INTEGRIN α6β4 PROMOTES PANCREATIC CANCER INVASION BY ALTERING DNA REPAIR-MEDIATED EPIGENETICS

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INTEGRIN α6β4 PROMOTES PANCREATIC CANCER INVASION BY ALTERING DNA REPAIR-MEDIATED EPIGENETICS

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By
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Lexington, Kentucky

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2016

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INTEGRIN α6β4 PROMOTES PANCREATIC CANCER INVASION BY ALTERING DNA REPAIR-MEDIATED EPIGENETICS

Integrin α6β4 is upregulated in pancreatic carcinoma, where signaling promotes metastatic properties, in part by altering the transcriptome. Such alterations can be accomplished through DNA demethylation of specific promoters, as seen with the pro-metastatic gene S100A4. I found that signaling from integrin α6β4 dramatically upregulates expression of amphiregulin (AREG) and epiregulin (EREG), ligands for the epidermal growth factor receptor (EGFR), and that these ligands promote pancreatic carcinoma invasion. To determine if AREG and EREG are regulated by DNA methylation, pancreatic cancer cells with low AREG and EREG expression were treated with the DNA methyltransferase inhibitor 5-aza-2’-deoxycytidine (5-Aza-CdR), resulting in stable overexpression of AREG and EREG, and this induction required signaling from integrin α6β4. Similarly, treatment of cells with high integrin α6β4 with the methyl donor S-adenosylmethionine inhibited gene expression of AREG and EREG. Whole genome bisulfite sequencing on pancreatic cancer cells revealed hypomethylation of the promoter regions of AREG and EREG when integrin α6β4 is high, and these regions correspond to H3K27Ac, indicative of enhancer location. Interestingly, I also observed genome-wide DNA demethylation, and a large proportion of altered CpGs correspond to potential enhancers. It is currently accepted that active DNA demethylation occurs via DNA repair. I tested this hypothesis by treating cells with Gemcitabine, which inhibits multiple components of DNA repair, including DNA demethylation mediated by GADD45A. Gemcitabine treatment resulted in marked reduction in AREG and EREG expression. To further test the involvement of GADD45A, I used RNAi-mediated knockdown or cDNA overexpression to alter GADD45A levels. In both instances, AREG and EREG expression positively correlated with GADD45A, particularly when integrin α6β4 is high, indicating that GADD45A is a rate-limiting step in AREG and EREG overexpression. Similarly, using stable shRNA, I show that Thymine DNA Glycosylase (TDG), and TET1 known modulators of DNA demethylation, are required for AREG and EREG expression in integrin α6β4 high cells, and nuclear localization of TDG is much higher in cells with high integrin α6β4. Using a specific inhibitor I found that AREG and EREG expression is dependent on Parp-1. Finally, I determined that integrin α6β4 signaling enhances cells ability to respond to and survive in the presence of DNA damage, and that
active DNA repair is required for integrin α6β4 mediated DNA demethylation. Taken together, these data indicate that DNA repair is required to maintain overexpression of AREG and EREG in response to signaling from integrin α6β4 and that integrin α6β4 promotes this overexpression by enhancing DNA repair.

KEYWORDS: Integrin α6β4, DNA Methylation, Base Excision Repair, Pancreatic Cancer, EGFR signaling

Brittany L. Carpenter

May 27, 2016
INTEGRIN α6β4 PROMOTES PANCREATIC CANCER INVASION BY ALTERING DNA REPAIR-MEDIATED EPIGENETICS

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May 27, 2016
For my grandmothers, Anna Lee and Sandy.
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CHAPTER 1: INTRODUCTION

1.1 Integrin Signaling and Function in Normal Tissues

Integrins were classified as a family in 1987 by Richard Hynes and since have become the most well studied cell surface receptors [1, 2]. Integrins are heterodimeric transmembrane proteins whose critical function is cellular adhesion to the extracellular matrix (ECM) and attachment to other cells. Through these contacts, integrins contact the ECM and directly bind the intracellular cytoskeleton, where these interactions modify actin and intermediate filaments [3, 4]. Additionally, integrins are highly utilized signaling molecules by cells as binding and integrin activation stimulates a vast number of signaling networks on the interior of the cell [5]. Integrin signaling affords the ECM the ability to alter cellular responses such as survival, proliferation, motility, tension on the ECM, and even ECM content [6].

Integrins are composed of a non-covalently linked single α and single β subunit, of which eighteen α and eight β subunits have been identified in mammals, and twenty-four combinations of integrin receptors have been characterized [1]. Every cell type has a unique set of integrin combinations that are expressed on the cell surface and contribute to cellular response to environmental stimuli. A vast number of ligands bind to integrin receptors including ECM proteins such as laminin, collagen, fibronectin, and vitronectin and combinations of integrins have overlapping substrate specificity [7].

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My study focused on a laminin binding integrin found on the surface of most epithelial tissues, integrin α6β4 [8]. The α6 integrin has two binding partners, integrin β1 and integrin β4, whereas the β4 integrin can only pair with integrin α6, therefore throughout this study, modulation of integrinβ4 subunit effectively alters the entire complex [9]. While most integrin receptors contain large extracellular domains and short cytoplasmic tails, the β4 subunit of integrin α6β4 has a long cytoplasmic signaling domain of nearly 1000 amino acids [10]. Although, integrins have no intrinsic enzymatic activity, the β4 cytoplasmic tail contains fibronectin type III repeats that allow this tail to act as a signaling platform for recruitment of complexes responsible for activation of downstream signaling pathways [11].

Integrin α6β4 is expressed in epithelial cells where its primary function is to maintain the integrity of epithelial monolayers through the formation of stable adhesive structures, termed hemidesmosomes [12]. Hemidesmosomes are responsible for attachment of cells to the basement membrane, and integrin α6β4, in cooperation with plectin, BP180, BP230 and tetraspanins serves as the core complex mediating adhesion between the extracellular matrix and cellular cytoskeleton [13]. Integrin α6β4 is the key component responsible for binding laminin in the basement membrane, while interactions with plectin mediate binding to intracellular intermediate filaments [3]. A single mutation in any one of the twelve different hemidesmosome genes result in skin blistering and loss of epithelial lining such as the gastrointestinal tract, trachea, and cornea, demonstrating their importance [14, 15]. Not only are hemidesmosomes critical for stable adhesion of cells but they are also necessary for maintaining apical-basal cell polarity [12]. During normal cellular processes such as differentiation and wound healing, hemidesmosomes are
dissolved and cells are released [12]. Additionally, this breakdown of hemidesmosomes occurs during carcinoma progression, when cells lose their ability to tightly bind, and now have a motile phenotype [14]. It is this loss of these stable contacts that releases integrin α6β4, allowing it to interact with the actin cytoskeleton and signal to promote tumorigenic properties.

1.2 Integrin α6β4 and Pancreatic Cancer Progression

Integrin signalling is a fundamental component of cancer progression, as it is critical for activation and integration of signaling networks through pro-oncogenic pathways. Of the 24 mammalian integrin receptors, integrin α6β4 is overexpressed in nearly all pancreatic carcinomas where it stimulates malignant progression by promoting tumor cell migration, invasion and cell survival [16, 17]. Pancreatic carcinoma is the fourth leading cause of cancer death among men and women in the US, with more than 90% of patients succumbing to their disease within 5-years [18]. This is due to an aggressive course of disease progression, as many patients are diagnosed with late stage metastatic disease, and lack of effective therapeutic options. Integrin α6β4 contributes to an overwhelming majority of the invasive characteristics of pancreatic carcinoma. Therefore, obtaining knowledge about the mechanistic function of this integrin will provide insight into the deadly nature of this disease.

The role of integrin α6β4 in tumorigenic properties of cancer cells is manifested in two ways, a mechanical function and a signaling function. During the progression of cancer to a metastatic phenotype, stimulation of the Hepatocyte Growth Factor (HGF) receptor, MET, and the EGFR by their respective growth factor ligands promotes disassembly of hemidesmosomes in most epithelial tumors, causing the loss of stable
contacts, and therefore allowing cells to detach and migrate [19]. During this process integrin α6β4 is liberated from these tight hemidesmosomal adhesions and relocates across mobile cells by interacting with F-actin to support lamellipodia and filapodia formation, thus facilitating malignant cells to migrate and invade [20]. Additionally, dynamic contacts between integrin α6β4 and the ECM allow carcinoma cells to have traction as it moves through the body during the process of metastasis [21].

The signaling capacity of integrin α6β4 has been widely demonstrated by the necessity for cooperative activation of integrin α6β4 with c-MET, RON, LPAR, and EGFR [22-24]. This activation of integrin α6β4 results in enhanced downstream signaling through growth factor receptor activated pathways such as PI3K, MAPK, Src family kinases, and the Rho family of small GTPases [25, 26], all of which promote pro-oncogenic properties such as invasion, angiogenesis, anoikis-resistance, cell survival, and proliferation (reviewed in [27]). Work done in the O’Connor lab has found previously that integrin α6β4 contributes to these properties, in part, through specific transcriptional activation of pro-tumorigenic genes [28-31].

1.3 Integrin Signaling and Transcriptional Regulation

Transcriptional regulation during tumorigenesis is one of the critical components in tumor initiation, progression, and colonization of metastatic sites. Downregulation of tumor-suppressors and activation of oncogenes have the capability to dramatically shift the transcriptome to a more permissive state for cancer cell survival, stem cell state, growth, angiogenesis, and metastatic potential [32]. Signaling from the tumor microenvironment can result in both genome-wide and gene-specific alterations in transcription that lead to malignant progression. Integrins, as the major sensor of the
tumor microenvironment, and the link between the ECM and internal cell networks, may hold the key to transcriptional alterations in response to environmental signals, specifically during metastatic cancer progression. Toker and colleagues established that integrin α6β4 signaling results in activation of the Nuclear Factor of Activated T-cells (NFATs), transcription factors, to promote gene expression, that in turn contributes to cancer cell invasion [33]. In support of this work, Chen et al. identified the first targets of integrin α6β4 mediated NFAT activation, S100A4 and Autotaxin in breast carcinoma [30, 34]. Furthermore, these studies demonstrated the robust ability of integrin α6β4 to dramatically alter the transcriptome, leading to the upregulation of key pro-invasion and metastatic genes. In my study, I found that integrin α6β4 stimulates the expression of EGFR ligands AREG and EREG, thus contributing to an invasive phenotype in pancreatic cancer cells.

1.4 Epidermal Growth Factor Receptor and Associated Ligands in Pancreatic Cancer

In normal tissues, EGFR signaling is required for development of epithelial structures including lung, pancreas, skin, gastrointestinal tract, and the mammary gland [35, 36]. EGFR and associated EGF-like ligands become overexpressed or mutated in many cancers, including pancreatic, head and neck, breast, colorectal, lung, prostate, kidney, ovarian, brain, and bladder cancers [37]. Signaling through the EGFR pathway mediates multiple processes involved in tumor progression, including angiogenesis, invasion, migration, proliferation, and evasion of apoptosis [38]. Consequently, particular attention has been given to the role of the EGFR pathway in the development of malignant phenotypes, resulting in this pathway being targeted by a substantial array of
chemotherapeutics. Use of receptor blocking antibodies and tyrosine kinase inhibitors specific for EGFR family members, as monotherapy or in combination with other therapeutic agents, have been successful for colorectal, lung, and head and neck cancers, in terms of increasing overall survival and progression free survival [39-41]

There are seven ligands known to bind and signal through EGFR: epidermal growth factor (EGF), transforming growth factor-α (TGFα), betacellulin, heparin-binding EGF-like growth factor, epigen, AREG, and EREG. Typically, after ligand binding, activated EGFR complexes are endocytosed, which leads to recruitment of the ubiquitin ligase c-Cbl. Recruitment of c-Cbl promotes ubiquitination, lysosomal targeting, and degradation of EGFR [42]. However, AREG and EREG are unique in their downstream signaling following ligand-receptor binding. Binding of AREG or EREG to EGFR results in a transient recruitment of c-Cbl to EGFR and a reduced level of ubiquitination. This property permits EGFR recycling back to the plasma membrane where it may be continually activated [43, 44]. As a result, AREG and EREG have been strongly implicated in tumor progression.

EGFR ligands are integral membrane pro-proteins that can participate in juxtacrine signaling. However, the more common mechanism is that once these ligands are cleaved and released into the extracellular environment, they act in a paracrine and autocrine manner [45]. For AREG, this occurs when disintegrin and metalloproteinase containing protein 17 (ADAM-17)/ tumor necrosis factor α converting enzyme (TACE) [46] or matrix metalloproteinase-1 (MMP1) [47] cleaves the membrane precursor pro-AREG. This release creates feedback loops in primary and metastatic sites to promote tumor progression. AREG may also enter the bloodstream and travel to distant organs,
acting as an endocrine signal [48], and thus potentially creating a favorable microenvironment [49]. This property allows tumors to maintain a high rate of proliferation with a reduced requirement for exogenously supplied growth factors [38]. Notably, AREG has been demonstrated to stimulate proliferation of pancreatic ductal cells and associate with an increased frequency of lymph node involvement in pancreatic cancer patients [50]. Lastly, AREG can induce EGF-independent cell growth by acting as a self-sufficient growth signal in serum-free conditions [51, 52]. Likewise, EREG expression is upregulated in pancreatic cancer and contributes to cell growth by binding to EGFR through paracrine and autocrine loops [53]. Similar to AREG, EREG is also cleaved at the cell membrane by ADAM-17/TACE [46]. Once released, EREG is unique in its ability to stimulate the majority of the ErbB heterodimer receptor combinations [54]. While the affinity of EREG to EGFR is lower compared to other EGFR ligands, its signaling potency is higher, thus making EREG a more effective signaling ligand [54].

1.5 Epigenetic Regulation

Every cell in the human body has the same genetic sequence, however, regulation of transcriptomic patterns defines cell type, cellular function and elicited cellular responses. Activation by transcription factors, in addition to stable changes in covalent modifications to DNA and packaging of DNA are critical for determining gene expression patterns, both in a normal developmental and in a diseased state. During differentiation, epigenetic regulation is responsible for establishing long-term gene expression patterns, which determine cell lineage and fate. Epigenetics is currently defined as heritable changes in gene expression, not associated with changes in gene sequence [55]. Epigenetic regulation allows for dynamic alterations in gene expression
associated with cellular environment. Furthermore, the reversible nature of epigenetic modification makes it an attractive target for therapies.

The human genome contains over 3 billion base pairs of DNA that must be efficiently packaged to fit in the nucleus of every cell [56]. While this compaction creates genomic stability, it also limits the ability of regulatory elements to bind and activate gene expression. Therefore, epigenetic modification allows for dynamic opening and closing of the chromatin, thus contributing to specific gene expression [57]. Nucleosomes are responsible for packaging the DNA into chromatin and consist of two each of histone proteins H2A, H2B, H3, and H4 [58, 59]. Nucleosomes have high affinity for DNA, wrapping 146 bps around each, and in cooperation with linker histones and architectural proteins compacts the DNA into higher order structures [60]. Movement of nucleosomes by ATPase dependent remodeling complexes alters nucleosome position and therefore overall chromatin compaction. Modifications of histone proteins within the nucleosome impact the affinity of DNA for the nucleosome and can recruit transcriptional regulators. Lastly, DNA methylation contributes to chromatin compaction in the form of covalent modifications to DNA bases. Together these three components are responsible for reversibly altering accessibility to transcription factors, and taken together make up the field of epigenetics. Establishment of these epigenetic factors is what ensures proper gene regulation during development, in different tissue types, during cellular responses, and in the context of this study, tumor progression and metastasis.

