point; a positive value indicates a gain of methylation over passage). P-values were corrected by multiplying the unadjusted p-value by 10 to account for multiple testing. Highlighted CpG loci are those that consistently show large changes in DNA methylation over passage for primary and immortalized bronchial epithelial cell lines.

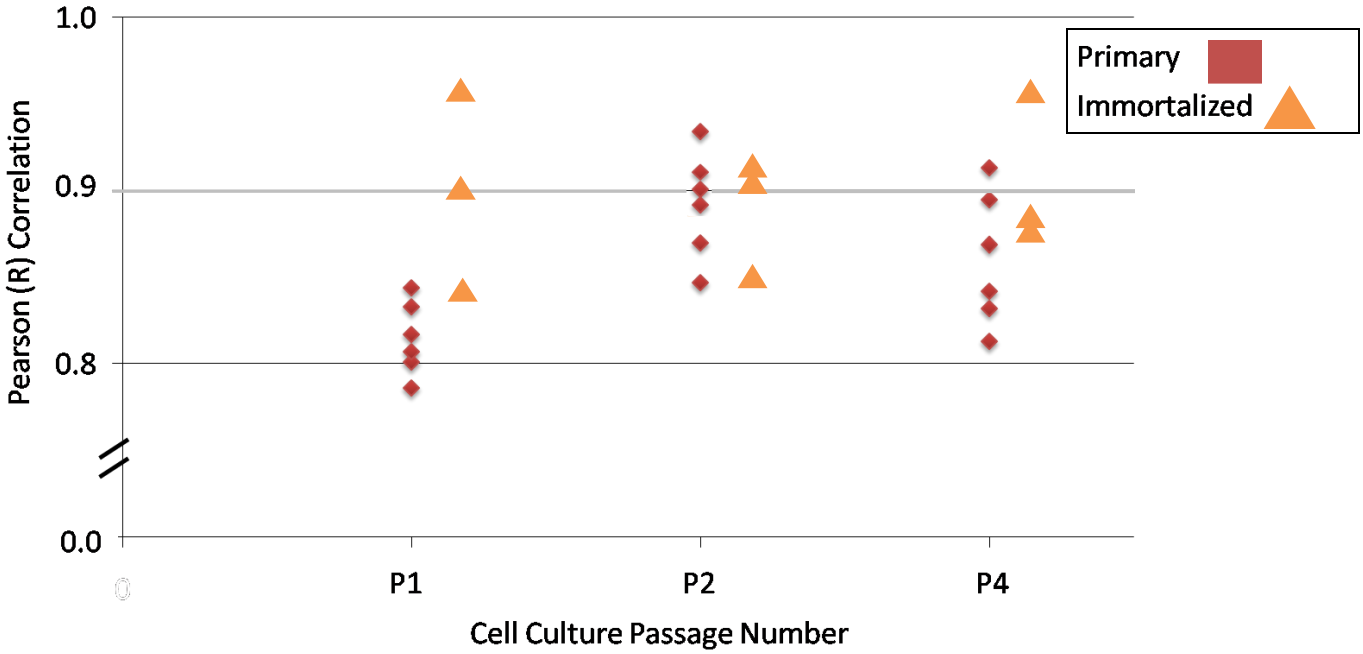

Figure 3.7 Inter-Individual and Inter-Replicate Pearson (R) Correlations in Primary and Immortalized Bronchial Epithelial Cells. The 1372 CpG loci were evaluated for their R Pearson correlations between passage 1,2,3,4 and passage 1,2 and 4 for immortalized and primary cell lines respectively. Correlations between the four primary cell lines 7220, 7179, 17036 and NHBE are represented by red diamonds. The orange triangles represent the R Pearson correlation for the three immortalized cell lines comparisons. The closer the R Pearson co-efficient value to 1 the higher the correlation between individuals at that passage number.

	CpG Methylation Status	Average Gene Expression Signal
Primary Samples (n=12)		
	Hypermethylated (n=319)	680.4
	Heterogeneous (n=204)	897.7
	Unmethylated (n=844)	1111.6
Immortalized Samples (n=12)		
	Hypermethylated (n=442)	1101.0
	Heterogeneous (n=299)	935.4
	Unmethylated (n=626)	1419.0

Table 3.3 Relationship between CpG Methylation Status and Gene Expression. CpG sites that were found to be less methylated showed a trend towards having higher levels of gene expression in the primary bronchial epithelial samples. However, the differences were not large enough to be significantly different ($F=2.35$, $p=0.095$). Similarly, in the immortalized bronchial epithelial cell samples, the highest levels of average gene expression occurred for the CpG sites that were unmethylated but there were no significant differences between groups ($F=2.030$, $p=0.130$).

CpG Methylation Status	CpG Loci	Rs Spearman Co-efficient
Hypermethylated		
	CRIP1_P874*	0.85
	HGF_P1293*	0.69
	CASP8_E474*	0.69
	LMTK2_P1034*	0.68
	PTPRH_E173*	0.67
Heterogeneous		
	IFNGR2_P377*	0.73
	NPR2_P1093*	0.73
	GSTM1_P266*	0.72
	NOS3_P38	0.64
	IL12B_P392	0.64
Unmethylated		
	PTK7_E317*	0.85
	GNMT_E126*	0.85
	TIMP2_E394*	0.81
	TJP1_P326*	0.80
	DUSP4_E61*	0.78

Table 3.4 Top 15 Positive DNA Methylation and Gene Expression Correlations for Primary Cells. The top 15 positive gene expression and CpG site correlations as determined by Rs Spearman co-efficient of correlation calculation and sorted by methylation status of the CpG sites in primary bronchial epithelial cells. All Spearman Rs greater than or equal to 0.65 are significant with the likelihood of the correlation occurring by chance set at 5% for 10 degrees of freedom (indicated by *).

CpG Methylation Status	CpG Loci	Rs Spearman
Hypermethylated		
	CD1A_P6*	-0.71
	PIK3R1_P307*	-0.69
	XRCC1_P681*	-0.69
	TNFSF8_E258	-0.64
	EPHA8_P456	-0.63
Heterogeneous		
	IL2_P607*	-0.84
	GRB10_P496*	-0.76
	CCKAR_E79*	-0.75
	KCNK4_P171*	-0.70
	HOXA9_P303*	-0.68
Unmethylated		
	IRAK3_P13*	-0.79
	FZD9_P175*	-0.76
	FRZB_E186*	-0.71
	TNFSF10_E53*	-0.69
	FGFR1_P204*	-0.69

Table 3.5 Top 15 Negative DNA Methylation and Gene Expression Correlations for Primary Cells. The top 15 negative gene expression and CpG DNA Methylation correlations as determined by Rs Spearman co-efficient of correlation and sorted by methylation status of the CpG sites in primary bronchial epithelial cells. All Spearman Rs greater than or equal to 0.65 are significant with the likelihood of the correlation occurring by chance set at 5% for 10 degrees of freedom (indicated by *).

CpG Methylation Status	CpG Loci	Rs Spearman
Hypermethylated		
	PODXL_P1341*	0.67
	WNT10B_P823	0.64
	GABRB3_P92	0.64
	GFAP_P1214	0.62
	IGF2AS_P203	0.61
Heterogeneous		
	CDH11_P354*	0.82
	MAPK4_E273*	0.71
	MMP7_E59*	0.67
	TEK_P479	0.61
	ITPR2_P804	0.57
Unmethylated		
	TJP2_P330*	0.79
	PPP2R1B_P268*	0.73
	IL8_E118*	0.72
	MDS1_E45*	0.71
	FN1_E469*	0.69

Table 3.6 Top 15 Positive DNA Methylation and Gene Expression Correlations for Immortal Cells. The top 15 positive gene expression and CpG site correlations as determined by Rs Spearman co-efficient of correlation and sorted by methylation status of the CpG sites in immortalized bronchial epithelial cells. All Spearman Rs greater than or equal to 0.65 are significant with the likelihood of the correlation occurring by chance set at 5% for 10 degrees of freedom (indicated by *).

CpG Methylation Status	CpG Loci	Rs Spearman
Hypermethylated		
	CYP2E1_E53*	-0.85
	DDR2_P743*	-0.76
	SFTPD_E169*	-0.68
	NPR2_P1093*	-0.66
	ALOX12_E85	-0.64
Heterogeneous		
	GSTM2_P109*	-0.87
	DCC_E53*	-0.66
	DCC_P177	-0.62
	GSTM2_P453	-0.61
	HGF_P1293	-0.60
Unmethylated		
	CAV1_P130*	-0.76
	WNT2B_P1195*	-0.69
	MEG3_E91*	-0.65
	EGR4_E70	-0.62
	ITGB4_E144	-0.62

Table 3.7 Top 15 Negative DNA Methylation and Gene Expression Correlations for Immortal Cells. The top 15 negative gene expression and CpG site correlations as determined by Rs Spearman co-efficient of correlation and sorted by methylation status of the CpG sites in immortalized bronchial epithelial cells. All Spearman Rs greater than or equal to 0.65 are significant with the likelihood of the correlation occurring by chance set at 5% for 10 degrees of freedom (indicated by *).