To produce full-blown metastatic disease, cancer cells require both genetic and substantial epigenetic changes. Initial evidence for the importance of epigenetic alterations was demonstrated by the dramatic differences in epigenetic modifications
between normal tissue, tumor tissue, and metastatic disease [61-63]. Aberrant epigenetic modification is a dynamic process that contributes to a continually changing transcriptome in cancer cells, and is ultimately the driving force for many oncogenes.

1.5.1 Chromatin Remodeling and Cancer

Chromatin remodeling is necessary for proper transcriptional regulation and maintenance of genomic stability. Chromatin exists in two states: condensed heterochromatin, which is transcriptionally silent and euchromatin, which is transcriptionally active and in a more dynamic, “breathable” state [64]. Currently, four families of chromatin remodelers have been identified: SWI/SNF, INO80, ISWI, and NuRD/Mi-2, all of which have the ability to interact with the histones and the DNA. These remodelers also have innate ATPase activity, as energy is required to move nucleosome position along DNA, and exchange histone variants within the nucleosome [65]. The contributions of each chromatin remodeling family to cancer progression are briefly summarized below.

The SWI/SNF complex provides access for the transcriptional machinery by destabilizing chromatin structures using ATP hydrolysis. The broad reaching impacts of the SWI/SNF complex in cancer is evidenced by the finding that more than 20% of all cancers harbor a mutation in this complex [66]. Interestingly, loss of the core component SNF5 of this 8-11 protein complex in mice resulted in T-cell lymphoma or rhabdoid tumor formation in 100% of mice with a median onset of only 11 weeks [67]. Also, studies done in vitro demonstrate that BRCA1 and pRB interact with the SWI/SNF components BRG1 and BRM to contribute to breast cancer [68]. However, while the
importance of SWI/SNF remodeling complexes has been well studied, the mechanism for how these complexes directly promote cancer progression is still under investigation.

INO80 and ISWI complexes function during DNA damage where chromatin remodelers are recruited to sites of damage to facilitate availability of DNA substrates for repair enzymes. INO80 and ISWI complexes act during double strand breaks where they facilitate homologous recombination and non-homologous end joining [69, 70]. INO80 is recruited to double strand breaks through binding of the histone variant γH2AX which is activated immediately after DNA insult [71]. ISWI containing remodelers also function during single strand lesion, which get repaired by either nucleotide excision repair (NER) or base excision repair (BER) [70]. This function of chromatin remodeling during the DNA damage response could play a major role in the ability of cells to respond to chemotherapies, as many of these elicit a DNA repair cascade.

Lastly, the nucleosome remodeling deacetylase (NuRD) complex contains seven subunits and has intrinsic ATPase dependent remodeling and histone deacetylase activity, making this complex unique in its ability to couple two independent epigenetic modifiers [72]. The metastasis-associated gene (MTA) is a component of the NuRD complex and is well known for its role in enhancing invasive properties of cancer, including breast, colorectal, gastric, lung, ovarian, prostate, and head and neck [73]. Additionally, NuRD has been found in complex with the TWIST transcription factor in breast cancer cells [74]. TWIST is known as a master transcription factor and for its ability to induce the epithelial to mesenchymal transition (EMT) and therefore promote metastatic growth [75]. The importance of NuRD in tumor carcinogenesis is also demonstrated by its ability to bind the retinoic acid receptor, target tumor-suppressor genes and recruit other
repressive epigenetic modifiers including the DNA methyltransferases [76]. Taken together, these examples of chromatin modifiers in cancer demonstrate the vast ability of epigenetics to impact tumor progression. Chromatin remodelers in turn are also dependent on and have the ability to impact histone modifications.

1.5.2 Histone Modifications and Cancer

As mentioned previously, nucleosomes are composed of a histone octamer, and post translational modifications of these histones impact the affinity of the nucleosome for DNA, as well as the ability of chromatin remodelers, DNA methyltransferases, and histone specific enzymes to bind [77]. Histones contain N-terminal tails available for modification at specific residues where they can be phosphorylated, methylated, acetylated, ubiquitinated, or sumoylated, all of which promote an active or repressive transcriptional state [78]. Initial studies examining global histone modifications in cancer found that acetylation of lysine 16 of histone H4 (H4K16ac) and tri-methylation of lysine 20 at histone H4 (H4K20me3) was lost in tumor vs. normal tissues, and this was common across many cancer types [62]. Other changes in histone patterns are more specific for cancer type, such as decreased H3K20 methylation in bladder cancer [79], reduced H3K4me2 and H3K9ac in breast cancer, and increased H3K9me2 in colorectal neoplastic cells compared to normal tissue [80]. Interestingly, these aberrant modification patterns are currently targets for epigenetic drugs such as the histone deacetylase inhibitors Trichostatin A (TSA) and Vorinostat, which attempt to reestablish a “normal” histone code. These altered epigenetic modifications do have the potential to act as predictive biomarkers for cancer progression, survival, and therapeutic response. When coupled with gene expression data a cancer’s unique histone code may provide a comprehensive
view of epigenetic alterations and provide insight into an appropriate course of action for individual patients.

1.5.3 DNA Methylation and Cancer

DNA hypomethylation was the first epigenetic event described in tumors and aberrant DNA methylation patterns are considered a hallmark of malignant disease [81]. DNA methylation involves the covalent addition of a methyl group to the 5th carbon position on cytosine, primarily in the context of a CpG dinucleotide [81]. 5-methylcytosine (5-mC) accounts for nearly 1% of all bases in the genome and 70-80% of all cytosines within the CpG context are methylated in somatic cells [82]. During embryonic development DNA methylation patterns are established by the de novo DNA methyltransferases (DNMTs), DNMT3a and DNMT3b [83]. DNA methylation is primarily maintained across multiple generations by the maintenance methyltransferase DNMT1, however recent studies have demonstrated that the DNMT3 family also contributes to maintenance methylation [84]. However, our traditional view of maintenance methylation is that DNMT1 is recruited to the replication fork where it recognizes hemi-methylated DNA, and replaces adjacent methyl groups on the nascent strand [83].

While other epigenetic modifications are critical for gene expression, DNA methylation is the most well studied and considered the primary form of epigenetic information. Cancer genomes, in particular, demonstrate a reduction in genome wide methylation patterns, thus resulting in destabilization of many regions of chromatin by creating instability of transposable elements [85, 86]. Gene specific DNA demethylation contributes to the activation of oncogenes that directly promote metastatic disease, and
are otherwise silenced in somatic cells [87]. However, most studies examining DNA methylation have done so in the context of CpG islands, which are regions of DNA more than 200 base pairs (bp) long and having a higher G+C content and higher frequency of CpG sites than the rest of the genome [88]. CpG islands typically occur at transcription start sites (TSS), and are found at nearly half of all genes with the remainder equally distributed intragenically and intergenically [89]. CpG islands located in gene promoters of major tumor suppressor genes are often hypermethylated in cancer, thus creating a stable, long term block on their activity, including pRB, P16\textsuperscript{INK4A}, VHL, hMLH1, and BRCA1 [90]. Furthermore, 5-mC has an increased rate of mutation to thymine, and accounts for about 1/3 of all disease causing mutations [91], including mutation of p53, thus acting as a major driver of cancer progression [92]. Interestingly, these findings implicate epigenetic mechanisms as causing some of the first “hits” in cancer initiation. Consequently, the importance of DNA methylation in carcinogenesis and it’s reversible nature makes DNA methylation ideal for targeted therapies [83]. Considering that epigenetic changes are reversible, DNA demethylating agents such as Azacitidine (the clinical equivalent of 5-aza-cytidine or 5-Aza) and Decitabine (5-Aza-2-deoxycytidine; 5-Aza-CdR; DAC) have become commonly used therapies for the treatment of several types of cancer [93-95]; however, because of their non-specific nature, resistance to these types of drugs can still be a major problem. A possible mechanism for this resistance may be the activation of pro-metastatic genes along with tumor suppressor genes. A potential solution to this problem is identification of invasion and metastasis promoting pathways that become activated in response to DNA demethylation, so that they may be targeted alongside these epigenetic drugs.
1.6 Goals and Hypothesis for Dissertation

It is well established that EGFR and associated ligands contribute to key steps in cancer growth and progression. Specifically, autocrine secretion of the ligands AREG and EREG increase the invasive and migratory capacity of cancer cells and can contribute to drug resistance [96, 97]. These molecules become upregulated throughout cancer progression and this is potentially the result of signaling through pro-metastatic pathways, such as those regulated by integrin α6β4. Integrin α6β4 is highly upregulated and activated in pancreatic carcinoma where it dramatically alters the transcriptome, in part through DNA demethylation of promoters of pro-tumorigenic molecules. DNA demethylation is most often achieved by enzymatic removal of the repressive methyl group coordinated by a DNA repair pathway. Therefore, considering that integrin α6β4 is a critical sensor of the tumor microenvironment, and contributes to gene regulation in cancer, DNA demethylation by DNA repair could provide insight into context dependent epigenetic alterations that contribute to metastatic disease. Consequently, the overall objective for this dissertation was to understand in mechanistic detail how integrin α6β4 alters the overall state of DNA methylation in pancreatic cancer and in particular how integrin α6β4 contributes to specific upregulation of two EGFR ligands, AREG and EREG. I therefore, hypothesized that integrin α6β4 contributes to genome wide DNA demethylation and mediates gene specific expression of AREG and EREG through active DNA demethylation of their respective promoters. Additionally, I hypothesized that a DNA repair pathway mediated this site-specific DNA demethylation of AREG and EREG promoters. The following data chapters will address three specific project goals:
1) Define the role of integrin α6β4 signaling in the transcriptional upregulation of AREG and EREG and determine the impact of DNA demethylating drugs on the expression of these genes.

2) Examine genome-wide and gene specific alterations in DNA methylation mediated by integrin α6β4 using whole genome bisulfite sequencing (WGBS).

3) Determine the involvement of DNA repair pathways on transcriptional upregulation of AREG and EREG, downstream of integrin α6β4 signaling.
CHAPTER 2: MATERIALS AND METHODS

2.1 Cell Lines and Cell Culturing

Panc1, Suit2, AsPC1 and Panc1 clones with variable expression of integrin α6β4 were used. The Panc1 cell line was previously sorted by FACS analysis for integrinβ4 subunit to establish stable populations with varying integrin α6β4 expression [98]. Suit2, AsPC1 and Panc1-3D7 cells were used as high expressers of integrin α6β4. Panc1-2G6 is a low integrin α6β4 expressing cell line and Panc1-β4ΔCyt cells express a dominant negative form of integrin α6β4 [99]. Suit2 (obtained from Dr. Takeshi Iwamura, Miyazaki Medical College, Miyazaki, Japan) and AsPC1 cells (from America Type Culture Collection, ATCC) were maintained in RPMI 1640. Clones generated from Panc1 cells (from ATCC) were grown in Dulbecco’s modified Eagle’s medium (DMEM; high glucose). HEK293-LTV cells (obtained from Dr. Tianyan Gao, University of Kentucky) were used for lentivirus production and maintained in Dulbecco’s modified Eagle’s medium (high glucose). All media were supplemented with 10% Fetal Bovine Serum (Sigma Aldrich, St. Louis, MO), 1% penicillin, 1% streptomycin, and 1% l-glutamine (GIBCO by Life Technologies, Grand Island, NY).

2.2 Drug Treatment

5-Aza-CdR (Sigma-Aldrich) was added to Panc1-2G6 (low β4), Panc1-3D7 (high β4), or Panc1-3D7 β4 shRNA cells in fresh medium daily at a final concentration of 1

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μM or 5 μM for 3 or 5 days, while control cells were treated with dimethyl sulfoxide (DMSO). Cells were collected either immediately or for indicated experiments, kept in culture for 10 days and two passages post 5-Aza-CdR removal, as indicated. Suit2 and AsPC1 cells were treated with 80 μM S-adenosylmethionine (SAM; New England Biolabs, Ipswich, MA) in fresh medium daily for 5 days. Control cells were treated with an equivalent volume of vehicle (0.005 M H2SO4 and 10% EtOH.) Pancreatic cancer cells were treated with 250-500 nM JQ1 (Bradner Lab; Dana-Farber Cancer Institute) or DMSO for 16 hours. For experiments using Gemcitabine (Sigma-Aldrich) or 3,4-Dihydro-5-[4-(1-piperidinyl)butoxyl]-1(2H)-isoquinolinone (DPQ; Sigma-Aldrich), Panc1-2G6 (β4 low) or Panc1-3D7 (β4 high) cells were treated with indicated concentration or equal volume vehicle control for 3 days. After treatment, cells were harvested and mRNA levels of AREG and EREG measured by Quantitative Real-Time Polymerase Chain Reaction (qPCR) as described below.

2.3 Gene Knockdown by RNAi

For siRNA treatment, cells from 70% confluent cultures were washed once with phosphate buffered saline (PBS), trypsinized, and washed three times by centrifugation with serum-free DMEM. Cells (3 x 10⁶) were resuspended in 400 μl serum-free DMEM and electroporated without or with 200 nM non-targeting siRNA (Dharmacon, Inc.), or 200 nM siRNA specific for NFAT5 or GADD45A as described previously [29]. Cells were then replated under normal culturing conditions and normal growth media was added 24 hours following electroporation. Cells were collected after 72 hrs and assessed for NFAT5, GADD45A, AREG and EREG expression by qPCR as described below.

For stable knockdown of AREG, EREG, TDG, XPA, XPG, or XPF lentivirus was
produced by combining MISSION constructs for packaging (pSAX2), envelope (pDM2G) vectors and targeting shRNA or a non-targeting vector (pLKO.1), at a 4:2:1 ratio (all vectors obtained from Sigma Aldrich, St. Louis, MO). Polyethylenimine (PEI; Polysciences) was combined with the DNA mixture at a 3:1, DNA to PEI ratio and added drop wise to 70% confluent HEK 293LTV cells that had been passaged 24 hrs prior to transfection. Conditioned media was collected 24 and 48 hrs post transfection, centrifuged at 2,000 x g for 10 mins and viral supernatant collected. Panc1-2G6 and Panc1-3D7 were passaged to 70% confluence in 10 cm dishes and 4 ml of viral supernatant added in combination with 8 μg/ml hexadimethrine bromide (polybrene, Sigma Aldrich). Viral supernatant was added again 24 hours later, and 48 hours later was replaced with normal growth media. Cells were placed under puromycin selection (2-4 μg/ml) for a minimum of three days, or until cell death stopped occurring due to selection. Gene expression was measured by qPCR 24 hrs following removal of puromycin, and migration and invasion assays performed immediately following confirmation of efficient knockdown of AREG and EREG.

2.4 RNA Extraction and qPCR

Cells from 70% confluent cultures were washed in 2 ml of PBS buffer (Gibco by Life Technologies) and harvested using 0.05% trypsin-EDTA. Total RNA was extracted using Trizol reagent and manufacturer’s protocol (Invitrogen, Carlsbad, CA). Chloroform was added and nucleic acids separated from protein by high-speed centrifugation (15,000 x g for 10 min.). The organic phase was removed and equal volume isopropanol added to precipitate RNA. Samples were centrifuged, washed with 70% ethanol, and redissolved in an appropriate volume of nuclease-free water. RNA quantity was confirmed using a
Nanodrop 2000 spectrophotometer (Thermo Scientific) and purity determined by OD 260:280 ratios, with a minimum requirement of 1.8. cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and 1 µg of total RNA. Expression of genes of interest was assessed by qPCR using available probes (Taqman; Applied Biosystems), reagents, and the StepOnePlus Real-Time PCR System from Applied Biosystems. Triplicate C_T values were analyzed in Microsoft Excel using the comparative C_T (ΔΔC_T) method as described by the manufacturer (Applied Biosystems). The amount of target cDNA (2^{-ΔΔCT}) was determined by normalizing to the endogenous reference (18S or β-Actin) and relative to one of the experimental samples, either control treated, or those transfected with non-targeting or empty vector for knockdown and overexpression studies.