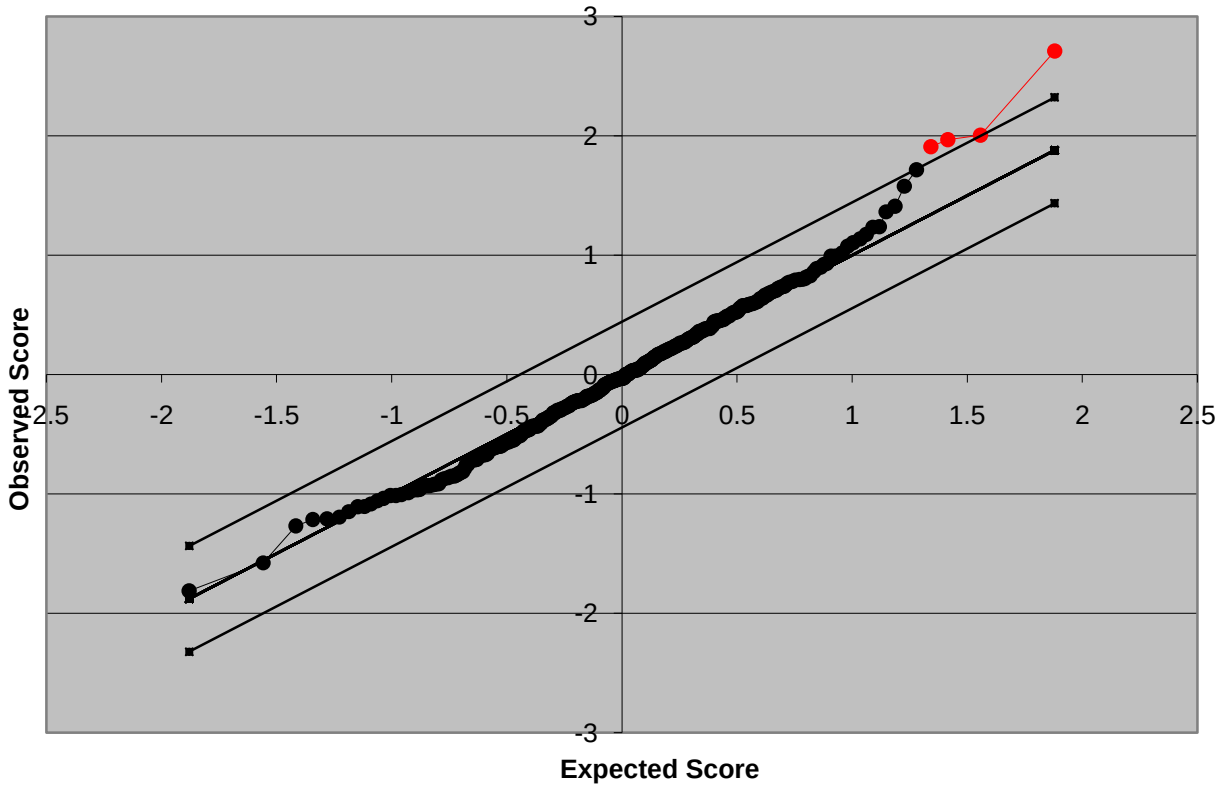

Figure 3.8 SAM Plot of a One Class Time-Course Slope Analysis. Significance analysis of microarrays software selects significant genes by performing multiple permutations of the data. In this case, for a one-class time course slope analysis, the CpG loci that deviate from the normal observed/expected score (in red) are those CpG loci that change over passage in a significantly linear manner that is more that would be expected to occur randomly.

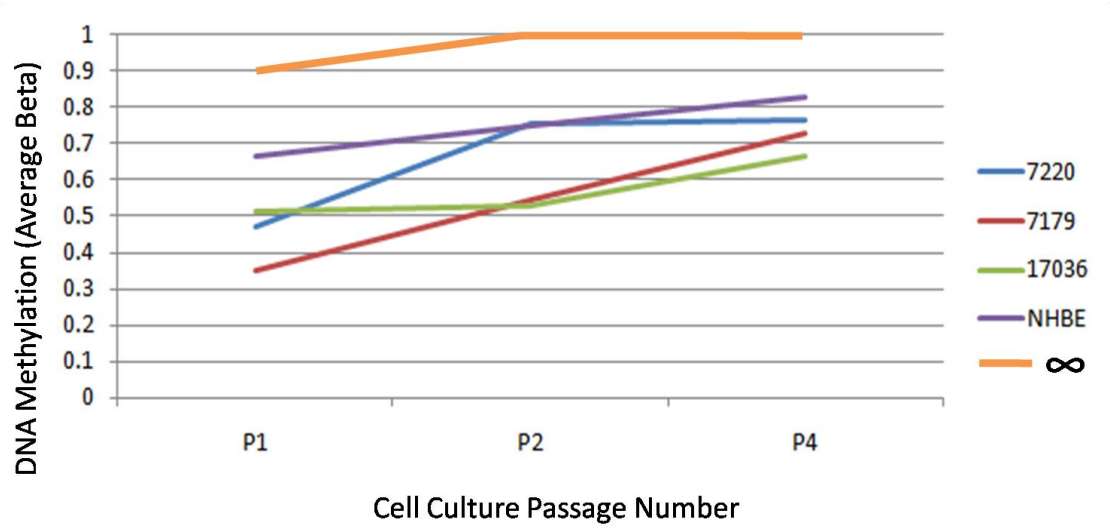

Figure 3.9 Keratin-13 (KRT13) DNA Methylation Changes over Passage. A comprehensive analysis by SAM software revealed that KRT13 DNA methylation significantly changes over passage in a linear manner (q-value=0%). The four primary cell lines 7220, 7179, 17036 and NHBE are represented by blue, red, green and purple lines, respectively. The orange line represents the average KRT13 DNA methylation for all three immortalized cell lines (∞) at each passage number. An average beta value of 1=methylated and 0=unmethylated. Primary cell KRT13 DNA methylation increases over passage approaching levels observed in the immortalized cell lines.

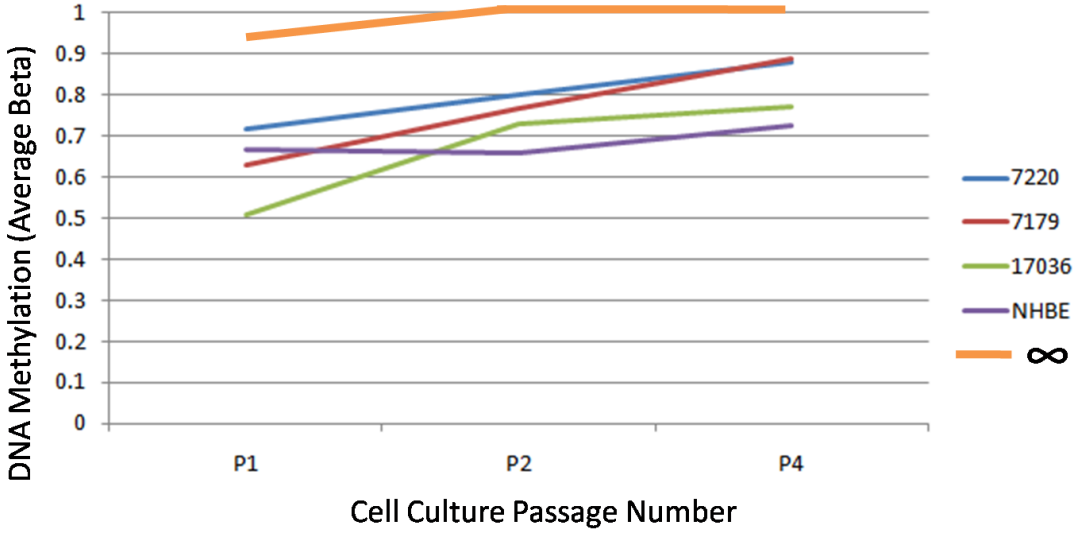

Figure 3.10 Claudin-4 (CLDN4) DNA Methylation Changes over Passage. A comprehensive analysis by SAM software revealed that CLDN4 DNA methylation significantly changes over passage in a linear manner (q-value=0%). The four primary cell lines 7220, 7179, 17036 and NHBE are represented by blue, red, green and purple lines, respectively. The orange line represents the average CLDN4 DNA methylation for all three immortalized cell lines (∞) at each passage number. An average beta value of 1=methylated and 0=unmethylated. Primary cell CLDN4 DNA methylation increases over passage approaching levels observed in the immortalized cell lines.

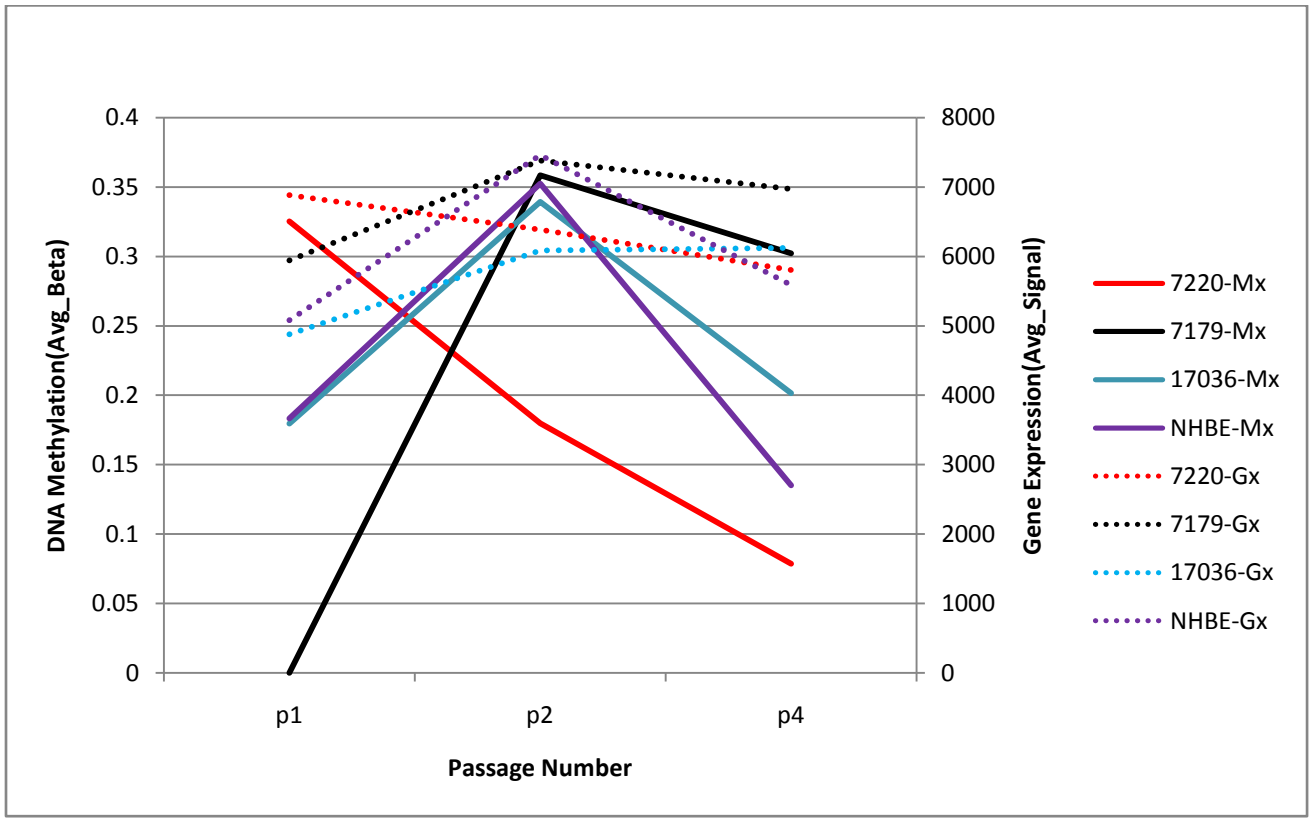

Figure 3.11 Interferon Gamma Receptor 2 (IFNGR2) DNA Methylation and Gene Expression. A comprehensive analysis by SAM software revealed that IFNGR2 DNA methylation significantly changes over passage in a dynamic manner (q-value=0%). The four primary cell lines 7220, 7179, 17036 and NHBE are represented by red, black, blue and purple lines, respectively. On the left y-axis is DNA methylation represented by the solid coloured lines. Corresponding gene expression levels are represented on the right y-axis by broken lines (---). An average beta value of 1=methylated and 0=unmethylated. IFNGR2 had a significant positive Rs Spearman correlation co-efficient of 0.73426 when comparing DNA methylation and gene expression levels at each passage.