2.5 Migration and Invasion Assays

Transwell chemotactic migration assays were performed as described previously (6.5-mm diameter, 8-µm pore size; Corning) [31]. Upper portions of Transwell chambers were coated with 15 µg/ml laminin-1 for migration assays or with 10 µg Matrigel (BD Biosciences) for invasion assays. For all assays, the bottom chambers of Transwells were coated with 15 µg/ml laminin-1. Following coating with laminin or Matrigel, wells were washed three times with serum-free media containing 250 µg/ml bovine serum albumin (BSA). Cells were trypsinized, collected from 70% confluent cultures, washed three times with serum-free media, and counted using a ViCell Coulter Counter (Beckman Coulter). A single cell suspension (5 x 10^4) was placed into the top Transwell chamber and allowed to migrate for 4 hrs or invade for 6 hrs towards 10 ng/ml HGF, which was placed in the bottom chamber. Cells that did not migrate or invade were removed from
the top chamber using a cotton-swab. Cells attached to the bottom of the chamber were fixed with 100% methanol for 20 minutes, stained using 3% crystal violet in 2% ethanol for 20 minutes, and counted visually using an inverted microscope. Results are reported as the mean number of cells migrated per mm² from triplicate determinations. Data reported are representative of at least three separate experiments.

2.6 Amphiregulin Enzyme-Linked Immunosorbert Assay (ELISA)

Pancreatic carcinoma cells (1.5-2.0 x 10⁵) were plated in 6-well plates in complete culture medium overnight. The following day, cells were rinsed twice with PBS and then placed in serum-free medium containing 250 µg/ml BSA. For HGF-stimulation experiments, cells were placed in new serum free medium for 4 hours, rinsed, and then stimulated with HGF (10 or 50 ng/ml, as noted). After 24 hrs, conditioned medium was harvested, cleared by centrifugation, concentrated by centrifugation in Ambion Ultra-15 Centrifugal filter units (Millipore), volume recorded, and AREG content analyzed by ELISA (Human Amphiregulin ELISA Kit, cat# ELH-AR-001, RayBiotech, Inc.) using recombinant human AREG as a standard. Cells were counted by hemocytometer at the conclusion of each experiment to confirm equal cell numbers. Data from triplicate determinations are reported as pg AREG/ml/10⁵ cells with standard deviation of the mean. For all experiments, data presented are representative of at least three separate determinations.

2.7 Whole Genome Bisulfite Sequencing (WGBS)

Pancreatic cancer cells were grown to 70% confluence and media changed 24 hours prior to harvest. Cells were washed with PBS, trypsinized, and collected by centrifugation. Whole genomic DNA was isolated using the GenElute Mammalian
Genomic DNA Miniprep Kit (Sigma-Aldrich). DNA quantity and quality were measured by a Nanodrop 2000 spectrophotometer. A minimum 260:280 ratio of 1.8 was required. DNA was processed for high-resolution methyl-seq by the NextGen Sequencing Core at the Norris Comprehensive Cancer Center. Whole genome sequencing was done on an Illumina NextSeq and each library was sequenced with paired-end runs for 150-bp read length analysis.

2.8 Read Alignment and Differential Methylation Analysis

Paired-end bisulfite-treated DNA reads were aligned against GRCH37 using Bismark [100] software version 0.14.3, permitting at most one mismatch, considering both sequence and bisulfite conversion mismatches. The methylation calls for each CpG were extracted using Bismark methylation extractor tool. Read alignment based on Bismark revealed that many reads could be mapped to regions in both AREG and the AREG pseudogene due to their high degree of homology. To allow mapping of AREG, the AREG pseudogene was masked during analysis, and AREG was masked when mapping the AREG pseudogene. As a result, some sequencing reads mapped to both the AREG and the AREG pseudogene, which was permitted in our analysis in Figure 4.1B and 4.1C only. Differential methylation analysis comparing Panc1-3D7 and Panc1-2G6 was performed using Bioconductor DSS software version 2.10.0 [101]. The differentially methylated loci (DML) were determined by > 0.99 posterior probability of the difference in mean methylation levels being greater than 0.3. Differentially methylated regions (DMR) were also detected using results from the DML detection. DMLs with p-value less than 0.01 were chosen and joined to construct DMRs. We required DMRs to have a minimum length greater than 50 bps, minimum number of DML greater than 3 and
greater than 50% of CpG sites with significant p-value (less than 0.01). Nearby DMRs with distance less than 100 bps were merged into longer ones. The DMLs and DMRs were annotated using methylKit [102] software version 0.9.5, where we defined the promoters as the 1000 bp upstream and downstream of the transcription start site and the CpG shores as the 2000 bp flanking regions on each side of the CpG island.

2.9 Western Blotting Analysis

Nuclei were isolated from Panc1-2G6, Panc1-3D7 NT, and Panc1-3D7 cells stably expressing TDG shRNA. Cells were collected by washing 10 cm culture dishes with cells attached, on ice with cold PBS two times, and scraping cells with 400 μl cold Buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF). Nuclei were lysed for 5 min on ice with 10% NP-40 and nuclear fraction collected by centrifugation. Supernatant containing cytosolic proteins were placed in new tubes and nuclear pellet was resuspended in cold Buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1mM DTT, 1 mM PMSF). Nuclear extracts were centrifuged and nuclear supernatant placed in new tubes. Extracts were separated using 10% SDS-PAGE, transferred and immunoblotted for TDG (GeneTex, GT622), and Lamin A/C (EMD Millipore).

2.10 Hydrogen Peroxide Treatment and MTT Assay

For hydrogen peroxide treatment (Sigma-Aldrich), cells were plated in a 96-well plate at a density of 2000 cells per well, and four wells per condition. Medium was changed each day to either normal growth medium or medium containing 500 μM H₂O₂. Cell density was measured each day for seven days. 20 μl of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT; Fisher Scientific) were added directly
to cell culture media and incubated for 3 hours. Media and MTT was removed and the reaction stopped by adding 100 μl of stop solution (90% isopropanol, 10% DMSO) to each well. Plates were incubated at room temperature for 20 minutes, rocking, in the dark, and read using a spectrophotometer at 590 nm.

2.11 DNA Repair analysis of 6-4 Photoproducts (6-4 PP)

Immuno-slot-blot analysis was performed as described previously [103]. Cells were plated in 10 cm dishes to 70% confluence. Media was removed, cells were washed 1x with PBS, exposed to 30 J/m² UV light and either harvested immediately or medium replaced. Cells were lysed (10 mM Tris pH 8.0, 1mM EDTA, 0.05% SDS, 100 μg/ml fresh proteinase K) at indicated time points and DNA isolated. DNA was bound to a nitrocellulose membrane using a Bio-Dot SF microfiltration apparatus (BioRad). Membranes were blocked for 1 hour using 5% non-fat milk in Tris-Buffered Saline and Tween-20 (TBST) and probed using antibody for 6-4PP (Cosmobio) overnight. Results are presented as percent repair compared to the amount of initial damage (0 hr time point).
CHAPTER 3: INTEGRIN α6β4 PROMOTES INVASION OF PANCREATIC CANCER CELLS BY UPREGULATING EGFR LIGANDS AREG AND EREG

3.1 Introduction

Integrins are essential components of tumor progression as they contribute to pro-oncogenic properties such as survival, proliferation, and cell motility [104]. Specifically, integrin α6β4 is a major driver of tumor metastasis as it is critical for invasion and motility of cancer cells [105, 106]. Activation of integrin α6β4 results in stimulation of downstream signaling pathways including PI3K, MAPK, Src family kinases, Rho family small GTPases, and the NFAT transcription factors [25, 26, 33]. Signaling through these pathways contributes to properties principal to tumor progression including invasion, angiogenesis, anoikis-resistance, cell survival, and proliferation (reviewed in [27]). Previous work has found that integrin α6β4 enhances these properties in part through specific transcriptional upregulation of pro-tumorigenic genes, including S100A4 in breast cancer cell lines [30]. Furthermore, Chen et al. demonstrated that integrin α6β4 promotes activation of the transcription factor NFAT5 thus driving S100A4 expression [30]. Uniquely this study was the first to reveal that integrin α6β4 could impact DNA methylation, as the S100A4 becomes actively demethylated within the first non-coding intron of the S100A4 gene in response to signaling from integrin α6β4 [30].

This chapter will explore the impact of integrin α6β4 signaling on gene expression of two EGFR ligands, AREG and EREG, and examine their necessity for

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pancreatic cancer cell migration and invasion. The importance of AREG and EREG in tumor progression, therapeutic resistance, and as a potential prognostic and predictive biomarker has been well established in multiple cancer types, including lung, ovarian, breast, head and neck, colon, liver, prostatic, stomach, bladder, and pancreatic cancers [48, 107]. Notably, EGFR signaling by AREG and EREG is enhanced in pancreatic carcinomas and contributes to the aggressive nature of the disease [50, 53].

Genes that are transcriptionally silent, such as those important for development, are traditionally characterized as having a repressive epigenetic state that compacts chromatin. These repressive epigenetic marks include non-acetylated histones, lysine methylation at H3K27 and H3K4 and cytosine methylation at CpGs [108]. Activation of gene expression during cancer progression constitutes loss of these repressive marks and a more dynamic chromatin state that may allow for situation specific gene repression or activation [109]. To determine if AREG and EREG could potentially be regulated by epigenetic modification, I examined the promoters of AREG and EREG as well as other transcriptional targets of integrin α6β4 signaling. I found that large CpG rich regions reside in the proximal promoters of these genes, providing ideal targets for transcriptional regulation by DNA methylation and epigenetic modification (Fig. 3.1) Additionally, in gastric cancer it has been demonstrated that EREG is differentially methylated in both cell lines and in tumor vs. normal tissue, and this is dependent on the activities of the de novo DNA methyltransferase DNMT3b [110].

Based on these observations, I hypothesized that integrin α6β4 contributes to pancreatic cancer progression, in part, through transcriptional upregulation of pro-oncogenic molecules AREG and EREG, and this is achieved through a similar
mechanism as S100A4 in breast cancer. I sought to test this hypothesis by utilizing well-characterized inhibitors of epigenetic modifiers in pancreatic cancer cell lines that have variable integrin α6β4 expression.
Figure 3.1 CpG rich regions overlap with regulatory regions of genes controlled by integrin α6β4.

CpG islands within the promoter regions of the sequences of genes known to be upregulated by integrin α6β4 were predicted using online software from the following websites: http://www.ebi.ac.uk/Tools/emboss/cpgplot/ and http://cpgislands.usc.edu/. CpG islands were defined as a DNA fragment with a GC content of more than 60%, an observed CpG/expected CpG ratio > 0.70, and length at least 200 base pairs.
3.2 Results

3.2.1 AREG and EREG expression positively correlate with integrin α6β4

Integrin α6β4 is associated with progression of many types of cancer in part through alteration of the transcriptome. Previous work in the O’Connor lab has demonstrated the widespread impacts of integrin α6β4 on gene transcription in both breast and pancreatic cancer [29, 31]. To determine if expression of AREG and EREG positively correlates with expression of integrin α6β4, qPCR was performed on cell lines with variable expression of integrin α6β4. Panc1-2G6 and Panc1-ΔCyt cells are derived from the Panc1 cell line and have very low integrin α6β4 expression or stably express a dominant negative form of integrin α6β4 respectively. Panc1-ΔCyt cells lack the 1000 amino acid cytoplasmic signaling domain of integrinβ4 subunit, allowing the integrin to bind to laminin but fail in its capacity to signal [99]. Panc1-3D7 cells, also derived from the Panc1 cell line, AsPC1 and Suit2 cells, (listed in order of increasing expression of integrin α6β4) are considered high expressers, with Suit2 having more than 100-fold higher expression of the β4 integrin compared to Panc1-2G6. As seen in Figure 3.2A Panc1-2G6 and Panc1-ΔCyt cell lines have very low AREG and EREG expression as compared to Panc1-3D7, AsPC1, and Suit2, which has incredibly high AREG and EREG expression. Thus indicating a positive correlation between integrin α6β4 and AREG and EREG expression. Additionally, the contribution of integrin α6β4 to AREG and EREG in Panc1-3D7 cells was confirmed by stably knocking down integrin β4 using a specific shRNA. This knockdown resulted in a corresponding decrease in AREG and EREG expression (Fig. 3.2B), demonstrating the dependence of AREG and EREG on integrin α6β4. Lastly, to confirm that these genes correlate with integrin α6β4 expression in human pancreatic tumors, I analyzed the cancer genome atlas (TCGA) pancreatic ductal
adenocarcinoma database, which contains gene expression data as measured by whole transcriptome shotgun sequencing, or RNA sequencing (RNA-seq). I found that both AREG (Fig. 3.2C) and EREG (Fig. 3.2D) positively correlate with expression of integrinβ4 subunit (ITGB4) as indicated by Pearson and Spearman Coefficients > 0.3. These data suggest that integrin α6β4 regulates expression of AREG and EREG in pancreatic carcinoma cells and these data are corroborated in pancreatic cancer patient samples.
Figure 3.2. Integrin α6β4 expression positively correlates with expression of AREG and EREG in Pancreatic Cancer.

(A) Expression of AREG and EREG was compared in Panc1-2G6 (low α6β4) and cells expressing a dominant negative α6β4 (Panc1-β4ΔCyt), Panc1-3D7, Suit2, and AsPC1 (high α6β4) cell lines. Cells are listed in order of increasing integrin α6β4 expression. (B) Expression of AREG and EREG was measured following shRNA knockdown of integrin β4 subunit. For these experiments RT-PCR was used to convert RNA to cDNA and qPCR was used to assess AREG and EREG expression. Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls, unless otherwise indicated. (C-D) Findings were validated in an external dataset (TCGA), where by linear regression, gene expression levels of integrin β4 subunit (ITGB4) were found to positively correlate with expression of AREG, EREG, in patient-derived pancreatic adenocarcinomas.
3.2.2 *AREG* and *EREG* are required for pancreatic cancer cell migration and invasion downstream of integrin α6β4

Integrin α6β4 promotes invasion, in part, by cooperating with growth factor receptors such as c-Met, LPAR, RON, and EGFR [111]. Notably, c-Met and its associated ligand, HGF, are important players in pancreatic carcinoma and cooperation of c-Met with integrin α6β4 promotes invasive growth [112]. Therefore, I examined the individual contributions of AREG and EREG to HGF-mediated pancreatic cancer cell migration and invasion. While it is well established that AREG and EREG contribute to cancer cell metastasis [53, 113], how autocrine EGFR secretion contributes to directed cell motility is less well defined. Therefore, I reduced AREG and EREG expression in cells with high integrin α6β4 (Panc1-3D7) using target-specific shRNA and performed HGF-stimulated chemotaxis and chemoinvasion assays. Of note, cells that have low integrin α6β4 are deficient in their ability to migrate and invade, and I therefore did not test these cells in this study [31]. Knockdown of AREG and EREG was confirmed by qPCR (Fig. 3.3E, 3.3F) the day prior to chemotaxis and chemoinvasion assays. Inclusion in chemotaxis and chemoinvasion assays required a minimum of 90% knockdown of AREG or EREG. As shown in Figure 3.3, AREG or EREG knockdown results in decreased migration (Fig. 3.3A, 3.3B) and invasion (Fig. 3.3C, 3.3D) of Panc1-3D7 cells. Interestingly, effective knockdown of one ligand resulted in decreased expression of the other ligand by about 50%, indicating their transcriptional dependence on one another. These data indicate that both AREG and EREG are required for HGF-mediated migration and invasion of pancreatic cancer cells with upregulation of integrin α6β4.
Figure 3.3. AREG and EREG are both required for chemotactic migration and invasion of pancreatic carcinoma cells.