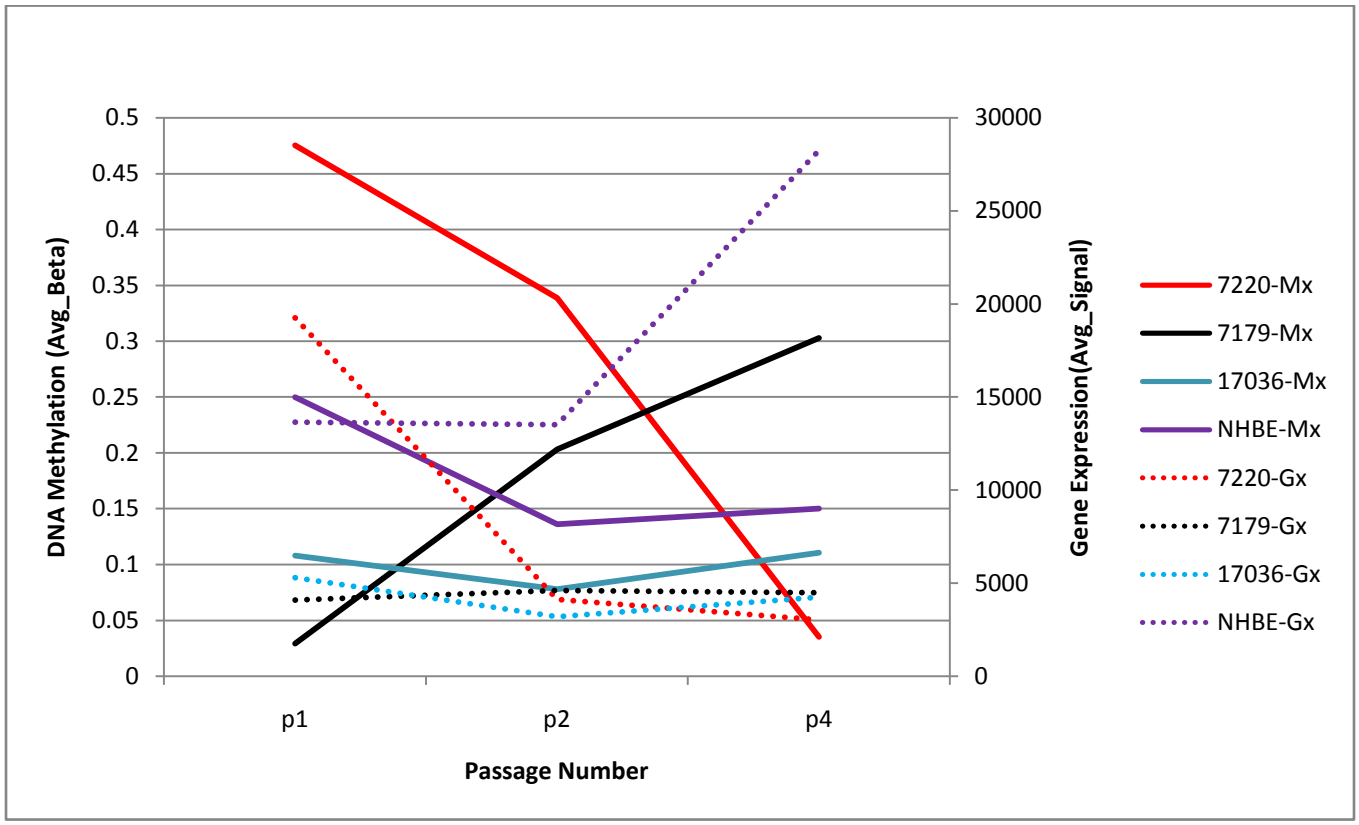

Figure 3.12 Transforming Growth Factor, Beta-Induced (TGFBI) DNA Methylation and Gene Expression. A comprehensive analysis by SAM software revealed that TGFBI DNA methylation significantly changes over passage in a dynamic manner (q-value=0%). The four primary cell lines 7220, 7179, 17036 and NHBE are represented by red, black, blue and purple lines, respectively. On the left y-axis is DNA methylation represented by the solid coloured lines. Corresponding gene expression levels are represented on the right y-axis by broken lines (---). An average beta value of 1=methylated and 0=unmethylated. TGFBI had an Rs Spearman correlation co-efficient of 0.566434 when comparing DNA methylation and gene expression levels at each passage.

Gene Symbol	Gene	Fold Change P1 to P2 (Array/qPCR)	Fold Change P2 to P4 (Array/qPCR)	p-value	Location
TGFBI	Transforming Growth Factor-Beta Induced	-1.36 / -1.05	1.28 / 1.46	0.418	5q31.1
INFGR2	Interferon-gamma Receptor 2	1.22 / 1.21	-1.11 / -1.03	0.746	21q22.11
CLDN4	Claudin-4	-1.26 / -1.13	-1.12 / -1.91	0.304	7q11.23

Table 3.8 Primary Bronchial Epithelial Cell Gene Expression Validation by Quantitative RT-PCR. For all genes, the fold change was calculated by dividing the expression value at one passage by the earlier passage expression value. If this number was less than one, the negative reciprocal is listed. P-values greater than 0.05 indicate there are no significant differences between qPCR and microarray fold-changes between passages.