AREG or EREG expression levels were reduced using lentiviral delivery of specific shRNAs. Upon stable selection with puromycin, cells were assessed for AREG (E) and EREG (F) expression by qPCR. Populations that achieved a minimum of 90% reduction in expression of AREG or EREG specifically were assayed for migration or invasion the following day (A-D). Cells (5 x 10^4) were placed into the top well of laminin1-coated Transwells for migration (A, C), or Matrigel coated wells for invasion (B, D) with either BSA containing DMEM (DMEM/BSA) alone or DMEM/BSA with 50 ng/ml HGF in the bottom well, as described in the Experimental Procedures. Cells were allowed to migrate for 4 hrs or invade for 6 hrs prior to harvest and quantification. Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05, and ** P<0.005.
3.2.3 NFAT5 is required for expression of AREG and EREG

Based on the correlations between integrin α6β4 and AREG and EREG, I sought to explain mechanistically how integrin α6β4 regulates their gene expression. It has previously been established that integrin α6β4 can signal downstream to transcription factors, including NFAT1 and NFAT5, to promote tumor cell invasion [30, 33]. Considering that NFAT consensus sites are found within the AREG and EREG sequences, I hypothesized that these genes could be targets for the NFATs. To determine if AREG and EREG are regulated by NFAT5 downstream of integrin α6β4 signaling, I used siRNA to specifically reduce expression of NFAT5. I found that both AREG and EREG expression are substantially decreased with NFAT5 knockdown in Panc1-2G6 cells (Fig. 3.4A) and even more robustly in Panc1-3D7 cells (Fig. 3.4B), as expected. Knockdown of NFAT5 by siRNA was confirmed by qPCR (Fig. 3.4C, 3.4D). Notably, knockdown of NFAT1 in pancreatic cancer cell lines did not impact expression of AREG and EREG (data not shown) indicating that gene regulation of AREG and EREG is specific for NFAT5. These data indicate that the NFAT5 transcription factor is required for expression of AREG and EREG in response to signaling from integrin α6β4.
Figure 3.4. Integrin α6β4 regulates gene expression of AREG and EREG via NFAT5.

Panc1 clones 2G6 and 3D7 were electroporated with siRNA specific for NFAT5 or non-targeting control. After 72 hrs in culture, RNA was harvested from treated cells and assessed for AREG and EREG expression (A, B) and for knockdown efficiency of NFAT5 (C, D) by qPCR. Experiments depicted here are representative of at least three separate experiments. Data represent the mean +/- standard deviation.
3.2.4 AREG and EREG expression are induced by 5-Aza-CdR and this induction is dependent on integrin α6β4

Prior work in the O’Connor laboratory demonstrated that integrin α6β4 contributes to upregulation of S100A4 in breast cancer via DNA demethylation and suggests a similar mechanism for upregulation of AREG and EREG in pancreatic cancer [28, 114]. Consequently, I sought to expand these ongoing studies by defining the contributions of DNA methylation to AREG and EREG expression and the impact of integrin α6β4 in this process. To determine if AREG and EREG expression could be induced in response to inhibition of DNA methylation, cells with low integrin α6β4 (Panc1-2G6) were treated with the DNA methyltransferase inhibitor, 5-Aza-CdR at either 1µM or 5 µM in fresh medium daily. Cells were harvested following 3 or 5 days of treatment, RNA was isolated, and analyzed by qPCR. I found that both AREG and EREG mRNA expression increased in a time and dose dependent manner (Fig. 3.5A) demonstrating the susceptibility of AREG and EREG to DNA methylation [28]. In addition, integrin α6β4 was either required for induction of AREG and EREG mediated by 5-Aza-CdR, or acts in a synergistic mechanism with 5-Aza-CdR to enhance gene expression, as knocking down the integrin β4 subunit in Panc1-3D7 cells hindered epigenetic induction of AREG and EREG expression (Fig. 3.5B).

Expanding on the finding that 5-Aza-CdR can induce AREG and EREG expression (Fig. 3.5A), I treated Panc1-2G6 and Panc1-Δcyt cells with 5 µM 5-Aza-CdR for five days to induce expression of AREG and tested the impact of integrin α6β4 on the process of AREG secretion. After treatment, cells were assessed for HGF-stimulated AREG secretion, by an ELISA on conditioned medium, and compared to the Panc1-3D7 cells. As shown in Figure 3.5C, 5-Aza-CdR induced AREG secretion to a level
approximately one-quarter of that of untreated Panc-3D7 cells. However, enhanced section following 5-Aza-CdR indicated that the gene expression data is mirrored at the protein level and has the potential for propagating EGFR signaling. In addition, the level of AREG in the medium from unstimulated and HGF-stimulated cells differed by 20 to 60-fold higher in Panc1-3D7 cells compared to the Panc1-Δcyt and Panc1-2G6 cells. This observation implicates integrin α6β4 signaling in the secretion of AREG in addition to regulation by DNA demethylation.

Considering that epigenetic changes are reversible and to confirm that AREG and EREG DNA demethylation is an active process, cells with high expression of integrin α6β4, and subsequently AREG and EREG, were treated with the methyl donor for DNA methyltransferases SAM in fresh media daily for 3 days to a final concentration of 80 μM. SAM is the rate-limiting molecule in the reaction facilitated by DNA methyltransferases. Following treatment, AsPC1 and Suit2 cells were collected and qPCR revealed roughly a 50% decrease in expression of AREG and EREG as seen in Figure 3.5D. Thus indicating that AREG and EREG can be epigenetically silenced when SAM, the methyl donor is added to cells in excess. Taken together these data support my hypothesis that AREG and EREG are controlled by DNA methylation and that active signaling from integrin α6β4 is required to maintain an unmethylated state and drive their expression.
Figure 3.5. AREG and EREG expression is mediated by DNA demethylation in response to signaling from integrin α6β4.

(A) Panc1-2G6 cells (low α6β4) were treated with vehicle only (control) or with 1 μM or 5 μM 5-Aza-CdR in fresh medium daily for 3 or 5 days. (B) Panc1-3D7 (high α6β4) and Panc1-3D7 cells stably expressing an shRNA for integrinβ4 were treated with 5 μM 5-Aza-CdR as described above. (C) Cells were incubated for 5 days with or without 5 μM 5-Aza-CdR in fresh medium daily. Cells were then replated at 1.5 x 10⁵ cells into 35 mm dishes on the fourth day. The following day, cells were serum starved for 4 hrs before medium was changed and HGF (0, 10 or 50 ng/ml, as noted) in serum-free medium was added. After 24 hrs, culture medium for each condition was assessed for AREG content by ELISA and cells counted to confirm equal cell numbers. Data are reported as pg of AREG per ml of culture medium per 1x10⁵ cells. (D) AsPC1 and Suit2 (high α6β4) were treated with vehicle only (control) or 80 μM S-adenosylmethione (SAM) in fresh media daily, for 5 days. Data are representative of at least three different experiments. Data are reported as the mean of triplicate determinations +/- standard deviation.
3.2.5 Integrin α6β4 stably upregulates AREG and EREG through epigenetic mechanisms

True epigenetic alterations are defined by changes that can be stably maintained across many cellular generations. Molecules such as 5-Aza-CdR can modify the epigenetic landscape, and it has been demonstrated that transient exposure to DNA demethylating agents can result in stable epigenomic rearrangement of select genes [115]. I sought to determine if transient 5-Aza-CdR treatment could recapitulate a stable epigenetic change in cells with low integrin α6β4. Pancreatic cancer cells were treated with 5-Aza-CdR at various concentrations, as indicated, for 24 or 72 hours in fresh media daily. 5-Aza-CdR was removed and cells were either harvested immediately or maintained in culture for 10 days and two passages. As seen in Figure 3.6A, expression of AREG and EREG when integrin α6β4 is low (Panc1-2G6), is not only induced and maintained in these cells following 5-Aza-CdR treatment but dramatically increases when kept in culture 10 days post 5-Aza-CdR removal. Cells with high integrin α6β4 (Panc1-3D7; Fig. 3.6B) only slightly increased in transcription of AREG and EREG, as these stable epigenomic rearrangements may have already taken place as a result of integrin α6β4 signaling. Based on these data, I confirmed that integrin α6β4 contributes to the stable upregulation of pro-tumorigenic molecules AREG and EREG, through epigenetic alterations.
Figure 3.6. Stable epigenetic alterations are required to maintain expression of AREG and EREG in response to integrin α6β4

Panc1-2G6 (A) and Panc1-3D7 (B) cells were treated with 2 μM 5-Aza-CdR for 3, or 5 days, 5-Aza-CdR was removed and cells were either collected immediately or maintained in culture for 10 days. Cells were collected and RT-PCR was used to convert RNA to cDNA and quantitative real-time PCR was used to assess AREG and EREG expression. Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls.
3.2.6 DNA methylation and not histone modifications are responsible for changes in AREG and EREG expression in pancreatic cancer cell lines

DNA methyltransferase inhibitors, while specific for epigenetic changes associated with methylation, often impact histone methyltransferase activity as well [116]. Histone methylation can be activating or repressive depending on the location of the methylation on the histone and the methyltransferase involved [117]. Likewise, changes in DNA methylation can impact the ability of histone modifying enzymes to bind and therefore alter chromatin compaction and transcriptional status [85]. Lysine acetylation is the most common form of histone modification associated with gene expression. Since DNA is negatively charged, acetylation of histone tails reduces charge-charge interactions between nucleosomes and DNA, and therefore opens DNA, allowing for transcription. Histone acetylation tends to coexist with activating marks of gene expression and a more “open” chromatin state, although exceptions do exist [118, 119]. The state of histone acetylation is dependent on the competing activity of histone acetyl transferases (HAT) and histone deacetylases (HDAC). While all epigenetic factors, including histone modifications, contribute to gene transcription, DNA methylation is considered the primary inhibiting factor [120]. To test the individual contributions of histone acetylation and DNA methylation to AREG and EREG gene expression, pancreatic cancer cells were treated with TSA, a histone deacetylase inhibitor, either alone or in combination with 5-Aza-CdR, and gene expression measured by qPCR. I found, as seen in Figure 3.7 that maintenance of histone acetylation alone (TSA only) cannot induce expression of AREG and EREG in either Panc-2G6 (Fig. 3.7A) or Panc-3D7 (Fig.3.7B). As expected TSA in combination with 5-Aza-CdR resulted in a synergistic transcriptional activation of AREG and EREG in Panc1-2G6 cells, therefore
indicating that a repressive chromatin state is present in the absence of integrin α6β4 signaling. Interestingly, in cells with high integrin α6β4, treatment with a histone deacetylase inhibitor negates the effects of 5-Aza-CdR, indicating the absence of a completely repressive epigenetic state.
Figure 3.7. DNA methylation and not histone modifications are responsible for changes in amphiregulin and epiregulin expression in pancreatic cancer cell lines

Panc1-2G6 (A) and Panc1-3D7 (B) cells were treated with either 5 μM 5-Aza-CdR (DAC) for 5 days, 1 μM Trichostatin A for 24 hours, or both. Cells were collected, RNA isolated, and gene expression of AREG and EREG measured by qPCR. Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls.
3.2.7 Enhancer elements drive expression of AREG and EREG

Alterations in DNA methylation have been shown to particularly impact the activity of enhancers. Enhancers are elements which are responsible for activating specific transcriptional profiles, in part through the recruitment of transcription factors which in turn interact with the mediator complex [121]. Enhancers can act in cis and be located within or adjacent to genes or in trans and regulate gene expression as far as several kilobases away [121]. Enhancers have the ability to impact transcription of many genes or gene networks and are therefore intriguing targets for cancer therapy. To determine if enhancer activity is required for AREG and EREG expression in pancreatic cancer cells, I treated cells with JQ1, a BET bromodomain inhibitor that is specific for bromodomain containing protein 4 (BRD4) [122]. BRD4 interacts with the elongating factor P-TEFB in Pol II complexes, and is required for both protein-coding and enhancer-derived noncoding RNAs [123]. When treating three different pancreatic cancer cell lines with high expression of integrin α6β4, I found that expression of AREG and EREG markedly decreased with low dose JQ1 treatment. These results indicate that AREG and EREG are transcriptionally dependent on enhancer function to maintain expression (Fig. 3.8).
Figure 3.8. JQ1 treatment reduces expression of AREG and EREG

Cells with high integrin α6β4 were treated with vehicle only (control) or 0.5 μM JQ1 overnight and harvested for analysis by qPCR. For all experiments RT-PCR was used to convert RNA to cDNA and quantitative real-time PCR was used to assess AREG and EREG expression. Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls.
3.3 Discussion

Integrin α6β4 is a pro-tumorigenic molecule that directly contributes to a malignant phenotype through the activation of tumor-promoting genes. In this study, I demonstrated the dependence of the EGFR ligands, AREG and EREG, on signaling from integrin α6β4, and determine that the NFAT5 transcription factor and DNA demethylation are the driving force for their upregulation.

The EGFR pathway is dysregulated in a variety of cancers where it contributes to aggressive properties of cancer cells. This pathway is a target for multiple chemotherapeutics either by small molecule blockade of the tyrosine kinase activity of EGFR, or function blocking monoclonal antibodies [124]. Unfortunately, EGFR targeted therapies while effective, often result in therapeutic resistance. Accordingly, understanding how this pathway may be regulated in a context specific manner could provide insight into mechanisms of tumor progression and chemotherapeutic resistance. In this study, I showed that integrin α6β4 positively regulates gene expression of AREG and EREG. In support of this finding, using the TCGA dataset on pancreatic ductal adenocarcinoma, I found that AREG and EREG correlated with expression of integrinβ4 subunit, which is indicative of integrin α6β4 expression. This finding confirmed the physiological relevance of these studies in patients, and the impact of integrin α6β4 on these genes, providing mechanistic insight into aberrant regulation of the EGFR pathway in pancreatic cancer.

To confirm the impact of EGFR ligands on aggressive properties of pancreatic cancer cells, I examined the phenotypic effects of AREG and EREG on integrin α6β4 high expressing cells. I found that the EGFR ligands AREG and EREG are required for
HGF-mediated invasion and migration of pancreatic carcinoma cells. Unexpectedly, knockdown of each individual ligand resulted in a corresponding decrease in migration and invasion. This coordinated gene regulation would indicate that, while AREG and EREG have somewhat overlapping functions, they are not completely redundant in their ability to activate downstream signaling, and both seem to be necessary for these phenotypic effects. Notably, the contributions of AREG and EREG as pro-oncogenic factors in pancreatic cancer have previously been established [50, 125]. Signaling from AREG, specifically autocrine secretion, has been implicated in chemoresistance, evasion of apoptosis, self-sufficiency in growth signals, proliferation, angiogenesis, invasion and metastasis [48]. In fact, AREG secretion levels in pancreatic cancer patients are elevated to the point that this ligand has been identified as a potential biomarker, which is associated with a worse outcome [126]. It may be that high levels of AREG are needed because of its low affinity for the receptor compared to other ligands. EREG, for example, is found at much lower concentrations but has a broader specificity for ErbB receptors, and very high binding affinity, thus resulting in more potent signaling capacity [54]. A more comprehensive understanding of how AREG and EREG contribute to pancreatic cancer may provide insight into the driving mechanism of tumor invasion, metastasis, and chemoresistance.