Gene Symbol	Gene	Fold Change P1 to P2 (Array/qPCR)	Fold Change P2 to P3 (Array/qPCR)	Fold Change P3 to P4 (Array/qPCR)	p-value	Location
TGFBI	Transforming Growth Factor-Beta Induced	1.99 / 2.00	1.11 / -1.26	-1.01 / -1.26	0.235	5q31.1
INFGR2	Interferon-gamma Receptor 2	1. 22 / 1.00	-1.20 / -1.16	1.14 / 1.13	0.303	21q22.11
CLDN4	Claudin-4	1.29 / 1.65	1.41 / 1.90	1.02 / 2.27	0.134	7q11.23

Table 3.9 Immortalized Bronchial Epithelial Cell Gene Expression Validation by Quantitative RT-PCR. For all genes, the fold change was calculated by dividing the expression value at one passage by the earlier passage expression value. If this number was less than one, the negative reciprocal is listed. P-values greater than 0.05 indicate there are no significant differences between qPCR and microarray fold-changes between passages.

4 DISCUSSION

4.1 Discussion of Results

4.1.1 DNA Methylation

In this thesis, several aims were addressed. First, I sought to characterize specific changes in DNA methylation at 1505 CpG loci in both primary and immortalized bronchial epithelial cell lines. In agreement with previous studies, the vast majority of methylation occured in LCPs or ICPs whereas HCPs are predominantly unmethylated. This is true even for immortalized bronchial epithelial cells that had a significantly higher proportion of hypermethylated CpG loci than primary cells across all passages. Interestingly, 38.3% of the CpG sites interrogated were methylated to some extent (greater than 20%) in the primary bronchial epithelial cell lines. The prevalence of methylation obvserved in this study is 6 times higher than other studies which report less than 5% of primary cell promoters as having any DNA methylation (67, 69). However, the large percentage of methylation observed in primary bronchial epithelial cells could be biased. This study focused upon specific CpG sites in 807 genes that were pre-selected for there epigenetic relevance to cancer whereas other studies have studied methylation across chromosomes and the genome in a less biased fashion.

Also, these results suggest that immortalized bronchial epithelial cell lines have unique DNA methylation profiles compared to primary bronchial epithelial cell lines as shown by their separation by unsupervised hierarchical clustering. The unique DNA methylation profile of immortalized cell lines that is easily identified by the higher prevalence of hypermethylated sites may be the cause or result of their continued survival during cell propagation. One

function of enhanced hypermethylation in immortalized bronchial epithelial cell lines could be to silence genes that are non-essential in the artificial culture environment and thus promote their survival. Also, immortalized bronchial epithelial cells had higher inter-passage and inter-sample correlations during propagation compared to primary cell lines. Interestingly, primary cultures showed a trend towards higher correlation between individual cell lines later in passage, indicating that there may be a loss of individual methylation profiles and a movement towards a convergent methylome in these cells in order to survive within the same *in vitro* cell culture environment.

4.1.2 DNA Methylation and Gene Expression

Second, the link between gene expression and DNA methylation in both a global and gene-specific context was explored. For the 1372 CpG sites interrogated, there was a trend towards a higher average normalized gene expression signal for unmethylated CpG sites. In addition, hypermethylated CpG sites in primary bronchial epithelial cells were associated with lower levels of gene expression. These trends across 807 genes are surprising considering the overall low reported rate of inverse correlation between gene expression and DNA methylation in the literature (67, 73, 210). Although the above findings were not statistically significant, it is interesting that a trend emerged considering the sample size limitations as well as the numerous factors and mechanims present in the cell culture environment that could affect gene regulation.

When studying specific CpG site and gene expression relationships, there were significant positive and negative correlations between gene expression and DNA methylation. Although

most research has focused upon DNA methylation as a gene silencer, the strong positive correlations suggest that there is more to DNA methylation than gene inhibition.

4.1.3 Candidate Genes

Third, an in-depth exploration of four candidate genes was undertaken. From these analyses, we observed that major gene-specific changes in DNA methylation occur only in the primary bronchial cell lines over passage. Few CpGs demonstrated consistent linear changes in DNA methylation over passage but those that did became more like immortalized cells by becoming hypermethylated. These findings are in disagreement with a recent study that showed similar DNA methylation profiles in cell culture when comparing the average DNA methylation of many CpG for selected genomic regions (67). In our study, since DNA methylation was studied at nucleotide resolution, we were able to observe DNA methylation changes that occur at individual CpG loci over passage. It is possible that because Eckhardt et al. (67) studied DNA methylation across the length of an amplicon that the small changes occuring at individual CpG sites were averaged out by the other CpGs of the amplicon. However, based on studies of IFN-y expression levels in Th cells, in some cases, methylation of one CpG site can result in a change in gene expression (138). An increase in DNA methylation in specific gene regions has been previously observed in other human cell types including fibroblasts, neural precursor cells and embryonic stem cells (73, 183, 187). Although, the signficant CpG sites that change linearly over passage all increased in methylation, it is important to recognize that this was not always related to gene expression. In fact, although KRT13 dramatically increased in methlation over passage in both primary and immortalized bronchial epithelial cell lines, its gene expression was

consistently very low as judged by our inability to detect KRT13 mRNA. Therefore, one must be careful when discussing any potential correlations or relationships between DNA methylation and gene expression.

4.2 Further Research

4.2.1 Studying the Asthmatic Airway Epithelium

This thesis provides the necessary background information to carry forward the larger intended project of studying differences in DNA methylation of bronchial epithelial cells obtained from asthmatic and non-asthmatic individuals. Although a large number of CpG loci in our study did not change over passage, there were enough sites and certainly large enough changes at individual CpG sites such as CLDN4 and INFGR2 in primary bronchial epithelial cells to warrant a warning regarding passaging bronchial epithelial cell lines since the largest changes in DNA methylation occurred after the first passage.