Our group [34] and others [33, 127, 128] have demonstrated that integrin α6β4 can activate important tumor promoting transcription factors such as NFAT1, NFAT5, NFκB and AP-1. These factors, along with epigenetic alterations of specific genes [30], alter the transcriptome to facilitate an invasive phenotype. In this study, I demonstrated that AREG and EREG, which are established contributors of tumor progression [48,
are upregulated by the transcription factor NFAT5, as siRNA-mediated reduction of NFAT5 resulted in a subsequent decrease in AREG and EREG. S100A4 was the first identified target for NFAT5 in cancer, and S100A4 activation is mediated by integrin α6β4 [30]. This common mode of gene activation by integrin α6β4 implies that this may be a widespread manner of transcriptional activation of tumor-promoting genes in advanced cancers.

This study also demonstrated the ability of DNA demethylating agents such as 5-Aza-CdR to stably upregulate AREG and EREG. Importantly, changes in gene expression are also reflected by secretion of AREG, indicating that active EGFR signaling is likely taking place. We find that integrin α6β4 is either required for induction by or acting in a synergistic manner with 5-Aza-CdR as knocking down or blocking signaling by integrin α6β4 inhibits complete upregulation of AREG and EREG by 5-Aza-CdR. Interestingly, in Panc1-2G6 cells long term upregulation of AREG and EREG can be achieved following short term 5-Aza-CdR treatment and removal, demonstrating that stable epigenetic modifications have taken place. Genes that are indeed epigenetically repressed via DNA methylation and repressive histone marks require both DNA demethylation and histone acetylation in order to fully achieve gene expression [120]. Therefore, synergistic activation of AREG and EREG in Panc1-2G6 cells using DNMT inhibitor in combination with HDAC inhibitor would also indicate reversal of a true epigenetically repressed state. This was not seen in Panc1-3D7 cells, and short-term 5-Aza-CdR treatment and removal does not induce a high level of gene expression. Based on these findings I concluded that downstream of integrin α6β4 signaling, chromatin structure surrounding AREG and EREG shifts to a more permissive, open state allowing
transcription to take place. These conclusions are of importance considering that DNMT inhibitors such as azacitidine and decitabine are currently used chemotherapeutic agents for cancer, most often for the treatment myelodysplastic syndromes, acute myeloid leukemia and chronic myelomonocytic leukemia [129]. However, only about 50% of hematological patients show a positive response. Likewise, a variety of clinical trials in solid tumors only showed a positive response rate for 3-35% of patients treated either alone or in combination with epigenetic therapies [130]. These low success rates are in part due to the nonspecific nature of these drugs and unexpected activation of tumor promoting genes such as AREG and EREG. Identification of molecules contributing to epigenetic upregulation, such as integrin α6β4 may help better predict patient response.

Interestingly, this study expands on others that have examined the impact of the tumor microenvironment on epigenetic regulation. Work in squamous cell carcinoma and MDA-MB-231 breast cancer cells demonstrated that ECM content, cell-cell interactions, and 3D environment impact the methylation state of the E-cadherin promoter and this dynamic epigenetic plasticity helps drive EMT [131, 132]. These observations collectively solidify the role of the tumor microenvironment in regulating DNA methylation. Considering that signaling through integrin α6β4 is activated during and contributes to metastasis, the context dependent, dynamic DNA demethylation of pro-oncogenic molecules downstream of integrin α6β4 could be a major contributing mechanism to advanced carcinomas. Thus placing the tumor microenvironment at a unique nexus between the metastatic phenotype and tumor epigenetics. By understanding how tumor promoting molecules such as integrin α6β4 cooperate with epigenetic drugs
and target other pro-oncogenic molecules for DNA demethylation we may be able more effectively use these therapies, specifically in the case of advanced stage metastasis.
CHAPTER 4: INTEGRIN α6β4 ALTERS GENE SPECIFIC AND GENOME WIDE DEMETHYLATION PATTERNS

4.1 Introduction

Appropriate DNA methylation patterns are crucial for establishment and maintenance of gene expression in normal functioning tissues. Abnormal DNA methylation patterns are both found in and contribute to the development of cancer and progression to metastatic disease. Additionally, studies done examining methylation patterns from normal and diseased tissues have led to the hypothesis that DNA methylation events prior to mutations are the driving force in tumor development. An established understanding of how the epigenome contributes to cancer is critical for the future of effective cancer therapies.

4.1.1 Genome Wide changes in DNA Methylation and Metastasis

Cancer cell invasion and metastatic disease is ultimately what leads to death of patients, as genetic and epigenetic changes have taken place between primary tumor and secondary sites. Additionally, the systemic nature of metastases makes treatment difficult and resistance to chemotherapeutic agents very common [133]. The metastatic cascade is characterized by invasion of tumor cells from the primary site through the basement membrane and stroma, intravasation into the lymphatic or blood vessel system, survival during transport, extravasation to a secondary site, survival in the new environment, and reestablishment of proliferation [133]. Each step in this process is rate limiting and reorganization of the chromatin to alter transcriptional profiles is necessary to adjust expression of genes required for this process [61].

Investigations in breast cancer of metastatic-specific alterations in DNA methylation, have revealed unique methylation patterns in breast cancer subtypes and
when comparing metastatic vs. primary tumors [61, 134]. Fang et al. identified a panel of genes that were differentially methylated in breast cancer, or a breast CpG island methylator phenotype (B-CIMP). The presence of B-CIMP was characteristic of tumor metastatic potential, prognosis, and survival, and therefore provides a potential biomarker and prognostic factor for metastatic disease. Similarly, work in gastric cancer has shown that specific epigenetic alterations in a panel of genes can predict metastasis and overall patient survival [135]. Integrin α6β4 is a major contributor to metastasis as it senses and coordinates cellular response to the tumor microenvironment. This includes enhanced cellular proliferation, directly promoting filapodia and lamellipodia formation to drive cell motility, upregulation of the MMPs that break down ECM and activate pro-proteins such as AREG and EREG, activation of pro-oncogenic signaling networks, and transcriptional activation of a variety of oncogenes [11, 25, 136-141]. My study examined the impact of integrin α6β4 on DNA methylation, as identifying critical components of this process provide insight into how pro-metastatic molecules become upregulated during tumor progression.

4.1.2 DNA Methylation of Enhancers

Enhancers are transcriptional elements that promote gene activation by acting as a hub for multiple transcription factors to interact with the mediator complex and in turn recruit RNA polymerase II [142]. Additionally, a single enhancer can impact expression of multiple genes or gene clusters, and many enhancers can act on a single gene depending on the context [143]. However, unlike promoters, enhancers are independent of orientation or location, as they can be found long distances from the TSS, downstream of promoters, and within introns [121]. Of the known transcriptional elements, enhancers are the most dynamic [144]. Heintzman et al. showed that enhancers impact cell-type and
context specific gene expression patterns and could therefore be critical for cancer type specific, or even metastasis specific transcriptional patterns [144]. Importantly, establishing how enhancer specific activation is achieved is critical for understanding and targeting key epigenetic components in cancer progression.

One mechanism that impacts the ability of enhancers to function is DNA methylation. Contrary to the preceding dogma, work done by Young and colleagues demonstrated that DNA methylation of enhancers correlates better with transcription of associated genes than do promoters [145]. Additionally, DNA methylation of enhancer elements is more dynamic, allowing for situation and context specific alterations in methylation and their associated gene expression changes. My study identified alterations in DNA methylation mediated by integrin α6β4, in association with potential enhancer elements.
4.2 Results

4.2.1 Integrin α6β4 promotes loci specific DNA demethylation at enhancer elements of AREG and EREG

To define DNA demethylation changes that drive expression of AREG and EREG in response to integrin α6β4 signaling, genomic DNA was isolated from pancreatic cancer cells with either high (Panc1-3D7) or low (Panc1-2G6) integrin α6β4 expression. DNA was exposed to sodium bisulfite conversion followed by high-resolution next-generation whole genome sequencing (WGBS). Alignment to the reference genome was achieved using Bismark, a methylation caller and aligner specific for Bisulfite Sequencing projects. As illustrated, cells with high integrin α6β4 (Fig. 4.1A and 4.1B; bottom panels) have reduced DNA methylation within intronic regions of both EREG (Fig. 4.1A) and AREG (Fig 4.1B), confirming my hypothesis that integrin α6β4 drives site-specific DNA demethylation within these genes. Importantly, we also found differentially methylated loci in an AREG pseudogene, which lies directly downstream of AREG (Fig. 4.1C). When comparing AREG and AREG pseudogene, as seen in Figure 4.1B and 4.1C, both the sequence structure similarity as well as the potential for regulatory similarity are noted. The sequences of AREG and AREG pseudogene are nearly identical, achieving 99% homology when blasted against the reference genome (GRCH37). Since Bismark only reports unique matches during the mapping step, the multi-mapping scenario of AREG and its pseudogene made it difficult to investigate the methylation alternations in these two regions. The analysis was possible by masking AREG pseudogene and mapping AREG, and vice-versa for AREG pseudogene. I attempted to investigate this further by using bisulfite conversion with methylation specific PCR to confirm altered CpGs within this region. However, the sequence
similarity between these two regions and difficulty designing unique primers for bisulfite converted DNA made this analysis technically not feasible.

A super-enhancer lies between AREG and the AREG pseudogene [145] within the range capable of impacting transcription of both AREG and EREG. Considering that AREG and EREG expression is robustly decreased in response to JQ1 treatment, we examined DNA methylation of this super-enhancer. We found no significant differences in super-enhancer DNA methylation (data not shown), indicating that it is unlikely that DNA methylation of this element is the major driver for enhanced AREG and EREG gene expression. However, more extensive investigations of this region would be required to confirm that the super-enhancer is not critical for AREG and EREG expression in response to signaling from integrin α6β4.

Interestingly, regions that have the greatest differences in DNA methylation in both AREG and EREG as a result of integrin α6β4 signaling corresponded to areas enriched in H3K27Ac marks (Fig 4.1A, 4.1B), as annotated in the UCSC genome browser. H3K27Ac has been shown to correspond to active enhancer elements in the genome [146]. While the analysis examining H3K27Ac is not specific for our chosen cell type, it does mark potential sites for regulation by enhancers, and very tightly associates with changes in DNA methylation. Taken together, these data, along with the observation that BRD4 is required for AREG and EREG expression, suggest that DNA methylation of an enhancer element localized within the proximal promoters of AREG and EREG are driving their expression in response to signaling from integrin α6β4 in pancreatic cancer cell lines. A more in depth analysis of these regulatory regions will need to be performed to confirm this hypothesis.
Figure 4.1 Loci specific changes in DNA methylation in AREG and EREG genes downstream of integrin α6β4 signaling

(A-C) Genomic DNA from Panc1-2G6 (β4 low; upper panels) and Panc1-3D7 (high β4; lower panels) was processed for high-resolution methyl-seq by the NextGen Sequencing Core at the Norris Comprehensive Cancer Center. Samples were analyzed bioinformatically and percent methylation shown for EREG (A), AREG (B), and the AREG pseudogene (C).
4.2.2 Integrin α6β4 contributes to genome-wide DNA demethylation

In addition to investigating targeted effects of integrin α6β4 on DNA methylation, we examined genome-wide effects using the data generated by WGBS on the Illumina NextSeq. A total of 236,371 Differentially Methylated Loci (DML) (207,168 hypomethylated and 29,203 hypermethylated) were identified comparing Panc1-3D7 vs. Panc1-2G6. Figure 4.2A illustrates the percentage of hypermethylated and hypomethylated events per chromosome as a percent of the number of DMLs. Of the DMLs that were identified, 87.6% were hypomethylated and 12.4% were hypermethylated, thus indicating that integrin α6β4 dramatically shifts chromatin to a more hypomethylated state.

More detailed analysis of these data revealed that only 3.1% of these loci were located in promoter regions, 2.1% in exonic regions, 31.1% in intronic regions and 63.1% were in intergenic regions, or regions that have otherwise not been classified as mapped to reference genome GRCH37 (Fig 4.2B). A total of 13,889 differentially methylated regions (DMR) were identified. Among them, only about 4% were located in CpG islands, and 5% in CpG shores (Fig. 4.2C). Additionally, when comparing all cytosines located at CpG sites in the genome, the Panc1-3D7 cells had roughly 12% more demethylated loci than integrin α6β4 low cells.
Figure 4.2 Distribution of differentially methylated loci and regions in response to signaling from integrin α6β4

(A) Percent hypomethylation (gray) and hypermethylation (black) per chromosome when comparing Panc1-3D7 vs. Panc1-2G6. E) Location of DMLs across the genome. F) Percent of DMRs located in CpG islands and shores.
4.2.3 Integrin α6β4 signaling dramatically impacts global enhancer methylation

The data thus far suggests that AREG and EREG are regulated by DNA demethylation of enhancer elements. Therefore, we examined alterations in DNA methylation patterns that correspond to H3K27Ac genome-wide, a mark of active enhancers. We found that 40,609 CpGs associated with H3K27Ac were hypomethylated as opposed to only 13,679 CpGs hypermethylated (Fig 4.3A). These events correspond to 4930 genes that have alterations in methylation within enhancer elements (Fig 4.3A). As seen in Figure 4.3B, the majority of these altered DML are localized to intronic and intergenic regions. This observation is typical of enhancer elements, as most enhancers are part of non-coding regions of the genome [147].
Figure 4.3 Integrin α6β4 promotes hypomethylation at potential enhancer elements

(A) Panc1-3D7 (high integrin α6β4) were compared to Panc1-2G6 (low integrin α6β4) cell lines and changes in DNA methylation graphed (left bar). Hypermethylated loci are indicated in black and hypomethylation in grey. DML that aligned with potential H3K27Ac sites are indicated by the right bar and represent 23% of all DMLs, representing 4930 genes. (B) All DML that mapped to H3K27Ac sites were identified and location characterized.
4.2.4 Global DNA demethylation by integrin α6β4 requires DNA repair

To determine if the DNA methylation changes found in pancreatic cancer cells were consistent in other models for integrin α6β4 signaling, the triple negative breast cancer cell line BT549 was used to analyze DNA methylation changes in response to integrin α6β4 signaling. The parental cell line and BT549 stably expressing a non-targeting shRNA construct were used as the control integrin α6β4 null cells. In the experimental samples the integrin β4 subunit was stably transfected into BT549 cells and a colony chosen, or a pooled population that overexpresses integrin α6β4. Of note, BT549 cells lack integrin α6β4 and when the integrin β4 subunit is stably transfected, cells gain an enhanced ability to migrate, invade, and proliferate (data not shown). When comparing integrin α6β4 high vs. integrin α6β4 low cells we saw very similar results as compared to the pancreatic cancer cell lines. Roughly 250,000 CpGs were altered in response to integrin α6β4 expression, of which 90% were hypomethylated and 10% were hypermethylated (Fig 4.4, middle bar); thus indicating that integrin α6β4 globally impacts DNA methylation levels, shifting the genome to a hypomethylated state, in multiple models of cancer.

A third model was chosen to examine integrin α6β4 signaling on DNA methylation, a triple negative breast cancer cell line lacking integrin α6β4, Hs578T. Of note, transfection with the integrin β4 subunit in to Hs578T cells does not confer an enhanced ability to invade, as seen with the other two models. The currently accepted mechanism for how active DNA demethylation is achieved is through enzymatic removal by DNA repair proteins. Hs578T cells are deficient in their ability to repair DNA damage. When examining whole genome DNA methylation following stable overexpression of integrin α6β4, there were very few alterations in DNA methylation
patterns (Fig 4.4, right bar) via hyper or hypomethylation. This would indicate that DNA repair is required for alterations in DNA methylation mediated by integrin α6β4. This concept will be explored further in the following chapter.
Figure 4.4 Integrin α6β4 promotes DNA demethylation in Breast and Pancreatic Cancer Cell lines

Genomic DNA was isolated from pancreatic cancer cell lines (Panc1) and triple negative breast cancer cell lines (BT549, Hs578T). Hs578T cells are deficient in their ability to repair damaged DNA. Whole genome bisulfite sequencing was performed on all cell lines and DNA methylation levels compared in integrin α6β4 high vs. integrin α6β4 low cells. Black bars represent number of differentially methylated hypermethylation events, and green bars represented hypomethylated events.
4.3 Discussion

The analysis of genome-wide DNA methylation patterns revealed that integrin α6β4 dramatically reshapes the epigenetic landscape, shifting global DNA methylation patterns to a more hypomethylated state. Interestingly, studies in breast cancer revealed that genome-wide alterations in DNA methylation occur surprisingly early on in cancer progression and may act as a driver of tumorigenesis [148]. My study proposes an additional shift in DNA methylation patterns from a benign to a malignant state, when integrin α6β4 is activated. Similarly, during cancer cell invasion, Herman and colleagues found that the E-Cadherin promoter is differentially methylated [132]. Additionally, Lujambio et al found that methylation of a large number of microRNAs specifically contributes to metastasis by altering expression of oncogenic target genes [149]. These studies support my hypothesis that a specific epigenetic signature is associated with and promotes invasive cancer growth. Importantly, specific epigenetic signatures could be used to as a diagnostic marker of metastatic disease and a potential target for therapies.

Furthermore, this study confirms that changes in specific CpG methylation within the AREG and EREG genes occur in intronic regions that are not defined by the presence of CpG islands. These sites of altered DNA demethylation corresponded to known sites of H3K27Ac as identified by the ENCODE project in the UCSC genome browser. My data demonstrating that AREG and EREG expression is highly dependent on BRD4 activity, suggests that enhancer elements are necessary to drive gene expression. In support of this concept, previous work on S100A4 yielded similar results as specific changes that control gene expression reside in an enhancer element located in a CpG rich region rather than a CpG island [114]. Similar to our gene specific data, the majority of
hypomethylation events induced by integrin α6β4 are not localized to CpG islands or promoter regions; but were instead found in intronic and intergenic elements. In addition, 23% of these regions corresponded to potential sites of H3K27ac, which is indicative of enhancer location [146]. These data provide evidence that integrin α6β4 is impacting methylation of regulatory elements as opposed to direct activation through demethylation of promoters. These changes in DNA methylation are not surprising as hypomethylation of enhancer elements is tightly linked to overexpression of cancer promoting genes and gene profiles, when compared to promoter methylation [150, 151]. Additionally, studies have shown that tissue specific intergenic CpG island methylation correlates with sites of alternative promoters imbedded within genes [152]. Therefore, these data suggest that this shift in methylation patterns mediated by integrin α6β4 is indeed a mechanism driving gene expression and progression to a more malignant phenotype in pancreatic cancer cells. While other evidence exists to suggest that the tumor microenvironment can influence epigenetics [131, 153, 154], this study is the first to identify a specific mediator of the microenvironment, integrin α6β4, as a regulator of this process.
CHAPTER 5: AREG AND EREG EXPRESSION REQUIRE BASE EXCISION REPAIR DOWNSTREAM OF INTEGRIN α6β4

5.1 Introduction

Genes that are transcriptionally silent, such as those important for development, are traditionally characterized as having a repressive epigenetic state that compacts chromatin. These repressive epigenetic marks include non-acetylated histones, lysine methylation at H3K27 and H3K4 and cytosine methylation at CpGs [108]. Activation of gene expression during cancer progression constitutes loss of these repressive marks and a more dynamic chromatin state that may allow for situation specific gene repression or activation. Specifically, removal of the repressive methyl group at 5-mC, or DNA demethylation, is required for activation of gene expression.

5.1.1. DNA Demethylation

DNA demethylation can occur through a passive or an active process. Passive removal occurs spontaneously over several rounds of replication as a result of loss or mistakes by the primary maintenance methyltransferase, DNA methyltransferase 1 (DNMT1) [155]. Spontaneous DNA demethylation results in stochastic changes eventually diluting out the methyl groups through rounds of replication. Active DNA demethylation is more tightly regulated and proceeds through either direct removal of the methyl group by a DNA demethylase or indirect enzymatic removal by the Ten-Eleven Translocation methylcytosine dioxygenase (TET) proteins and DNA repair. However, strong evidence for a vertebrate DNA demethylase does not currently exist, and a mechanism for targeting by direct removal has not been identified. Moreover, the C-C bond found in 5-mC is difficult to break energetically, making active direct DNA demethylation unlikely [156]. Active indirect removal is the more probable mechanism
involved in upregulation of pro-tumorigenic genes. Importantly, two different DNA repair pathways have been implicated in active DNA demethylation, BER and NER [157, 158]. These pathways have partially overlapping functions in terms of damage repair and DNA demethylation. Consequently, it is still unknown under what conditions each pathway becomes activated to target specific CpG sites for DNA demethylation.

5.1.2. DNA Demethylation by Base Excision Repair

My study focused on DNA demethylation mediated by the BER pathway, which is described in detail below. Modification of 5-mC by TET has been identified as a crucial step in DNA demethylation as this protein recognizes specific 5-mC to be targeted for removal by DNA repair and conversion from 5-mC to 5-hydroxymethyl cytosine (5-hmC) [159]. 5-hmC can be further oxidized by the TET proteins to produce 5-carboxylcytosine (5-caC) and 5-formylcytosine (5-fC). These derivatives are found less often in the genome and while their complete function is still being characterized, it is clear that these modifications are playing a critical role in epigenetic regulation [160].

5-hmC and other oxidized 5-mC products are subsequently identified by the protein growth arrest and DNA damage inducible protein alpha (GADD45A). GADD45A activity impacts multiple cellular functions including cell cycle, apoptosis, cellular stress response, and most recently DNA demethylation [161]. Figure 5.1 summarizes how enzymatic, active DNA demethylation mediated by GADD45A is achieved based on my understanding of the current literature. GADD45A is responsible for recruitment of other repair factors to CpG sites for removal of methyl groups, and has been implicated as a necessary step in DNA demethylation by DNA repair as it provides the link between epigenetics and DNA repair [162, 163]. GADD45A can in turn recruit activation induced
cytidine deaminases such as the AID and APOBEC proteins [163]. AID is responsible for deaminating 5-hmC to 5-hydroxymethyl Uracil (5-hmU), in the process generating a G-U mismatch. This mismatch is identified and subsequently cleaved and removed by the DNA glycosylase, TDG. Methyl-binding protein 4 (MBD4) is also a DNA glycosylase that can contribute to active DNA demethylation. However, MBD4 does not require the activity of GADD45A or AID and can directly act on TET created derivatives of 5-mC. Notably, it has been demonstrated that GADD45A mediated DNA demethylation is dependent on TDG, as they occur in complex together where DNA demethylation is actively taking place [164]. This cleavage activates the normal functions of the BER pathway including cleavage of the DNA backbone by AP-endonuclease (APE1) and repair back to a non-methylated cytosine by X-ray Repair Cross Complementing Protein (XRCC1), Poly-ADP Ribose Polymerase (PARP1), DNA ligase, and DNA polymerase. Therefore, considering that AREG and EREG are activated by DNA demethylation, I chose to investigate the influence of DNA repair on transcription of AREG and EREG downstream of integrin α6β4.
Figure 5.1 GADD45A mediated DNA demethylation by Base Excision Repair
5.2 Results

5.2.1 DNA repair is required for transcription of AREG and EREG

Gemcitabine is a currently used chemotherapeutic that has multiple proposed mechanisms of action, one of which is inhibition of ribonucleotide reductase. This inhibition causes depletion of deoxynucleotide triphosphates necessary for DNA synthesis, and completion of DNA repair [165]. Interestingly, Gemcitabine has been shown to specifically inhibit GADD45A mediated gene activation via DNA demethylation and DNA repair [166]. Therefore, to investigate the role of GADD45A mediated DNA repair in expression of AREG and EREG, Panc1-2G6 and Panc1-3D7 cells were treated with 10 μM gemcitabine for 72 hours and their RNA harvested for qPCR. As demonstrated in Figure 5.2, AREG and EREG expression decreases by 63% and 90% respectively in cells with high integrin α6β4 in response to gemcitabine treatment. These data indicate that a functioning DNA repair pathway, possibly mediated by GADD45A is required to maintain expression of AREG and EREG.
Figure 5.2 DNA repair is required for expression of AREG and EREG in integrin α6β4 high expressing cells

Panc1-2G6 and Panc1-3D7 cells were treated for 72 hours with 10 μM gemcitabine. RNA was isolated and gene expression of AREG and EREG determined by qPCR. Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls.
5.2.2 GADD45A is the rate-limiting molecule in upregulation of AREG and EREG

GADD45A has been demonstrated to be the recognition step for DNA demethylation by both NER and BER, and is found in complex with molecules from each pathway [162, 164]. Therefore, to confirm the contribution of this molecule to AREG and EREG expression, I specifically modulated GADD45A in pancreatic cancer cells. I reduced GADD45A expression in pancreatic cancer cells by using siRNA to knockdown or overexpressed GADD45A by adenoviral infection. Efficient knockdown or overexpression was confirmed by qPCR (Fig. 5.3C, F). As seen in Fig. 5.3A & 5.3B, knockdown of GADD45A resulted in decreased expression of AREG and EREG in cells with both integrin α6β4 low and high expression. Similarly, overexpression of GADD45A resulted in a further increase in AREG and EREG expression, only when integrin α6β4 is high (Fig 5.4D, E). Taken together these data indicated that GADD45A is a rate-limiting step in gene activation of AREG and EREG and requires integrin α6β4, as induction was not achieved in Panc1-2G6 cells. Based on these data, I concluded that GADD45A mediated DNA demethylation proceeds through the BER pathway in my system and tested this hypothesis further.
Figure 5.3 GADD45A is necessary and required for expression of AREG and EREG

Changes in AREG and EREG expression downstream of overexpression or knockdown of GADD45A were measured by qPCR in Panc1-2G6 (A & D) and Panc1-3D7 (B & E). Transient knockdown of GADD45A was achieved using electroporation and specific siRNA (C). Adenovirus was used to overexpress GADD45A in Panc1-2G6 and Panc1-3D7 cells (F). Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls.
5.2.3 **TET1 mediates DNA demethylation of AREG and EREG**

Discovery of TET proteins allowed for a possible mechanism for active DNA demethylation, as the TETs are solely responsible for conversion of 5-mC to 5-hmC, 5-fC and 5-caC in mammalian DNA [167]. 5-hmC is the key component for active DNA demethylation by DNA repair, as it provides a substrate for further processing to a cytosine by the DNA glycosylases and BER. Three TET proteins have currently been identified with enzymatic activity for 5-mC. Therefore, using specific shRNAs, I depleted TET1 in cells with high integrin α6β4 expression (Fig. 5.4A). As demonstrated in Figure 5.4B, AREG and EREG expression is robustly decreased following a 70% reduction in TET1.
Figure 5.4 TET1 is required for expression of AREG and EREG

(A) Transient knockdown of TET1 was achieved using lentiviral infection, followed by puromycin selection in Panc1-3D7 cells, and reduced expression confirmed by qPCR. (B) Changes in AREG and EREG expression were measured by qPCR. Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls.
5.2.4 Integrin α6β4 enhances nuclear localization of TDG to promote DNA demethylation

DNA glycosylases TDG and MBD4 have both been implicated as required molecules for active DNA demethylation by DNA repair, in different contexts. Specifically, TDG has been found in complex with AID and GADD45A during the process of active DNA demethylation [157]. Furthermore, genome-wide analysis reveals that depletion of TDG results in accumulation of TET induced 5-mC derivatives [168]. MBD4 is unique in that it can directly excise 5-hmC without the assistance of other molecules such as GADD45A [169, 170]. Therefore, I stably knocked down either MBD4 or TDG to assess the contributions of known DNA glycosylases to AREG and EREG expression. Figure 5.5A, demonstrates that stable knockdown of TDG is achieved in Panc1-3D7 cells, as visualized by nuclear fractionation and western blot analysis. Additionally, it can also be appreciated in Figure 5.5A that there is much higher nuclear expression of TDG when integrin α6β4 is high as compared to low expressing cells (Panc1-2G6). This would indicate that integrin α6β4 causes preferential nuclear localization of TDG, potentially for enhanced DNA demethylation or DNA repair, which will need to be investigated further. As seen in Figure 5.5B, this stable knockdown of TDG resulted in marked downregulation of AREG and EREG in integrin α6β4 high expressing cells, indicating that TDG is necessary to maintain expression of these genes when integrin α6β4 is upregulated. Interestingly, knockdown of MBD4 in both Panc1-2G6 (Fig. 5.5C) and Panc1-3D7 (Fig 5.5E) cells had no effect on expression of AREG and EREG (Fig. 5.5 D & 5.5F); thus indicating that DNA demethylation of AREG and EREG is specific for TDG mediated DNA repair.
Figure 5.5 TDG is preferentially expressed in the nucleus downstream of integrin \( \alpha 6\beta 4 \) and is necessary and specific for AREG and EREG expression.

Stable knockdown of TDG (A) and MBD4 (C & E) was achieved using lentiviral infection, followed by puromycin selection in Panc-2G6 cells for MBD4 and Panc1-3D7 cells for TDG and MBD4, and reduced expression confirmed. Western blot was used to examine nuclear protein levels of TDG in Panc1-2G6 cells, and Panc1-3D7 cells stably expressing a NT vector or TDG shRNA. Changes in AREG and EREG expression were
measured by qPCR following stable knockdown of TDG (B) and MBD4 (D & F). Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls.
5.2.5 PARP-1 is a mediator of integrin α6β4 upregulation of AREG and EREG

PARP-1, a required molecule for BER has been shown to modulate chromatin patterns including DNA methylation [171]. PARP-1 has been implicated in genome-wide and locus specific active DNA demethylation in part through epigenetic regulation of TET1 [172]. Using a PARP-1 inhibitor, DPQ, I saw a dramatic decrease in AREG and EREG expression in high integrin α6β4 expressing cells (Panc1-3D7; Fig. 5.6B). However, in cells with low integrin α6β4, expression of AREG and EREG was relatively unaffected by PARP-1 inhibition (Fig. 5.6A), indicating that PARP-1 is a mediator of AREG and EREG transcriptional induction regulated by integrin α6β4.
Figure 5.6 PARP-1 activity regulates AREG and EREG expression mediated by integrin α6β4

Panc1-2G6 (A) and Panc1-3D7 (B) cells were treated with 1 or 10µM DPQ for three days to inhibit PARP-1 function. Gene expression of AREG and EREG were measured by qPCR. Data depicted here are representative of at least three different experiments and represent the mean +/- standard deviation. Statistical significance was calculated using a one-tailed t-test in which * denotes P<0.05 as compared to controls.
5.2.6 NER is not required for expression of AREG and EREG

Since my data indicate that AREG and EREG DNA demethylation is an active process, and considering that both BER and NER have been implicated as mediators of DNA demethylation, I chose to examine the NER pathway. The NER pathway, including the Xeroderma Pigmentosum proteins, XPA, XPG, and XPF, have been implicated in active DNA demethylation by DNA repair [158, 173]. To test if NER is required to maintain AREG and EREG expression, I targeted molecules critical for and specific to the NER pathway and examined their impact on AREG and EREG expression. I hypothesized that manipulation of NER proteins would not impact AREG and EREG expression, as NER is responsible for repairing helix-distorting lesions on a stretch of DNA. This is different from BER, which repairs single nucleotide mismatches.