4.2.2 Building Connections

The main purpose of this thesis was to explore how one epigenetic mechanism, DNA methylation, and its potentially related gene expression changed over passage in cell culture. However, it is important to recognize that DNA methylation is only one epigenetic mechanism that plays a role in regulating gene expression. In addition, although a number of analyses in this thesis focused on how DNA methylation was related to its CpG context, there is abundant research in other cell types indicating that DNA methylation is strongly correlated with posttranslational modifications on associated histone proteins (73). These studies have yet to

be carried out in the bronchial epithelium but it would be very interesting if a future study were to compare DNA methylation levels with histone modifications in the bronchial epithelium. It was not possible to conduct these additional tests at the present time since bronchial epithelial cells are difficult to obtain and quantitative analyses of histone methylation and other posttranslational modifications require copious amounts of sample compared to DNA methylation studies. Also, in this thesis, DNA methylation was only compared with gene expression data at the level of mRNA analysis. Although much can be learned from this comparison, it would be important to relate changes in DNA methylation to changes at the protein level in future studies in order to learn more about how an altered DNA methylome is influencing the basic functioning of bronchial epithelial cells.

To study these intricate connections in the bronchial epithelium will require the development of new technology that can analyze more while requiring less starting material. The rapid development of cheaper technology that is capable of interrogating and integrating information for multiple facets of a cell's characteristics is well on its way and paving the way for new strategies that will surely produce profound insights about the links between DNA methylation, histone modifications, gene expression and protein levels.

4.2.3 Cell Culture Studies

There are a number of additional studies that could be done in cell culture to fully understand how cell culture influences the DNA methylation marks of primary and immortalized cell lines.

1) In the present study, DNA methylation information was collected starting at passage 1 when cells had already been exposed to cell culture conditions for several days during initial processing. It would be interesting to compare cells that have been collected directly from donor lungs with those that have been cultured to compare whether even at passage 1, culture has already radically altered the DNA methylation and gene expression profile of the bronchial epithelial cells.

2) It is common for researchers to immortalize some of their primary cell cultures so that they can create viable and easily sustainable in vitro model systems. Little is known about how the methylation profiles of primary cells compares to their immortalized state for specific individual cell lines. One study in Epstein Barr virus transformed lymphoblastoid cell lines found that DNA methylation of the cell lines was not always representative of DNA methylation present in the original B-lymphocytes of the patient (211). In this study, we compared primary bronchial epithelial cell lines from four individuals with an SV40 immortalized cell line that was derived from a different individual and found significant differences in their profiles. Therefore, another future cell culture study could focus upon measuring changes in DNA methylation before, during and after immortalization of primary bronchial epithelial cell lines using various techniques to see which one best maintains the individual's methylation profile.

3) Another suggestion would be to carry out similar cell culture studies in other tissues. The majority of research does not focus upon primary cell culture even though these cells are regularly used and phenotyped. This thesis focused solely upon studying the DNA methylation profiles of bronchial epithelial cells lines over passage. It would be important to see if these

findings can be extrapolated to other similar cell types such as fibroblasts or are they specific to bronchial epithelial cell lines. It would be preferably to compare different tissue samples from the same individual.

4) In this thesis, primary bronchial epithelial cells were grown under standard cell culture conditions on a tissue flask. However, a number of researchers grow bronchial epithelial cells on an air-liquid interface that allows the differentiation and stratification of cell types to emerge in vitro similar to the in vivo airway epithelium. One of the limitations of growing primary bronchial epithelial cells obtained from donor lungs in monolayer culture is that it is mainly the basal epithelial cells grow while the ciliated cells do not attach efficiently or proliferate. Therefore, the methylation status represents a heterogenous mixture of mainly basal cells and some other airway epithelial cell types without much being learned about the differentiated cilated cells that play an important role in trapping and removing particles from the lungs. Laser capture mircroscopy of air-liquid interface cultures to separate out the different types of differentiated cells from basal to ciliated would allow the comparison of their DNA methylation and gene expression profiles.

5) Another study that could be conducted would be to compare brochial epithelial cells obtained from bronchial epithelial cell brushings, in which the cells are surgically removed from a living person's airway epithelium, and cells obtained from donor lungs. These are both common ways of obtaining and growing bronchial epithelial cell lines but there is little current research exploring the common and distinct characteristics of each method – especially with regards to the impact upon epigenetic marks.

6) Lastly, since we observed changes in DNA methylation and gene expression over passage it would be interesting to explore possible ways to stabilize the cell culture system and limit such alterations. In the cell culture of human embryonic cells, the stabilization of epigenetic marks is often discussed since dysfunctional epigenetic marks could influence the development of a child who has been conceived via in vitro feritlization or other reproductive technological methods.

4.3 Limitations

4.3.1 Sample Size

One of the major limitations of this study is the sample size. Due to the large number of data points collected from the DNA methylation and gene expression arrays and the small sample size it was difficult to produce statistically significant results. However, this thesis serves as a small pilot project from which the results can be extrapolated to achieve a given False Discovery Rate (FDR) and Expected Discovery Rate (EDR) for future experiments in the airway epithelium. The FDR is an estimate of the expected proportion of genes declared significant that is in fact not differentially expressed, e.g. “false discoveries” (207). The EDR is the expected proportion of genes that is truly different between two conditions that are found to be significantly different.