XPA is a critical component of the NER system as it is recruited to sites of DNA damage where it allows for appropriate assembly of the NER machinery specifically proteins required for incision [174]. When knockdown of XPA (Fig. 5.7A) was achieved, transcription of AREG and EREG in both Panc-2G6 (low α6β4; Fig. 5.7B) and Panc-3D7 (high α6β4; Fig. 5.7C) remained unaffected. Additionally, XPG and XPF are both crucial for completion of NER as they are responsible for incision at the junction of single and double stranded DNA at the 5’ and 3’ ends, respectively, allowing for subsequent removal of the DNA fragment containing the helix distorting lesion [175, 176]. Interestingly, XPF and XPG have the ability to promote gene expression through chromatin looping and DNA demethylation [173]. I hypothesized that if NER were critical for AREG and EREG expression, then completion of this incision process would be vital for the complete removal of repressive methyl marks. Using specific shRNAs I knocked down the Excision Repair Cross Complementation Group 4 (ERCC4) gene
(XPF protein; Fig. 5.7D) and ERCC5 (XPG; Fig. 5.7G) in pancreatic cancer cell lines, and demonstrated that effective knockdown of NER genes had relatively little or no effect on AREG and EREG expression (Fig 5.7E-F, I-J). Taken together these data indicate that NER is not required to maintain expression of AREG or EREG, regardless of expression of integrin α6β4. Therefore, I concluded that integrin α6β4 promotes gene specific upregulation of AREG and EREG through a BER mediated process.
Figure 5.7 NER is not required for expression of AREG and EREG

Using lentiviral transfection stable knockdown of XPA (A), ERCC4 (D), and ERCC5 (G) was achieved in Panc1-2G6 (low α6β4) and Panc1-3D7 (high α6β4) cells as confirmed by qPCR. AREG and EREG expression was examined following knockdown in cells with both low α6β4 (B, E, H) and high α6β4 (C, F, J) expression. Data depicted are representative of at least three different experiments and represent the mean +/- standard deviation.
5.2.7 Integrin α6β4 promotes DNA repair of oxidative stress and UV-induced DNA damage

The data to this point indicates that integrin α6β4 utilizes DNA repair mechanisms to maintain a hypomethylated state in the AREG and EREG promoters. I rationalized that if integrin α6β4 is using the BER pathway to activate specific genes, integrin α6β4 may also enhance DNA repair in response to DNA damage. Accordingly, I induced oxidative damage, which would subsequently be repaired by the BER pathway, by exposing cells to 500 μM H₂O₂ over seven days and measured the number of viable cells each day via MTT assay. I observed a modest decrease in cell number with high integrin α6β4 (Panc1-3D7); however, increase in cell number was nearly abolished in cells with low integrin α6β4 (Panc1-2G6) in response to H₂O₂ treatment. This lack of cellular proliferation in response to H₂O₂ treatment indicated a decreased ability to survive and tolerate insult induced by oxidative stress (Fig 5.8A). To measure DNA repair more directly, and to synchronize the repair process, I examined NER dependent DNA repair by exposing cells to 30 J/m² UV light and measuring resolution of 6-4 photoproducts over time. As illustrated in Figure 5.8B, cells with high integrin α6β4 resolve UV induced lesions more rapidly than cells with low integrin α6β4, with a difference in half-life of about 1 hour. Taken together, this indicates that integrin α6β4 can both utilize DNA repair, and enhance the ability of cells to respond to, repair, and survive in the presence of DNA damage.
Figure 5.8 Integrin α6β4 promotes DNA repair and cell survival in response to DNA damage

(A) Cells were treated with 500 μM H₂O₂ in fresh media daily for 7 days. Each day cell proliferation was measured by MTT colorimetric assay. (B) Cells were exposed to 30 J/m² UV light and DNA isolated at indicated time points. Slot blot assay was performed using antibody for 6-4 photoproducts and percent repair compared to damage achieved immediately after exposure (0 Hr).
5.3 Discussion

Mounting evidence places the BER pathway as the most common, and context dependent mediator for active DNA demethylation [157, 168]. My data support this concept, as I have demonstrated that modulation of multiple components of the BER pathway, including GADD45A, TET1, TDG, and PARP-1, impact transcriptional upregulation of AREG and EREG. Additionally, the confirmation that AREG and EREG enhancers become actively demethylated downstream of integrin α6β4, support active DNA demethylation by DNA repair as the mechanism for transcriptional upregulation by integrin α6β4. More specifically, GADD45A acts as a rate-limiting step in the activation of AREG and EREG and in accordance with the literature, is the coordinating molecule for specific DNA demethylation by BER. Interestingly, one study demonstrated that GADD45A does not promote DNA demethylation [177]. However, Pfeifer and colleagues examined genome-wide DNA demethylation in oocytes and zygotes and this broad scale type of regulation seems unlikely for a single molecule [177]. In my study, I examine a very specific locus, and foresee GADD45A as a context specific activator of gene expression.

Importantly, I show that TDG specifically, and not MBD4, is required for gene expression of AREG and EREG. I also show that recruitment of TDG to the nucleus is amplified in cells with high integrin α6β4 expression, suggesting that integrin α6β4 coordinates steps in this pathway, potentially through nuclear recruitment or specific targeting of repair factors. Interestingly, I also examined gene expression levels of DNA repair proteins using Qiagen’s DNA damage response qPCR gene array (Appendix B). I found that integrin α6β4 does not impact transcription of DNA repair proteins when
comparing Panc1-3D7 to Panc1-2G6. This finding supports the hypothesis that integrin α6β4 utilizes the DNA repair pathway as opposed to directly modulating transcription of the DNA repair components. These data implicate integrin α6β4 as a critical amplifier of DNA repair mediated DNA demethylation, identifying a novel mode of transcriptional upregulation in response to the integrin.

Finally, I found that not only can integrin α6β4 utilize BER to promote transcriptional upregulation, this integrin also enhances the ability of pancreatic cancer cells to respond to and survive in the presence of DNA damage, through both the BER and NER pathways. These data support previous studies that demonstrate that tissue architecture mediated by integrin α6β4 promotes resolution of double strand breaks [178]. Taken together these studies demonstrate that integrin α6β4 contributes to a multitude of DNA repair pathways, and may be a key component for connecting the extracellular environment with DNA repair mediated nuclear events. These findings have major implications considering that the majority of chemotherapeutics currently utilized either cause DNA damage or inhibit DNA repair. A better understanding of how integrin α6β4 promotes the DNA damage response may assist us in choosing the most effective therapies for cancer patients. Therefore, considering the widespread impact that DNA repair has on both DNA methylation and cancer progression, I have established integrin α6β4 as a key mediator of these tumor-promoting pathways.
CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

Integrin α6β4 is as a mediator of pro-tumorigenic events in many types of cancer including breast, bladder, cervical, head and neck, and pancreas [179]. Additionally, many of the downstream signaling pathways that contribute to integrin α6β4 mediated invasive properties have been well established. However, aside from studies exploring transcription factors such as the NFATs, mechanisms for how integrin α6β4 mediates changes in transcriptional profiles have not been explored. In this study, I demonstrated that integrin α6β4 contributes to an invasive phenotype of pancreatic cancer cells by dramatically upregulating the EGFR ligands AREG and EREG. I found that this upregulation is the result of signaling to the NFAT5 transcription factor and active DNA demethylation. Interestingly, this DNA demethylation occurred at putative enhancer elements in the first intronic region of AREG and EREG. Likewise, using WGBS my study revealed that DNA demethylation mediated by integrin α6β4 was not confined to site specific changes as seen with AREG and EREG, but promoted genome-wide DNA demethylation, a common theme seen throughout cancer progression. Importantly, this observation was also manifested in breast cancer cells, but was completely abolished in a cell line that lacked the ability to respond to DNA damage. These observations indicated that widespread epigenetic regulation mediated by integrin α6β4 occurs in breast and pancreatic cancer, two cancers highly dependent on integrin α6β4 signaling [16, 24, 30, 31]. Therefore, it is reasonable to conclude that DNA demethylation is a common mechanism for integrin α6β4’s contribution to an invasive phenotype in cancer cells.

The loss of alterations in DNA methylation without an intact DNA repair pathway provided a plausible mechanism of integrin α6β4 mediated epigenetic events. A thorough
examination of the impacts of DNA repair on expression of AREG and EREG revealed a dependence on the BER signaling network to promote gene expression. This upregulation of AREG and EREG mediated by integrin α6β4 included the molecules TET1, GADD45A, PARP1, and TDG. My evidence suggested that this was achieved by specific targeting of indicated repair molecules such as TDG to the nucleus. Therefore, I concluded that gene specific DNA demethylation, downstream of integrin α6β4 signaling, is an active process requiring the BER pathway. Additionally, I demonstrated that integrin α6β4 enhances the ability of cancer cells to survive in the presence of oxidative damage and repair UV induced DNA damage. These findings have major implications for cancer treatment. The majority of currently used therapies either cause DNA damage or inhibit DNA repair, and therefore integrin α6β4 could play a role in chemotherapeutic resistance or patient response.

My study thoroughly explores the dependence of AREG and EREG on DNA demethylation by integrin α6β4. However, to fully elucidate the contributions of integrin signaling to the metastatic transcriptome, a more comprehensive approach is necessary. Therefore, it will be essential to couple whole genome methylation analysis with whole genome RNA-Seq, which is a high throughput method for measuring gene expression, where mRNA is isolated, converted to cDNA and whole genome sequencing performed. While we can draw conclusions from regions of DNA that become demethylated in response to signaling from integrin α6β4, it is critical to compare this information with transcriptomic analysis to identify other gene targets that may be similarly regulated. By combining these two methods, we can determine if promoter DNA demethylation drives gene expression or if changes in more discrete regions, such as non-coding regions, are
required as seen with AREG and EREG. This study can be expanded further by identifying common mechanisms for gene activation within the breast and pancreatic cancer data sets. These common mechanisms for transcriptional regulation by DNA demethylation can potentially be identified by exploring shared patterns of altered DNA methylation. Additionally, by mining data from different cancer types generated from WGBS and RNA-Seq, common signaling pathways that may be upregulated due to DNA demethylation could be identified. These data would be critical for establishing common mechanisms mediated by integrin α6β4 that contribute to cancer progression and tumorigenesis.

Interestingly, a large proportion (~23%) of the changes in DNA methylation that were identified reside in potential enhancer elements, as mapped to acetylation of histone H3 at lysine 27 from the Encyclopedia of DNA Elements (ENCODE) project and visualized on the UCSC genome browser [180]. However, as previously described epigenetic modifications of enhancer elements are dynamic and cell type specific [144]. Pancreatic cancer cell lines were not represented by ENCODE, and therefore may not be an accurate representation for mapping acetylation patterns. Therefore, to validate the findings that integrin α6β4 impacts enhancers, chromatin immunoprecipitation and sequencing (ChIP-Seq) will need to be performed for the active enhancer marks H3K27Ac and H3K4me1, in cell lines with variable expression of integrin α6β4 and compared to marks of DNA demethylation. I expect that alignment of DNA methylation with matched ChIP-Seq data will yield even higher numbers of overlapping differentially methylated loci with active enhancer elements in integrin α6β4 high expressing cell lines. The hypothesis that integrin α6β4 impacts enhancer elements is supported by our
investigations of AREG and EREG in pancreas, and S100A4 in breast as DML aligned perfectly with potential enhancers identified by ENCODE [30]. There is also the likelihood that genes regulated by integrin α6β4 are, at least in part, regulated by super-enhancers. This idea is supported by the finding that AREG and EREG were associated with super-enhancer methylation in multiple myeloma and treatment with low dose BRD4 inhibitor, JQ1, dramatically reduced gene expression when integrin α6β4 signaling was active [145]. Utilizing ChIP-Seq for BRD4 and the Mediator complex would provide a comprehensive analysis of activation of enhancers and super-enhancers by integrin α6β4, as BRD4 and Mediator occupancy is much higher in super-enhancers [181]. Super-enhancers are especially intriguing in cancer because a single super-enhancer can regulate entire transcriptional profiles that can mediate cell identity and tumorigenesis [145, 182]. Identification of integrin α6β4 as a direct activator of enhancers and potentially super-enhancers will have far reaching implications as these regulatory elements control gene expression profiles relevant to cancer, including the Myc oncogene [145].

Enhancer elements regulate gene expression through recruitment of transcription factors and the mediator complex, often times from long distances [150]. This transcriptional regulation comes as the result of chromatin looping, which brings together enhancers and promoters, and is dependent on CCCTC-binding factor (CTCF) binding at insulator elements [183]. Interestingly, DNA methylation at CTCF binding sites reduces the affinity for CTCF binding and therefore decreases chromatin looping, thus reducing potential interactions between promoters, enhancers, transcription factors, and epigenetic modifiers [184]. Further analysis of our WGBS data revealed 6757 DML, of 259,980
CTCF binding sites that contain at least 1 CpG site as identified by ENCODE. However, DNA methylation is not the only factor impacting CTCF binding. Therefore, investigating both DNA methylation and CTCF binding ability, downstream of integrin α6β4 signaling, by ChIP-seq, will provide a more comprehensive view of DNA demethylation induced transcriptional regulation. Also, to address the impact of integrin α6β4 on chromatin looping, chromosome confirmation capture (3C) methods could be performed. 3C allows for identification of regions of chromatin that are connected by large protein complexes, such as CTCF, and therefore determine chromatin regions that are in close proximity. This technique is of interest to my study because of the broad scale impact of integrin α6β4 on the epigenome. 3C will also allow us to identify what enhancer elements are in close proximity to integrin α6β4 regulated genes such as AREG and EREG. I expect that integrin α6β4 will have broad impacts on the epigenome, including chromatin looping and enhancer interactions. Therefore, if enhancers or CTCF binding sites are indeed a common target of DNA demethylation by integrin α6β, these alterations in DNA demethylation may have much broader reaching impacts on transcription than were originally anticipated. These findings would therefore have the potential to identify integrin α6β4 as a critical epigenetic modifier specific for metastatic growth. Broad reaching changes in the epigenome mediated by a sensor of the tumor microenvironment, integrin α6β4, could have major implications for targeting advanced stage disease, and therefore patient mortality.