The number of samples that would be required for a full-scale study is calculated by estimating the variability in the dependent variable, in this case DNA Methylation and/or gene expression levels. Traditional experimental designs for statistical analysis were based on controlling type I

errors for detection of a specifically sized difference at alpha = 0.05 and 80% power. However, using these parameters was not possible in this study because the variance was not known beforehand and a functionally relevant change in DNA methylation is not known. After determining the standard deviation of the dependent variables, it was possible to determine the sample sized required when using a Bonferroni correction for multiple testing method. In this case, the sample size required to determine significant differentialy methylated/expressed genes from genome-wide arrays would be more than 250 per independent group since Bonferroni corrections are extremely conservative. For example, in Table 3.1, standard Student's t-tests of changes in DNA methylation over passage for KRT13 and other CpG sites was not significant when p-values were adjusted conservatively for multiple testing. However, a different statistical approach using significance analysis of microarray (SAM) software that controls the false discovery rate (212) and the expected discovery rate with a permutation analysis showed that KRT13 was a site significantly changing over passage in primary bronchial epithelial cell lines.

A post-hoc power analysis of changes in DNA methylation over passage indicates that for this sample size, standard Student t-tests were only powered between 20 and 60%. Based on a review of the literature and the post-hoc analysis, an appropriate sample size for a full-scale study would be between 15 and 40 individual cell lines since the primary bronchial epithelial cell lines are relatively non-heterogeneous, and of similar age (213).

4.3.2 DNA Methylation Platform

Our understanding of DNA methylation and how it changes during cell differentiation *in vivo* and *in vitro* in human tissues remains limited. Here we reported the generation of DNA methylation profiles at nucleotide resolution for immortalized and primary bronchial epithelial cells which serves as a first step towards improving our knowledge regarding the epigenetics of the epithelium. It is important to recognize that at the time this study was conducted the best technology available was the Illumina Methylation GoldenGate Platform that interrogates 1505 CpG sites with the majority of these located in CpG Islands. Current technology now allows the interrogation of 20 times more CpG sites at nucleotide resolution although this still proportionally represents a small proportion of the entire methylome (214). Another limitation of the Illumina GoldenGate platform is the type of CpG loci interrogated. There has been convincing evidence in the literature recently that supports an important role for gene-body methylation in regulation gene transcription (74, 79, 98). This aspect of DNA methylation could not be analyzed in this study because the Illumina GoldenGate platform utilized here is limited to CpG sites located in gene promoter regions.

4.4 Concluding Remarks

In summary, we have provided the first systematic profile of CpG DNA methylation and related gene expression in primary and immortalized bronchial epithelial cell lines during standard culture conditions that are frequently used by researchers throughout the world. We found extensive differences between the DNA methylomes of primary and immortalized bronchial epithelial cell lines, revealing the importance of this epigenetic modification in cell culture.

Essential future studies will need to explore the specific functional implications of these distinct methylomes in a larger sample size in order to fully understand the role DNA methylation plays in the adaptation of bronchial epithelial cell lines to culture conditions.

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APPENDIX A

PROVIDENCE HEALTH CARE
Research Institute

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Providence Health Care
Institutional Certificate of Final Approval

Principal Investigator:	Department:	Reference Number:
P. Pare	Medicine – iCapture	PHC REB H07-01116
Sponsoring Agencies: Canadian Institutes of Health Research (CIHR)		
Project Title: Genetic and Epigenetic Determinants of Asthmatic Epithelial Cell Phenotype		
Date Ethical Approval: July 3, 2007		

The UBC-PHC Research Ethics Board granted ethical approval for the above-referenced research project on the date stated above. I am now pleased to inform you that all necessary hospital department/facilities approvals and institutional agreements/contracts are now in place and that you have permission to begin your research.

Dr. Yvonne Lefebvre
Vice President Research and Academic Affairs, Providence Health Care
President, Providence Health Care Research Institute

Aug 1 2007
Date

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ETHICS CERTIFICATE OF EXPEDITED APPROVAL: ANNUAL RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC-PHC REB NUMBER:
Peter D. Paré	CAPTURE	H07-01116

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Providence Health Care	St. Paul's Hospital

Other locations where the research will be conducted:
Australia - SirCharles Gairdner Hospitaland Princess Margaret Hospital

CO-INVESTIGATOR(S):

Andrew J. Sandford
Michael S. Kobor
Darryl Knight

SPONSORING AGENCIES:

Canadian Institutes of Health Research (CIHR)

PROJECT TITLE:
Genetic and Epigenetic Determinants of Asthmatic Epithelial Cell Phenotype

EXPIRY DATE OF THIS APPROVAL: April 9, 2010

APPROVAL DATE: April 9, 2009

CERTIFICATION:

1. The membership of the UBC-PHC REB complies with the membership requirements for research ethics boards defined in Part C Division 5 of the Food and Drug Regulations of Canada.
2. The UBC-PHC REB carries out its functions in a manner fully consistent with Good Clinical Practices.
3. The UBC-PHC REB has reviewed and approved the research project named on this Certificate of Approval including any associated consent form and taken the action noted above. This research project is to be conducted by the principal investigator named above at the specified research site(s). This review of the UBC-PHC REB have been documented in writing.

The UBC-PHC Research Ethics Board Chair or Associate Chair, has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal.

Approval of the UBC-PHC Research Ethics Board or Associate Chair, verified by the signature of one of the following:

Dr. Kuo-Hsing Kuo, Chair	Dr. J. Kernahan, Associate Chair	Dr. I. Fedoroff, Associate Chair