Expanding on the requirement of effective DNA damage repair to achieve DNA demethylation downstream of integrin α6β4 signaling, my data revealed that AREG and EREG expression is dependent on an intact BER signaling network. To determine if
integrin α6β4 regulated gene expression of DNA repair molecules. I examined gene expression levels by qPCR array and found no significant alterations in gene expression mediated by integrin α6β4 (Appendix B). This lack of transcriptional regulation on DNA repair proteins is surprising when considering the broad impact that integrin α6β4 has on gene expression and its utilization of DNA repair to activate DNA demethylation. Therefore, I hypothesize that integrin α6β4 is contributing to DNA demethylation by targeting the DNA repair molecules to specific regions for demethylation. This concept could be examined first at the AREG and EREG enhancers by looking for occupancy of DNA repair proteins by ChIP analysis, within their respective regions of altered DNA methylation. If continued signaling were required to maintain a hypomethylated state then I would expect that at least TET, TDG, and GADD45A would reside in some of these sites for DNA demethylation. Targeted localization of DNA repair proteins by integrin α6β4 could be expanded further, as I would anticipate genome-wide occupancy downstream of integrin α6β4, considering the broad impact integrin α6β4 has on DNA demethylation. This hypothesis can be tested by utilizing cellular fractionation experiments, where proteins are isolated from the cytoplasm, nucleus, and chromatin and western blot analysis used to detect protein levels. Additionally, ChIP-Seq analysis for DNA repair proteins downstream of integrin α6β4 would determine if integrin α6β4 upregulated genes are indeed targets for these DNA repair complexes. Coupling these data with RNA-Seq and WGBS will provide confirmation that targeting of the BER machinery is indeed what drives expression of integrin α6β4 upregulated genes. I expect that a proportion of these genes will indeed align with altered sites in DNA methylation. However, because other DNA repair pathways have been implicated in DNA
demethylation, it is likely that genes are regulated differently in response to different situations or stimuli.

In this study, I showed that knockdown of TET1 resulted in a corresponding decrease in AREG and EREG expression. However, this decrease was not always consistent. Importantly, three TET proteins have currently been identified, TET1, TET2, and TET3, all of which have also been implicated in DNA demethylation, as they too have enzymatic activity towards 5-mC [185]. However, how the TET family of proteins differs in their substrate specificity has not been fully elucidated, specifically in cancer. We do know that TET proteins are commonly mutated in blood-derived cancers and less often in solid tumors, but evidence suggests that these mutations can indeed act as drivers of tumor progression [186]. Additionally, a decrease in expression of TET proteins, and subsequent 5-hmC reductions, has been implicated in tumor growth and metastasis [187], indicating that regulation of these proteins may offer a potential explanation for integrin α6β4 mediated DNA demethylation. In my system, expression levels of the different TET family members will need to be elucidated, in order to more completely understand how integrin α6β4 impacts the DNA demethylation cascade.

While levels of TET proteins are certainly important, the field is just beginning to understand how 5-mC derivatives are localized across the genome and how they contribute to cancer. 5-hmC has been preferentially found at enhancers and gene body elements in embryonic stem cells [188-190]. Considering that about 23% of the DML found in response to integrin α6β4 are located at potential enhancer elements, 5-hmC may therefore be of importance for integrin α6β4 facilitated DNA methylation and DNA demethylation. Importantly, bisulfite sequencing cannot distinguish between 5-mC and 5-
hmC, and 5-fC and 5-caC appear as unmethylated cytosines. This is an important observation, as my study fails to accurately capture dynamic modifications of 5-mC taking place in response to integrin α6β4. Fortunately, with the recent development of state of art techniques to discriminate between 5-mC derivatives. Use of antibody based sequencing methods to specifically test 5-hmC, 5-fC, and 5-caC localization across the genome will help to uncover the interplay between such DNA demethylation reactions. Lastly, knockdown of the individual TET molecules by shRNA and assessment of methylation patterns by WGBS downstream of integrin α6β4 may help to reveal which of these proteins is indeed responsible for DNA demethylation downstream of integrin α6β4 signaling. The current literature demonstrates the importance of all three TET proteins, but how they differ in their activation of gene expression is still unknown. We may be able to unveil a context dependent targeting mechanism of the TET proteins if integrin α6β4 mediated DNA demethylation proceeds through a specific TET, or targets DNA demethylation to a specific region through the TET proteins.

Lastly, the TET proteins and DNA glycosylases have an interdependent relationship, as cooperativity between these molecules is required for active DNA demethylation by DNA repair [164, 168, 191, 192]. Interestingly, TDG has a preference for binding and removal of 5-caC and 5-fC but has no intrinsic enzymatic activity towards or ability to bind 5-hmC [191]. TDG’s preference for these substrates supports my data that GADD45A is necessary for removal of 5-mC, as it must act as the intermediate step between TET and TDG [162, 164]. Additionally, this would suggest that the demethylation reaction of AREG and EREG can proceed through all three 5-mC intermediates, as TDG can rapidly remove 5-fC and 5-caC without the activity of
GADD45A [192]. This reliance of TDG on GADD45A for removal of 5-hmC explains why other DNA glycosylases may also be required as MBD4 can directly act on 5-hmC, and would therefore be a more efficient process for removal [163, 169]. I did test the dependence of AREG and EREG on MBD4 and found no change in gene expression, thus supporting my hypothesis that GADD45A is required. Considering that the TETs are responsible for initiating the process of DNA demethylation and the newly found importance of 5-mC derivatives in cancer, it is possible that signaling to these molecules could indeed be how gene specific DNA demethylation, and subsequent gene activation is achieved in response to integrin α6β4 signaling [159, 167, 186, 188, 191].

The majority of investigations examining the role of DNA repair on DNA methylation have done so in the context of genome wide reprogramming or by using in vitro reporter systems [163, 193, 194]. My study, demonstrating the dependence of AREG and EREG on the BER pathway in response to a specific stimulus, integrin α6β4, is unique in that it assessed context dependent DNA demethylation by DNA repair in pancreatic cancer cell lines. A previous study done in MDA-MB-231 breast cancer cells examined the pS2 promoter in response to Estrogen Receptor alpha signaling and revealed a dual role for the de novo methyltransferases DNMT3a/3b, during cyclical methylation and demethylation following stimulation with estradiol [195]. Expectedly, during a repressive state DNMT3a/3b acted in cooperation with DNMT1 to cause gene methylation, and therefore silencing [196]. However, during demethylation, DNMT3a/3b and TDG were coordinately recruited to CpG sites where the methyltransferases acted as a deaminating agent at 5-mC similar to the AID and APOBEC proteins. This deamination in turn recruited the remainder of the BER complex of proteins including PARP-1,
resulting in active DNA demethylation. This study is particularly interesting considering that AID, the most common mediator of cytosine deamination [197], is not detectable in any of the pancreatic cancer cell lines that I examined (data not shown). These findings that DNMT3a/3b can act as the deaminating agent, may provide an explanation for the missing link in my study for how rapid, context dependent DNA demethylation could be achieved. This study is also pertinent to my work with the finding that integrin α6β4 recruits TDG to the nucleus, and work done by Yun et al. demonstrating that EREG is epigenetically silenced by DNA methylation in gastric cancer by DNMT3b [110]. Therefore, this switch in repressive and activating functions mediated by TDG and DNMT3a/3b could be a potential mechanism for activation of AREG and EREG in metastatic cancer cells. Further analysis of the dependence of AREG and EREG on DNMT3a/3b by knockdown and overexpression experiments, as well as ChIP analysis examining TDG and DNMT3a/3b localization to promoter regions, downstream of integrin α6β4 would be required to confirm this hypothesis.

In conclusion, this study examines a very specific sensor of the tumor microenvironment, integrin α6β4, and provides an exciting new role for this molecule in promoting tumor progression. The data presented in this dissertation offer a novel mechanism for the upregulation of tumor promoting genes, alterations in the epigenome, and utilization of DNA repair, and taken together places integrin α6β4 as a major regulator of cancer epigenetics. These findings have far reaching impacts on our understanding of pancreatic carcinoma. Further analysis of integrin α6β4’s role in these processes could yield a broad understanding for how the tumor microenvironment impacts gene regulation in cancer.
### APPENDICES

#### Appendix: List of Acronyms and Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>3C</td>
<td>Chromatin Conformation Capture</td>
</tr>
<tr>
<td>5-Aza-CdR</td>
<td>5-Aza-2’-Deoxycytidine</td>
</tr>
<tr>
<td>5-caC</td>
<td>5-Carboxylcytosine</td>
</tr>
<tr>
<td>5-fC</td>
<td>5-Formylcytosine</td>
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<td>5-hmC</td>
<td>5-hydroxymethylcytosine</td>
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<td>5-mC</td>
<td>5-methylcytosine</td>
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<td>6-4PP</td>
<td>6-4 Photoproducts</td>
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<td>ADAM</td>
<td>Disintegrin and metalloproteinase domain-containing protein</td>
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<tr>
<td>AID</td>
<td>Activation Induced Cytidine Deaminase</td>
</tr>
<tr>
<td>APE</td>
<td>Apurinic/Apyrimidinic Endonuclease</td>
</tr>
<tr>
<td>APOBEC</td>
<td>Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like</td>
</tr>
<tr>
<td>AREG</td>
<td>Amphiregulin</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>B-CIMP</td>
<td>Breast – CpG Island Methylator Phenotype</td>
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<td>BER</td>
<td>Base Excision Repair</td>
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<tr>
<td>bp</td>
<td>Base Pairs</td>
</tr>
<tr>
<td>BRD4</td>
<td>Bromodomain-containing protein 4</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>ChIP-Seq</td>
<td>Chromatin Immunoprecipitation and Sequencing</td>
</tr>
<tr>
<td>CTCF</td>
<td>CCCTC-Binding Factor</td>
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<tr>
<td>DAC</td>
<td>5-Aza-2’Deoxycytidine</td>
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<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
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<td>DML</td>
<td>Differentially Methylated Loci</td>
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<td>DMR</td>
<td>Differentially Methylated Region</td>
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<td>DNMT</td>
<td>DNA methyltransferase</td>
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<td>DPQ</td>
<td>5-(4-Piperidin-1-ylbutoxy)-3,4-dihydro-2H-isoquinolin-1-one</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
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<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
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<tr>
<td>ENCODE</td>
<td>Encyclopedia of DNA Elements</td>
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<td>EREG</td>
<td>Epiregulin</td>
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<tr>
<td>GADD45A</td>
<td>Growth Arrest and DNA Damage Inducible Protein Alpha</td>
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<td>HAT</td>
<td>Histone Acetyltransferases</td>
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<td>HDAC</td>
<td>Histone Deacetylases</td>
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<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<td>HR</td>
<td>Homologous Recombination</td>
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<td>ITGB4</td>
<td>Integrin Beta 4</td>
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<td>MBD4</td>
<td>Methyl-binding Protein 4</td>
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<td>Description</td>
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<tr>
<td>MET</td>
<td>Mesenchymal Epithelial Transition Factor</td>
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<td>MMP</td>
<td>Matrix Metalloproteinase</td>
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<tr>
<td>MTA</td>
<td>Metastasis-Associated Gene</td>
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<td>MTT</td>
<td>3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide</td>
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<tr>
<td>NER</td>
<td>Nucleotide Excision Repair</td>
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<td>NFAT</td>
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<td>NHEJ</td>
<td>Non-homologous End Joining</td>
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<td>NuRD</td>
<td>Nucleosome Remodeling Deacetylase</td>
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<td>PARP</td>
<td>Poly ADP Ribose Polymerase</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>qPCR</td>
<td>Quantitative Real-time PCR</td>
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<tr>
<td>RNA-Seq</td>
<td>RNA Sequencing (Whole Transcriptome Shotgun Sequencing)</td>
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<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription PCR</td>
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<td>SAM</td>
<td>S-adenosylmethionine</td>
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<td>shRNA</td>
<td>Short Hairpin RNA</td>
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<tr>
<td>TACE</td>
<td>Tumor necrosis factor α converting enzyme</td>
</tr>
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<td>TBST</td>
<td>Tris Buffered Saline with Tween-20</td>
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<td>TCGA</td>
<td>The Cancer Genome Atlas</td>
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<td>TDG</td>
<td>Thymine DNA Glycosylase</td>
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<tr>
<td>TET</td>
<td>Ten Eleven Translocase Methylcytosine Dioxygenase</td>
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<td>TGFα</td>
<td>Transforming Growth Factor alpha</td>
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<td>TSA</td>
<td>Tricostatin A</td>
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<td>TSS</td>
<td>Transcription Start Site</td>
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<td>WGBS</td>
<td>Whole Genome Bisulfite Sequencing</td>
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<td>XPA</td>
<td>Xeroderma Pigmentosum Complementation Group A</td>
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<tr>
<td>XRCC</td>
<td>X-Ray Repair Cross Complementing Protein</td>
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## APPENDIX B: Expression Changes in DNA Repair Genes Altered in Panc1-3D7 Compared to Panc1-2G6 Cell Lines

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<td>C- abl oncogene 1, non-receptor tyrosine kinase</td>
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<td>ATM</td>
<td>Ataxia telangiectasia mutated</td>
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<td>ATR</td>
<td>Ataxia telangiectasia and Rad3 related</td>
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<td>ATRIP</td>
<td>ATR interacting protein</td>
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<td>Alpha thalassemia/mental retardation syndrome X-linked</td>
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<td>BAX</td>
<td>BCL2-associated X protein</td>
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<td>Bloom syndrom, RecQ helicase-like</td>
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<td>CDC25A</td>
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<td>CDK7</td>
<td>Cyclin-dependent kinase 7</td>
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<td>CHK2 checkpoint homolog (S.Pombe)</td>
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<td>CIB1</td>
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<td>CRY1</td>
<td>Cryptochrome 1 (photolyase-like)</td>
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REFERENCES


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Education:
2010-2016 Graduate Student, Department of Cellular and Molecular Biochemistry, University of Kentucky
2008-2010 Graduate Student, Department of Biology, Murray State University
2004-2008 Undergraduate Student, Biology, Georgetown College

Degrees Awarded: Bachelor of Science in Biology, Georgetown College

Professional Experience:
2015 Instructor – Research Methods, Health Researchers Youth Academy, University of Kentucky
2012 Teaching Assistant – Medical Biochemistry, University of Kentucky, College of Medicine
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Academic Awards and Honors:
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2015 Travel Award, University of Kentucky Graduate School, AACR Chromatin and Epigenetics
2015 Oral Presenter, Markey Cancer Center Annual Research Day
2015 Travel Award, University of Kentucky Graduate School, AACR 104th Annual Meeting
2014 Travel Award, University of Kentucky Graduate School, Keystone, Cancer Epigenetics
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2012 Travel Award, ASBMB, Experimental Biology National Conference
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Publications:

Carpenter BL, Chen M, Knifley T, Davis KA, Harrison SMW, Stewart RL and O’Connor KL. Integrin α6β4 promotes autocrine EGFR signaling to stimulate migration and invasion towards HGF. Journal of Biological Chemistry. 290 (45): 27228–27238. 2015.


Abstracts:


Carpenter BL, Chen M, Knifley T, Davis KA, Harrison SMW, Stewart RL, and O’Connor KL. “Integrin α6β4 promotes autocrine EGFR signaling to stimulate migration and invasion toward HGF.” AACR 2015 Annual Meeting, Philadelphia, PA, April 18-22, 2015.


Carpenter BL, Chen M, and O’Connor KL. “Integrin alpha6beta4 promotes directed DNA demethylation of EGFR ligand promoters.” Cancer Epigenetics, Santa Fe, NM, February 4-9, 2014.


Carpenter BL, Chen M, O’Connor KL. Integrin α6β4 promotes DNA demethylation of amphiregulin and epiregulin promoters. AACR 2012 Annual Meeting, Chicago, IL, March 31-April 4, 2012.

Oral Presentations:
Oct, 2015  “Integrin α6β4 promotes pancreatic cancer cell invasion by altering DNA repair mediated epigenetics”
Student Seminar Presentation
Department of Molecular and Cellular Biochemistry, University of Kentucky

May, 2015  “Integrin α6β4 regulates expression of amphiregulin and epiregulin through DNA repair-dependent mechanisms”
Selected Student Speaker
Markey Cancer Center Annual Research Day, University of Kentucky

May, 2015  “Integrin α6β4 regulates expression of amphiregulin and epiregulin through DNA repair-dependent mechanisms”
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Tumor Microenvironment and Metastasis Meeting
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